MOLECULAR GENETICS OF NEUROFIBROMATOSIS TYPE 1 (NF1)

A thesis submitted for the degree of Philosophiae Doctor

Claudia Consoli

2006

Institute of Medical Genetics

Cardiff University

UMI Number: U488227

UMI®

Dissertation Publishing

UMI U488227

ProQuest®

Contents

Chapter 8. General Discussion   **171**

Summary

Neurofibromatosis type 1 (NF1) is a complex autosomal dominant neurogenetic disorder affecting 1 in 3500 individuals worldwide. The main clinical features of the disease include café-au-lait (CAL) spots and cutaneous neurofibromas. The *NF1* gene, which spans ~ 350 kb of chromosome 17q11.2, contains 60 exons and encodes a protein, neurofibromin, which is involved in the downregulation of Ras activity by the conversion of active Ras-GTP to inactive Ras-GDP.

Patients exhibiting signs of NF1 limited to one or more body segments (segmental NF1; SNF1) are rare and are assumed to represent somatic mosaicism of the *NF1* gene. Although the majority of cases of SNF1 are sporadic, there are reports of NF1 families in which only one parent exhibits segmental features of the disease but whose children manifest typical NF1. The possible different molecular mechanisms underlying these two types of SNF1 were explored. DNA samples from 30 sporadic and 9 familial cases of SNF1 were screened for mutations in the *NF1* gene using several different approaches. In one family nonsense mutation (R1947X) in exon 31 of the the *NF1* gene was identified in the lymphocyte DNA of the affected child by DHPLC and PCR/direct sequencing. DNA sequence analysis failed, however, to identify the R1947X mutation in peripheral lymphocytes, and in keratinocytes and fibroblasts cultured from affected and unaffected skin in the mother. DNA fragments containing exon 31 were then cloned from each cell line and these clones were screened using allele-specific PCR. The R1947X mutation was identified in 29 of 146 clones derived from keratinocytes from the affected region and in 12 of 136 clones derived from fibroblasts from the affected region, but in no clones derived from clinically unaffected tissues. These findings confirm for the first time that gonosomal mosaicism can occur in SNF1. In addition, a nonsense mutation (R1513X) in a leukocyte DNA sample and a novel single base-pair substitution at nucleotide position +8 in intron 32 (IVS32+8 C→G) in both leukocyte and fibroblast DNA samples in two unrelated sporadic SNF1 patients were identified.
A pilot expression profiling study based on fibroblast RNA derived from affected and unaffected skin did not reveal any significant difference in the expression pattern, suggesting that fibroblasts may not be the optimal cells to be targeted for such gene expression changes in SNF1.

Some families with NF1-related syndromes (viz. Watson syndrome, familial café-au-lait spots and familial spinal neurofibromas) share clinical features. The question arises therefore as to whether underlying NF1 gene mutations are responsible. Four families and 10 sporadic NF1 patients were selected who had either presented with atypical NF1 or with different disorders with some similarity or clinical overlap with NF1. DHPLC analysis of the *NF1* gene in DNA from these patients succeeded in identifying three micro-deletions, two of which were in-frame (2970-2972 del AAT, 4312del GAA, 3525-3526del AA), a single frameshift insertion (4095ins TG), a nonsense mutation (R1513X) and a missense mutation (H553R). The 2970-2972 del AAT in-frame micro-deletion was identified in NF1 patients without neurofibromas in two different families. The difficulty in correlating specific *NF1* mutations with a particular clinical phenotype suggests that modifying genes are involved in the clinical variability of NF1. This conclusion is strongly supported by the observation that identical *NF1* gene lesions can cause very different clinical phenotypes in unrelated NF1 patients.

In an attempt to correlate the occurrence of specific *NF1* gross deletion breakpoints with the clinical phenotype, 21 NF1 patients known to be carrying deletions of the entire *NF1* gene were analysed in order to determine the frequency of the common deletion breakpoints. A specific 3.4 kb junction fragment was identified in 10 of 21 DNA samples, confirming the previously reported frequency (46%). However, owing to the paucity of clinical data, further studies will be required to assess a potential relationship between the extent of the deletion and clinical features.

Promoter hypermethylation has been shown to inactivate a considerable number of tumour suppressor genes in a wide variety of tumours. In this study, the *NF1* promoter region was screened for hypermethylation using the bisulphite conversion method in a panel of 29 tumours (benign and malignant tumours) from NF1 patients. However, no hypermethylation was noted in a ~120-bp region flanking the *NF1* gene transcriptional start site suggesting that epigenetic inactivation of the *NF1* gene promoter is not a common event in NF1-related tumorigenesis.

Publications

Consoli C, Moss C, Green S, Balderson D, Cooper DN, Upadhyaya M (2005). Gonosomal Mosaicism for a Nonsense Mutation (R1947X) in the *NF1* Gene in Segmental Neurofibromatosis Type 1. *J Invest Dermatol* 125: 463-466.

Oktenli C, Gul D, Deveci MS, Saglam M, Upadhyaya M, Thompson P, **Consoli C**, Kocar IH, Pilarski R, Zhou XP, Eng C (2004). Unusual features in a patient with neurofibromatosis type 1: Multiple subcutaneous lipomas, a juvenile polyp in ascending colon, congenital intrahepatic portosystemic venous shunt, and horseshoe kidney. *Am J Med Genet*127A: 298-301.

Upadhyaya M, Han S, **Consoli C**, Majounie E, Horan M, Thomas NS, Potts C, Griffiths S, Ruggieri M, von Deimling A, Cooper DN (2004). Characterization of the somatic mutational spectrum of the neurofibromatosis type 1 (*NF1*) gene in neurofibromatosis patients with benign and malignant tumors. *Hum Mutat* 23:134-146.

Oktenli C, Saglam M, Demirbas S, Thompson P, Upadhyaya M, **Consoli C**, Ulucan H, Koz C, Durukan AH, Bozkurt A, Koc B, Kocar IH, Gul D (2003). A large deletion (1.5 Mb) encompassing the neurofibromatosis type 1 (*NF1*) gene in a patient with sporadic NF1 associated with dysmorphism, mental retardation, and unusual ocular and skeletal features. *Clin Dysmorphol* 12:199-201.

Upadhyaya M, Majounie E, Thompson P, Han S, **Consoli C**, Krawczak M, Cordeiro I, Cooper DN (2003). Three different pathological lesions in the *NF1* gene originating *de novo* in a family with neurofibromatosis type 1. *Hum Genet* 112:12-17.

Acknowledgements

Abbreviations

A	Adenine
C	Cytosine
CALs	Café-au-lait spot
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary DNA
CMT1	Charcot-Marie-Tooth disease
CNS	Central nervous system
CSA	Comparative Sequence Analysis
CSRD	cysteine/serine-rich domain
DHPLC	Denaturing High Performance Liquid Chromatography
EMSA	Electrophoretic mobility shift assay
ESE	Exonic splicing enhancer
EST	Expressed sequence tag
EVI	Ecotropic viral integration site
FISH	Fluorescent *in situ* hybridisation
FSNF	Familial spinal NF
G	Guanine
GABA	Gamma-aminobutyric acid
GAP	GTPase activating proteins
GDP	Guanosine diphosphate
GRD	GAP related domain
GTP	Guanosine triphosphate
HA	Heteroduplex analysis
HNPCC	Hereditary non-polyposis colon cancer
LCRs	Low copy repeats
LOH	Loss of heterozygosity
MAPKs	Mitogen activated protein kinases.
MMR	Mismatch repair
MPNST	Malignant Peripheral Nerve Sheath Tumour
mRNA	Messenger RNA
MSI	Microsatellite instablility
mTOR	Mammalian target of rapamycin
NAHR	Non-allelic homologous recombination
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
NHEJ	Non homologous end joining
NS1	Noonan syndrome
OMGP	Oligodendrocyte myelin glycoprotein
PCR	Polymerase chain reaction
PKA	Protein kinase A
PTT	Protein truncation test
RB	Retinoblastoma
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleotide acid
RT-PCR	Reverse transcriptase PCR
SNF1	Segmental NF1
T	Thymine
TSC	Tuberous sclerosis complex
UTR	Untranslated region
VHL	Von Hippel Lindau
WS	Watson syndrome

Chapter 1. General Introduction

The neurofibromatoses are congenital disorders that cause tumours to grow along various types of nerves and, in addition, can affect the development of non-nervous tissues such as bones and skin. Only neurofibromatosis type 1 (NF1) (MIM 162200), also known as von Recklinghausen or peripheral NF, and neurofibromatosis type 2 (NF2) (MIM 101000), also known as bilateral acoustic NF, have been fully described. Both forms of NF are transmitted as autosomal dominant conditions with NF1 being at least 20-fold more prevalent than NF2. The *NF2* gene has been identified on chromosome 22q12.2 (Rouleau *et al.* 1993, Troffatter *et al.* 1993) which is therefore quite distinct from the *NF1* gene located on chromosome 17q11.2 (Cawthon *et al.* 1990b, Viskochil *et al.* 1990, Wallace *et al.* 1990).

1.1 Neurofibromatosis type 1

1.1.1 Clinical aspects

1.1.1.1 History
Descriptions of cases almost certainly representing neurofibromatosis type 1 (NF1) (MIM 162200) can be found through many centuries of history. A thirteenth century drawing of a man with skin nodules by a Cistercian monk named Heinricus seems to depict a patient with neurofibromatosis (Zanca and Zanca, 1980; Morse, 1999). In 1642, Ulisse Aldrovandi, an Italian physician and naturalist, described a man of short stature with large tumours growing from the left side of his head and upper trunk resembling plexiform neurofibromas (Zanca and Zanca, 1980; Morse, 1999). However, the first English language clinical report was produced by Mark Akenside (1768), a British physician, who described several individuals within one family with multiple dermal tumours (Akenside, 1768; Ober, 1978). In 1845, the Irish pathologist Robert William Smith cited 75 references in an NF1 literature review, presented two further cases and postulated, but was unable to prove, that the tumours arose from the fibrous connective

1

tissue of small nerves (Smith, 1849). In 1882, Von Recklinghausen, a German pathologist, gave the first full description of the disorder, including the observation that the tumours arose from the fibrous tissue surrounding small nerves and coined the term neurofibroma (von Recklinghausen, 1882). The condition subsequently became known as von Recklinghausen disease. In 1918, Preiser and Davenport demonstrated the autosomal dominant inheritance pattern with the iris nodule, a crucial diagnostic feature, being defined in 1937 by the Viennese ophthalmologist Lisch (Lisch, 1937; Preiser & Davenport, 1918).

1.1.1.2 Clinical features

The most frequent clinical features of NF1 are *café-au-lait spots* (CALs), neurofibromas, skinfold frecklings, Lisch nodules, optic gliomas, but learning disabilities, malignant peripheral nerve sheath tumours (MPNST), and characteristic osseous lesions can also be present (Friedman, 2002).

CALs are the first clinical feature of NF1 and they are often present at birth or develop within the first two years of life. They increase in number in early childhood, but tend to fade with age (Huson & Hughes, 1994; Ruggieri & Huson, 1999). CALs, present in approximately 99% of all NF1 patients, are hyperpigmented macules having a diameter between 0.5 to 50 cm and ovoid in shape, and characterized histologically by the presence of giant melanosomes within the melanocytes (Benedict *et al.*, 1968). They are not unique to NF1 patients. Indeed, 11%-25% of individuals in the general population have 1-3 CALs; it is the increased number that is significant (Crowe & Schull, 1953; Burwell *et al.*, 1982).

Skinfold freckling involves hyperpigmented macules resembling CALs that are 1-3 mm in diameter and which are unique to NF1. Axillary and inguinal freckling occurs in 80% of children by the age of 6 (Obringer *et al.*, 1989). In addition, freckling may also be observed under the neck and breasts and over the trunk (Riccardi, 1992).

Lisch nodules are benign hamartomas located within the iris that do not have any effect on visual function and are an important diagnostic feature of NF1 (Lubs *et al.*, 1991; Lewis & Riccardi, 1981; Riccardi, 1981). They occur in fewer than 10% of patients

younger than 6 years, but are found in the majority of patients older than 10 years (Gutmann & Collins, 2001; Cnossen *et al.*, 1998; McGaughran *et al.*, 1999).

Neurofibromas are benign tumours of peripheral nerves and are composed of Schwann cells (80%) intermingled with smaller numbers of fibroblasts, mast cells, perineurial cells and endothelial cells (Lott *et al.*, 1981). These nerve sheath tumours manifest themselves as fleshy nodules in skin (dermal neurofibromas), circumscribed masses in nerves (intraneural or nodular neurofibromas), or lesions growing diffusely through multiple fascicles of large nerves or nerve plexuses (plexiform neurofibromas). Dermal neurofibromas usually appear during adolescence and increase in number with age, such that they are present in almost all adults with NF1 (Huson *et al.*, 1988; Riccardi, 1992). Diffuse plexiform neurofibromas are present in approximately one third of individuals with NF1. They usually appear in early childhood and are believed to be congenital lesions (Waggoner *et al.*, 2000; Huson & Harper, 1988; Korf, 1999). Diffuse plexiform neurofibromas can occur superficially or within deeper tissues of the face, neck, trunk, or limb. Diffuse plexiform neurofibromas and, less commonly, deep nodular neurofibromas can become transformed into highly aggressive sarcomas known as malignant peripheral nerve sheath tumours (MPNSTs) (Korf, 1999; Waggoner *et al.*, 2000; Woodruff, 1999). MPNSTs are aggressive and highly metastatic transformed cells, which originate from peripheral nerves and invade surrounding tissues (Sangueza & Requena, 1998). MPNSTs are often lethal because they form secondary metastatic sites in the lung, lymph nodes, and liver. Patients with NF1 have an increased incidence of developing MPNST. Recently, Evans *et al.* (2002) estimated that 8-13% of NF1 patients develop MPNSTs.

Optic nerve gliomas are pilocytic astrocytomas which cause expansion of the optic nerve. They occur in about 15% of children with NF1, but only one third to one half of these patients ever develop associated symptoms (Listernick & Gutmann, 1999; Listernick *et al.*, 1999).

Other features of NF1 are variable, with the phenotypic expression being characteristic of a given patient. Cognitive impairments are prevalent in children with NF1, resulting in specific learning disabilities in 30-65% of patients (North *et al.*, 1997). A variety of sensory or motor deficits can result from central nervous system (CNS) tumours or from

neurofibromas involving spinal nerve roots or major peripheral nerves. NF1 patients are also at increased risk for the development of certain malignancy, including pheochromocytomas and childhood leukaemias (Side *et al.*, 1998). Other NF1 complications, such as seizures, macrocephaly, short stature, scoliosis, pseudoarthrosis, and malignancy occur in a small percentage of cases (Huson *et al.*, 1989a; 1989b; Riccardi, 1981; Ruggieri & Huson, 1999). Clinical complications of NF1 are summarised in Table 1.1

Table 1.1: Clinical complications in NF1 with their frequency and age of appearance

	Frequency (%)	Age
Skin		
Plexiform neurofibromas	30-39	<5 years
Juvenile xanthogranuloma	1-2	Infancy
Eye		
Optic glioma	15	Infancy
Symptomatic	2-4	Infancy
Bones		
Pseudoarthrosis	3-4	Childhood
Scoliosis requiring surgery	2-4.4	Childhood, adolescence
Nervous system		
Learning difficulties	33-70	Childhood
Epilepsy	6-7	Childhood
Hydrocephaly	1.5-2.6	Childhood
Cancers		
Neurofibrosarcoma	3-4	Adolescence, adulthood
Leukaemia	<0.1	Infancy
Carcinoid tumour	0.6-1.5	Adolescence, adulthood
Arterial hypertension		
Hypertension	5	Adulthood
Pheochromocytoma	<1	Adulthood
Renal artery stenosis	1	Childhood, adulthood

(Derived from Pinson, 2002)

1.1.1.3 Diagnosis

NF1 is diagnosed using clinical criteria developed by a National Institutes of Health (NIH) Consensus Conference in 1987 (Stumpf *et al.*, 1988) and further reaffirmed by Gutmann *et al.* (1997). To meet the NIH Diagnostic Criteria for NF1, an individual must exhibit at least two of the features listed below:

1) Six or more café-au-lait macules over 5 mm in greatest diameter in pre-pubertal individuals and over 15 mm in greatest diameter after puberty.

2) Two or more neurofibromas of any type or one plexiform neurofibroma.

3) Freckling in the axillary or inguinal regions.

4) An optic glioma.

5) Two or more Lisch nodules.

6) A distinctive, osseous lesion such as sphenoid wing dysplasia or thinning of the cortex of the long bones (with or without pseudoarthrosis).

7) A first-degree relative (parent, sibling or offspring) with NF1 by the above criteria.

1.1.1.4 Classification

NF is a heterogeneous condition and several attempts have been made to classify the disease into distinct categories (Riccardi 1982, 1884, Carey *et al.*, 1986). As yet, only NF1 and NF2 have been confirmed as genetically distinct diseases, as a result of the cloning of the respective genes. In 1992, Viskochil and Carey proposed a classification in which the different forms of neurofibromatosis were divided into two categories: *alternate*, which comprises forms having some of respective clinical features of either NF1 or NF2, and *related*, which comprises forms having classic clinical features of NF in addition to distinctive clinical features not typically seen in either NF1 or NF2. However, the most widely used classification continues to be that recommended in 1987 by the National Institutes of Health (NIH) Consensus Development Conference that was based on distinctive clinical features of NF. This is a numerical classification and only NF1 and NF2 were distinguished.

1.1.1.5 Neurofibromatosis type 2

Neurofibromatosis type 2 (NF2) (MIM 101000), also known as bilateral acoustic neurofibromatosis is a disorder clinically and genetically distinct from NF1, in which affected individuals develop schwannomas, meningiomas and ependynoma (Evans *et al.*, 1992).

Diagnostic criteria for NF2 are based on the presence in an individual of one of the following clinical features:

1) Bilateral vestibular schwannomas.

2) First-degree relative with NF2 plus

2a) one vestibular nerve schwannoma

or

2b) two of the following: meningioma, schwannoma, glioma, neurofibroma, posterior subcapsular lens opacity.

The *NF2* gene, which has been mapped to chromosome 22q12, is a tumour suppressor gene spanning 110 kb (Rouleau *et al.*, 1987; Seizinger *et al.*, 1986). It encodes a protein, termed merlin or schwannomin, which has sequence similarity to a family of proteins that link the actin cytoskeleton to cell surface glycoproteins (Rouleau *et al.*, 1993; Trofatter *et al.*, 1993). NF2 exhibits autosomal dominant inheritance with an estimated birth incidence of 1/33 000 (Evans *et al.*, 1992). *NF2* manifests a high degree of penetrance and about 50% of cases represent new mutations. The hallmark of NF2 is the development of bilateral schwannomas on the vestibular portion of the eighth cranial nerve which occurs in more than 90% of patients by 3 years of age (Eldridge, 1981). However, other tumours of cranial and cervical nerve roots are also common. Individuals with NF2 often have a small number of café-au-lait spots, whereas fewer than 1% of NF2 patients present with six café-au-lait spots (Evans *et al.*, 1992; Parry *et al.*, 1994).

Both NF1 and NF2 show ophthalmological diagnostic features with NF1 patients presenting with Lisch nodules of the iris and NF2 patients presenting with cataracts (Short *et al.*, 1994). A patient who presented with both NF1 and NF2 simultaneously has been reported (Sadeh *et al.*, 1989). This individual had a father with NF1 and a mother with NF2, indicating that both NF1 and NF2 can be transmitted together and manifest equally in the same person without exclusion of the other. Recently, another individual who met the criteria for the diagnosis of both NF1 and NF2 was described, but he did not present with a family history of neurofibromatosis (Pandey *et al.*, 2002).

1.1.2 NF1-related syndromes

There are several related or alternate forms of NF1, such as Watson syndrome (MIM 193520) (Tassabehji *et al.*, 1993; Ahlbom et al., 1995), Noonan syndrome (MIM 163950) (Tassabehji *et al.*, 1993; Bahuau *et al.*, 1996), autosomal dominant multiple CLS (MIM 114030) (Brunner *et al.*, 1993; Abeliovich *et al.*, 1995), and LEOPARD syndrome (MIM 151100) (Wu *et al.*, 1996). They are considered as related or alternate

6

forms of NF1 because they show some but not all of the classical symptoms of NF1 and do not adhere to the NIH criteria strictly. However, there exists some controversy as to whether the molecular basis of these conditions involves the *NF1* gene.

1.1.2.1 Familial spinal neurofibromatosis

Familial spinal NF (FSNF) (MIM 162210) has been considered an alternative form of NF1 since patients generally lack dermal neurofibromas and Lisch nodules, both typical hallmarks of NF1, and because symptomatic and generalised spinal neurofibromas are uncommon in classical NF1. FSNF has been reported in a small number of families in whom affected individuals have presented with multiple spinal cord tumours, occasional cervical, thoracic and lumbar region tumours and multiple café-au-lait spots (Pulst *et al.*, 1991; Poyhonen *et al.*, 1997; Ars *et al.*, 1998). Three of these FSNF families with CAL spots were shown to be linked to markers surrounding the *NF1* locus (Poyhonen *et al.*, 1997 ; Pulst *et al.*, 1991; Ars *et al.*, 1998) whereas, in the family presenting with spinal neurofibromas without CAL spots, linkage to the *NF1* locus was excluded (Pulst *et al.*, 1991). However, in only one FSNF family has a frameshift mutation 8042insA in *NF1* exon 46 been documented (Ars *et al.*, 1998). Recently, Messiaen *et al.* (2003) described the identification of an *NF1* mutation in two FSNF families, originally described by Pulst *et al.* (1991) and Poyhonen *et al.* (1997), whose phenotype was known to be linked to chromosome 17q11.2 markers. They identified a novel substitution at the splice donor site of exon 39 (7126+3A>C), leading to the skipping of exon 39 in one family, and a missense mutation L357P in exon 8 in the other. These findings emphasise that FSNF (with CAL spots) is caused by mutations in the *NF1* gene, but do not support the hypothesis that it is caused by a unique type of *NF1* mutation.

1.1.2.2 Segmental NF1 (SNF1)

Segmental, or regional NF1, is sometimes diagnosed in individuals who have features of NF1 restricted to one part of the body and whose parents are both unaffected (Friedman & Riccardi, 1999; Ruggieri & Huson, 2001; Listernick *et al.*, 2003). In some cases, the unusual distribution of features is probably just a chance occurrence in an individual with NF1. In other individuals, segmental NF1 represents mosaicism for a somatic *NF1* mutation (Tinschert *et al.*, 2000; Vandenbroucke *et al.*, 2004). Individuals with segmental NF whose children have full-blown NF1 have been reported (Moss &

7

Green, 1994; Oguzkan *et al.*, 2004). Age at presentation of NF1 lesions in SNF1 patients varies according to the presence of pigmentation anomalies only (birth to 2 years of age) or neurofibromas alone (from around puberty to young adulthood). Affected individuals manifest the NF1 clinical features in exactly the same way as when they occur in the full-blown disease. The calculated prevalence of segmental NF1 in the general population is one in 36 000–40 000 individuals (Huson & Ruggieri, 2000; Ingordo *et al.*, 1995; Wolkenstein *et al.*, 1995; Ruggieri & Huson, 2001) but it is probably underdiagnosed because of highly variable signs or symptoms in most of the affected individuals.

1.1.2.3 Neurofibromatosis-Noonan syndrome

Noonan syndrome (NS1) (MIM 163950) is an autosomal dominant condition characterized by webbing of the neck, unusual facies, short stature, and congenital heart disease (often pulmonic stenosis). A gene for Noonan syndrome has been mapped to 12q24. The Noonan syndrome phenotype occurs in about 12% of individuals with NF1 (Buehning & Curry, 1995; Colley *et al.*, 1996). The features may include ocular hypertelorism, down-slanting palpebral fissures, low-set ears, webbed neck, and pulmonary stenosis. Relatives of such individuals who are affected with NF1 may or may not have concomitant features of Noonan syndrome. The NF1-Noonan phenotype appears to have a variety of causes, including the segregation of two different relatively common autosomal dominant traits in some families and segregation as an NF1 variant in others (Carey, 1998). Features of Noonan syndrome, often without a cardiovascular malformation, have been observed in about 12% of individuals with NF1 (Buehning & Curry, 1995; Colley *et al.*, 1996). This relative high frequency of co-occurrence seems rather unlikely if NF1 and Noonan syndrome are truly independent. In some families, NF1 and Noonan syndrome have been shown to segregate as independent autosomal dominant traits, and Noonan syndrome is not linked to the *NF1* locus in families without features of NF1. In other instances, features of both Noonan syndrome and NF1 appear to result from mutations of the *NF1* gene, and these phenotypes segregate together (Colley *et al.*, 1996). Recently, the molecular concurrence of both diseases has been described in a patient exhibiting mutations in both the *NF1* and the *PTPN11* genes (Bertola *et al.*, 2005). It appears that the concurrence of NF1 and Noonan syndrome may have several different causes, including the segregation of two different relatively

common autosomal dominant traits in some families and segregation as an NF1 variant in others (Carey, 1998).

1.1.2.4 Watson syndrome

Watson syndrome (WS) (MIM 193520) is characterised by short stature, café-au-lait spots, cognitive impairment and pulmonary stenosis. Lisch nodules and neurofibromas are not invariably present in individuals affected by Watson syndrome. However, WS patients display an increased frequency of these clinical features. Allanson *et al.* (1991) demonstrated that WS is linked to markers flanking the *NF1* gene and more recently, mutations in the *NF1* gene have been identified in WS families. Upadhyaya *et al.* (1992) identified an 80kb deletion of the *NF1* gene, while an in-frame tandem duplication of 42bp in exon 28 was detected by Tassabehji *et al.* (1993). These findings have led to the hypothesis that WS may be allelic to NF1.

1.1.2.5 Autosomal dominant familial café-au-lait spots

The occurrence of café-au-lait spots in the absence of other features of NF1 have been reported in several families. Linkage studies in two families did not confirm linkage to the NF1 locus, suggesting that the heritable possession of café-au-lait spots is due to the action of a dominant gene genetically distinct from *NF1* (Charrow *et al.*,1993; Brunner *et al.*, 1993). However, Abeliovich *et al.* (1995) identified close linkage between familial café-au-lait spots and the *NF1* locus in three generations of a family and concluded that at least in some families, the trait was allelic to *NF1*.

1.1.2.6 Leopard syndrome

Leopard syndrome (MIM 151100) is an autosomal dominant disorder characterized by multiple lentigines, cardiac abnormalities, variable mental retardation, and typical craniofacial features. Leopard syndrome displays considerable clinical overlap with NF1, but linkage analysis has suggested that the phenotype does not segregate with markers from the *NF1* gene (Ahlbom *et al.*, 1995). However, a *de novo NF1* mutation in a patient with features of Leopard syndrome and a muscular outflow tract stenosis of the left cardiac ventricle has been reported (Wu *et al.*, 1996).

1.1.3 Association between NF1 and other syndromes

1.1.3.1 Tuberous sclerosis

Tuberous sclerosis complex (TSC) (MIM 191100) was described in 1880 by Bourneville. TSC is an autosomal dominant neurocutaneous disorder occurring in 1 in 6,000 with spontaneous mutations representing approximately two thirds of cases (Bourneville, 1880; Cheadle *et al.*, 2000). Clinical features of TSC include seizures, mental retardation, and cutaneous lesions; patients are also at risk for developing neoplasms including ependymomas and giant cell astrocytomas. There are two genes known to be responsible for TSC; these map to 9q34 (*TSC1*) (MIM 605284) and 16p13 (*TSC2*) (MIM 191092) respectively. *TSC1* codes for hamartin, whilst *TSC2* codes for tuberin. Both proteins are widely expressed in the brain and appear to interact as part of the insulin pathway and lead to inhibition of S6K activity (Inoki *et al.*, 2002). Occurrence of both NF1 and TSC in a single individual is rare with only a few patients reported in the literature. In three reported cases, inheritance of one condition, and sporadic mutation of the other was assumed (Schull and Crowe, 1953; Picccoli *et al.*, 1977; Lee *et al.*, 1994). In three other cases, authors assumed the *de novo* occurrence of both conditions (Sicilia *et al.*, 1968; Gould, 1991; Phillips and Rye, 1994). Recently, a girl who inherited both NF1 and TSC from her mother and father respectively was reported (Wheeler & Sadeghi-Nejad, 2005).

Apparently pathogenic *NF1* mutations have been demonstrated in a few individuals or families who do not have NF1 according to the NIH Diagnostic Criteria. This includes three families with multiple spinal neurofibromas but no café-au-lait spots (Kaufman *et al.*, 2001; Kluwe *et al.*, 2003b), a man with an optic glioma but no other diagnostic features of NF1 (Buske *et al.*, 1999), and a child with encephalocraniocutaneous lipomatosis (Legius *et al.*, 1995). The relationship of these *NF1* mutations to the unusual phenotypes in these families is not understood.

1.1.3.2 Hereditary non-polyposis colon cancer (HNPCC)

Hereditary non-polyposis colon cancer (HNPCC) (MIM 120435) is an autosomal dominant disorder associated with the early onset of colon cancer. Heterozygous mutations in the mismatch repair (MMR) genes, especially *MSH2* and *MLH1*, account for most cases. Patients with HNPCC and clinical features of NF1 have been described

10

(Puisieux, 1999; Ricciardone *et al.*, 1999; Wang *et al.*, 1999; Trimbath *et al.*, 2001; Whiteside *et al.*, 2002; Gallinger *et al.*, 2004; Raevaara *et al.*, 2004). DNA sequence analysis documented homozygous *MLH1* mutations in these individuals. The phenotype is remarkably similar to NF1. However, the kind of tumour that occurs in these homozygotes is early onset and more typical of HNPCC.

These cases strongly suggest that homozygous mutations in MMR genes lead to a mutator phenotype and also that mutations in both the MMR and *NF1* genes probably predispose to the development of cancer in these patients. Furthermore, the malignant phenotype appears to be somewhat different from that expected, with some of the patients demonstrating non-Hodgkin's lymphoma (NHL) rather than the expected myeloid malignancies and/or brain tumours. Although NHL has been reported in neurofibromatosis, it is less common than the other malignancies. In addition, since 50% of all NF1 patients have *de novo* mutations without a family history, it could be that many individuals have been predisposed to *NF1* gene mutation by the presence of either heterozygous or homozygous abnormalities in their MMR genes.

1.1.3.3 MoyaMoya disease
MoyaMoya disease (MIM 252350) is characterized by spontaneous occlusion of the temporal portions of the bilateral internal carotid arteries, the circle of Willis, with a high incidence in the Japanese population.
MoyaMoya disease is a definitive clinical entity of unknown cause. However, it has been hypothesized that MoyaMoya disease is most probably inherited in a polygenic mode or an autosomal dominant fashion with a low penetrance (Osawa *et al.*, 1992). There are several lines of evidence indicating that MoyaMoya disease is related to genetic factors in familial cases. Ikeda *et al.* (1999), demonstrated that a gene for familial MoyaMoya disease maps to chromosome 3p24.2-p26. However, more recently, Yamauchi *et al.* (2000) localized the gene abnormality to chromosome 17q25 in affected individuals from MoyaMoya families.

Arterial abnormalities associated with NF1 have been described (Muhonen *et al.*, 1991). Such lesions were seen in the vessels of the retina, endocrine glands, heart, gastrointestinal tract and brain. However, abnormalities of the intracranial arteries are rare (Muhonen *et al.*, 1991; Sobata *et al.*, 1988). The characteristic lesions of

MoyaMoya disease are occasionally associated with NF1, and more than 50 cases of such an association have been reported so far (Osawa *et al.*, 1992; Barrall & Summers, 1996; Edwards-Brown & Quets, 1997; Erickson *et al.*, 1980; Woody *et al.*, 1992).

Table 1.2 Examples of other genetic disorders associated with NF1

Genetic disorder	Reference
Charcot-Marie-Tooth disease (CMT1)	Lupski *et al.*, 1993 ; Bosch *et al.*, 1981
Multiple sclerosis	Perini & Gallo, 2001; Fernet *et al.*, 1995; Masson & Colombani, 1997; Johnson *et al.*, 2000 ; Feuillet *et al.*, 2004
Gilbert syndrome	Kocer *et al.*, 2003
Weaver syndrome	Van Asperen *et al.*, 1998
McCune Albright syndrome	Gonzalez *et al.*, 2000
Jaffe Campanacci syndrome	Olby & Saul, 2003
Ullrich-Turner	Schorry *et al.*, 1996
Coeliac disease	Biagi *et al.*, 2005
Breast cancer	Guran & Safali, 2005 ; Nakamura *et al.*, 1998 ; El-Zawahry *et al.*, 1989
Retinoblastoma	Koestenberger *et al.*, 2003

1.1.4 Genetic aspects of NF1

Neurofibromatosis type 1 (NF1) is one of the most common familial tumour syndromes in human (Huson & Hughes, 1994). NF1 is an autosomal dominant disorder affecting approximately 1 in 3500 individuals, without preference of race or sex (Gutmann *et al.*, 1997; Parada, 2000; Gutmann, 2001). The average age at death among people with NF1 is about 15 years earlier than in the normal population (Rasmussen *et al.*, 2001), with malignancies (Gutmann *et al.*, 1997; Riccardi, 1992) and cardiovascular disease being the most common causes of premature death.

1.1.4.1 Prevalence and Penetrance

It is difficult to quantify accurately the incidence of NF1 mostly because it is often not diagnosed at birth. Multiple population studies from the United States (Crowe *et al.*, 1956), Russia (Sergeyev, 1975), Denmark (Samuelsson & Axelsson, 1981) and Wales

(Huson *et al.*, 1989) have estimated the disease prevalence to be approximately 1 in 2500 to 1 in 5000. The Russian study provided a lower estimate of 1 in 7800, but this may have underestimated the true frequency of NF1. Owing to disease-related mortality and the under-ascertainment of mildly affected cases, the disease prevalence is probably around 1 in 4500 (Huson & Hughes, 1994). Approximately 50% of individuals with NF1 lack a family history and are presumed to represent new mutations (Crowe *et al.*, 1956; Samuelsson & Axelsson, 1981; Huson et al., 1989). The penetrance of NF1 is complete, but the manifestations are extremely variable, although they generally increase with age (Friedman & Birch, 1997; DeBella *et al.*, 2000).

1.1.4.2 Mutation rate

The mutation rate for the *NF1* gene (~ 10^4 per generation) is among the highest known for any gene in humans (Lynch & Gutmann, 2002). Thus, ~50% of all NF1 patients lack a family history of the disease (Huson & Hughes 1994). It has been speculated that the high mutation rate is caused by the large size of the gene and the complexity of its processing (Upadhyaya *et al.*, 1994). Most constitutional mutations in the *NF1* gene are nonsense or frameshift mutations that lead to premature truncation of neurofibromin (Viskochil, 1999; Von-Deimling *et al.*, 1995).

Recent advances in detecting mutations of the *NF1* gene have made it possible to identify >95% of the mutations in a given cohort of patients (Messiaen *et al.*, 2000). There are no general 'hotspot' regions for mutations in the *NF1* gene, but exons 10 and 37 have been shown to have the highest mutation rate and account for ~30% of the mutations (Messiaen *et al.*, 2000). Recently, Ars *et al.* (2000) identified 44 different mutations, of which 32 were novel, in 80 unrelated patients with NF1. Mutations were identified in 87% of the familial cases and in 51% of the sporadic cases. Since mutations resulted from splicing alterations are the most common molecular defects in *NF1*, it has been suggested that part of the clinical variability in NF1 could be related to mutations affecting mRNA splicing. The high proportion of aberrantly spliced transcripts detected in NF1 patients emphasizes the importance of studying mutations at both the genomic and the RNA level.

Large deletions (>1Mb) have also been reported in patients with NF1 (Kayes *et al.*, 1992). These deletions, identified in patients manifesting typical NF1 symptomatology as well as significant mental retardation, were found to extend beyond the *NF1* gene and possibly include other genes on chromosome 17q11.2 (Lopez-Correa *et al.*, 2001).

1.1.4.3 Expressivity

The *NF1* gene is highly pleiotropic and is expressed in a diverse set of tissues and organs (Riccardi, 1999; Carey & Viskochil, 1999). The wide variability of the NF1 phenotype, even in individuals with the same *NF1* gene mutation, suggests that other factors are involved in determining clinical manifestations. Several different factors, including allelic heterogeneity, 'second-hit' mutations, somatic mosaicism, epigenetic effects such as imprinting, alternative splicing posttranscriptional control of the NF1 protein, and environmental factors, have been postulated to contribute to the clinical variability of NF1 (Carey & Viskochil, 1999; Friedman & Riccardi , 1999; Riccardi, 1993)

In 1993, Easton evaluated variation of the NF1 phenotype with the degree of relatedness. This study revealed a strong correlation between monozygotic twins, a weaker correlation between first-degree relatives, and the weakest correlation of all among more distant relatives. These results supported the postulate that modifying genes may influence the NF1 phenotype.

It has also been suggested that environmental factors may influence the NF1 phenotype. However, no convincing evidence has been presented to support the involvement of any particular environmental factor. Riccardi (1993), has suggested that mechanical trauma may often precede the development of neurofibromas but no evidence for this has so far been presented.

The role of stochastic factors in the occurrence of some NF1 manifestations has also been hypothesized. Chance may therefore be involved in determining which cells are affected by a somatic mutation and at what point in development somatic mutation occurs. It is likely that the NF1 genotype, modifying genes, environmental and stochastic factors all play a role, in combinatorial fashion, in the clinical manifestations of *NF1* gene mutations.

Contiguous genes responsible for variations in expressivity have also emerged as a potential explanation for NF1 variation (Kayes *et al.*, 1992). Large deletions involving the entire *NF1* gene and flanking DNA appear to result in a discrete phenotype that is characterised by early onset of many cutaneous neurofibromas, minor anomalies and

14

learning or developmental disabilities (Wu *et al.*, 1995; Cnossen *et al.*, 1997; Leppig *et al.*, 1997; Valero *et al.*, 1997; Upadhyaya *et al.*, 1998)

A further explanation for phenotypic variation relates to the notion of somatic mosaicism. Riccardi and Lewis (1988) proposed this idea when they noticed that the patients from earlier generation of multigenerational families presented with a milder phenotype, suggesting that some progenitors may have had somatic mosaicism that could account for the phenotypic difference. Somatic mosaicism has recently been identified as the causative factor for segmental NF1 development (Tinschert *et al.*, 2000).

1.2 The *NF1* gene

1.2.1 Mapping of the NF1 gene

The *NF1* gene was mapped in 1987 to the pericentromeric region of chromosome 17q by linkage analysis (Barker *et al.*, 1987; Seizinger *et al.*, 1987). Subsequently, cytogenetic studies of two NF1 patients identified balanced translocations involving band 17q11 (O'Connell *et al.*, 1989; Cawthon *et al.*, 1990b). In further work, balanced translocations in the two unrelated individuals with NF1 were isolated in hybrid cell lines, and the breakpoints were physically mapped to the same region on chromosome 17. In one patient, the balanced translocation was located between chromosome 1 and 17, t(1;17)(p34.3;q11.2) (Schmidt *et al.*, 1987), whereas in the other patient it was located between chromosome 17 and 22, t(17;22)(q11.2;q11.2) (Ledbetter *et al.*, 1989). Four candidate genes were identified within the translocation breakpoints by positional cloning, *OMGP* (Viskochil *et al.*, 1991), *EVI2A* (Cawthorn *et al.*, 1990), *EVI2B* (Cawthorn *et al.*, 1991) and *TBR* (Viskochil *et al.*, 1991). However, mutations in genomic DNA from affected individuals involved only the fourth gene which was established to be responsible for the clinical sequelae (Cawthon *et al.*, 1990a; Vischocil *et al.*, 1990; Wallace *et al.*, 1990).

1.2.2 The NF1 gene structure

The *NF1* gene spans ~ 350 kb of genomic DNA in the chromosomal region 17q11.2. It contains 60 exons, of which three are alternatively spliced as in-frame insertion exons, and encodes a 12 kb mRNA transcript (Li *et al.*, 1995) (Figure 1.1). The 8457 bp open reading frame predicts a protein, neurofibromin, of 2818 amino acids with an estimated molecular mass of 327 kD (Marchuk *et al.*, 1991; Viskochil *et al.*, 1990; Upadhyaya & Cooper, 1998; Wallace *et al.*, 1990; Xu *et al.*, 1990a). The sizes of the exons and introns are listed in Table 1.3, with intron 1 being as large as 140 kb. Intron 27b is also large and contains the three embedded genes, *EVI2A, EVI2B* and *OMGP* (see section 1.2.2.3) along with two variable-repeat sites, separated by approximately 31 kb of sequence (Lazaro *et al.*, 1994).

The *NF1* gene is highly conserved between species. The protein product, neurofibromin, displays ~98% homology between mouse and human, with the 3' untranslated segment highly conserved, and ~60% homology between *Drosophila* and human (Bernards *et al.*, 1993, The *et al.* 1997). Sequence analysis of the *NF1* transcript revealed amino acid homology with bovine p120GAP extending from exon 21 to 27a. Furthermore, a peptide homology between neurofibromin and the yeast inhibitory regulator proteins Ira1 and Ira2, covering exons 16 through 40, has also been identified (Xu *et al.*, 1990).

The genomic structure of the *NF1* gene of the pufferfish *Fugu rubripes* has been characterized (Kehrer-Sawatzki *et al.*, 1998). This gene is 13 times smaller than its human counterpart, spanning only 27 kb, mainly due to reduced intron sizes. An overall similarity of 91.5% in amino acid sequence was found, with only exon 12b and the two alternatively spliced exons, 9br and 48a, missing.

Table 1.3: Sizes of the coding exons and introns of the *NF1* gene

Exon No.	Size (bp)	Introns (kb)	Exon No.	Size (bp)	IVS (kb)
1	60	20-140 ?	23-2	136	4
2	144	3.1	23a	63	6
3	84	4	24	159	0.53
4a	195	6.5	25	98	1.25
4b	103	2	26	147	1.27
4c	68	0.22	27a	147	3.4
5	76	0.8	27b	111	45-50
6	158	17.7	28	433	1.3
7	174	0.4	29	341	2.7
8	123	0.25	30	203	4.3
9	75	1.6	31	194	1.55
9a	30	3.1	32	141	0.15
10a	142	8.1	33	280	0.4
10b	135	4	34	215	0.24
10c	114	2.5	35	62	0.15
11	80	0.54	36	115	0.57
12a	124	1.5	37	102	1.7
12b	156	1.2	38	141	2.4
13	250	0.49	39	127	6
14	74	0.23	40	132	0.93
15	84	1.3	41	136	2
16	441	0.83	42	158	4
17	140	0.28	43	123	0.53
18	123	0.46	44	131	0.18
19a	84	1.2	45	101	1.1
19b	117	0.55	46	143	0.35
20	182	0.12	47	47	1.4
21	212	2.2	48	217	605
22	162	0.14	48a	54	6.7
23-1	104	13	49	153	

(Adapted from *Li et al.*, 1995).

1.2.2.1 The promoter/5'-untranslated region

The *NF1* gene is most highly expressed in brain and spinal cord, although low levels of mRNA can be found in nearly all tissues. The *NF1* gene promoter is embedded in a CpG-rich region. The 5' ends of the human and murine *NF1* genes are highly conserved. While no discernible TATA or CCAAT box sequences are seen, transcription initiates at identical sites in both species, corresponding to 484 nucleotides upstream of the ATG initiation codon in the human gene (Hajra *et al.*, 1994). The human and mouse *NF1* genes share particularly high sequence homology (95%) between nucleotides -33 and +261 and contain several perfectly conserved transcription factor binding site motifs,

including a cAMP response element, several AP2 consensus binding sites, and a serum response element. The high degree of evolutionary conservation displayed by these sequences indicates that they are likely to be significant in the regulation of *NF1* gene expression (Hajra *et al.*, 1994).

1.2.2.2 The 3'-untranslated region (3'UTR)

The 3'untranslated region of *NF1* mRNA shares strong homology between human and mouse, suggesting that it is important for mRNA stability (Bernards *et al.* 1993). A few proteins have been identified that bind to the *NF1* 3'untranslated region, including the human RNA-binding protein (HuR), which is known to bind mRNAs of proto-oncogenes, cytokines and transcription factors (Haeussler *et al.*, 2000). Furthermore, *NF1* mRNA has recently been identified to be targeted towards the cell-cell adhesion zone (Yla-Outinen *et al.*, 2002).

1.2.2.3 Embedded genes

Three genes, *EV12A* (Cawthon *et al.*, 1990a), *EV12B* (Cawthon *et al.*, 1991) and *OMGP* (Viskochil *et al.*, 1991) are embedded within intron 27b of the *NF1* gene. Each of these genes is transcribed in the opposite orientation to the *NF1* gene. A pseudogene, *AK3* (adenylate kinase 3) occurs in intron 37 and is transcribed in the same orientation as *NF1* (Xu *et al.*, 1992).

The *EV12A* and *EV12B* genes are human homologues of the murine putative proto-oncogenes, *Evi-2A* and *Evi-2B* respectively, which are implicated in retrovirally-mediated leukaemogenesis. *EV12A* gene contains two exons and encodes a transcript of 1.6 kb found in bone marrow, peripheral blood and brain tissue. *EV12B* also consists of two exons and encodes a transcript of 2.1 kb found in bone marrow, peripheral blood and fibroblasts (Cawthon *et al.*, 1991). The 5' exon of *EV12B* lies 4kb downstream from the 3' exon of *EV12A*. The protein products of both the *EV12A* and *EV12B* genes appear to be glycosylated with single membrane-spanning domains that also contain a leucine zipper domain, which may interact with other membrane-bound proteins.

The oligodendrocyte-myelin glycoprotein gene (*OMGP*) encodes the oligodendrocyte-myelin glycoprotein (Omgp), which is a membrane glycoprotein that appears in the human central nervous system (CNS) at the time of myelination (Mikol *et al.*, 1990).

18

The *OMGP* gene also comprises two exons and the 5' end of the *OMGP* cDNA maps 5kb centromeric to the 3' end of *EV12B*. Omgp can be detected immunohistochemically in CNS myelin and on the surface of cultured oligodendrocytes, as well as at the paranodal region of myelin in peripheral nerve (Apostolski *et al.*, 1994). The glycoprotein is anchored to the outer leaflet of the myelin membrane through a glycosylphosphatidyl inositol lipid molecule, as is the cell surface form of the neural cell adhesion molecule N-CAM (Jessel, 1988). Structurally, therefore, Omgp has the potential to function as an adhesion molecule and could contribute to the interactions between the plasma membranes of oligodendrocytes and axons required for myelination or survival of myelinated axons. Furthermore, it has been shown that Omgp is an important inhibitor of neurite outgrowth acting through the Nogo receptor (NgR) and its associated receptor complex. Thus, interfering with the Omgp/NgR pathway may allow damaged axons to regenerate after injury *in vivo* (Wang *et al.*, 2002).

The existence of three genes within an intron of another gene is fairly unusual and complicated arrangement. Although a considerable number of genes embedded within other genes have now been described, the biological significance of this arrangement remains unknown. Habib *et al.* (1998) demonstrated that structurally unrelated products of two genes, one of which is embedded within the other, may fulfil closely related functions. Large deletions that encompass multiple *NF1* exons and the embedded genes exhibit no apparent genotype-phenotype correlation associated with the inactivation of the *NF1* gene coupled with the loss of any of the embedded genes. Therefore, haploinsufficiency of these genes is not apparently a requirement for any of the characteristic features of NF1.

Figure 1.1 The *NF1* gene
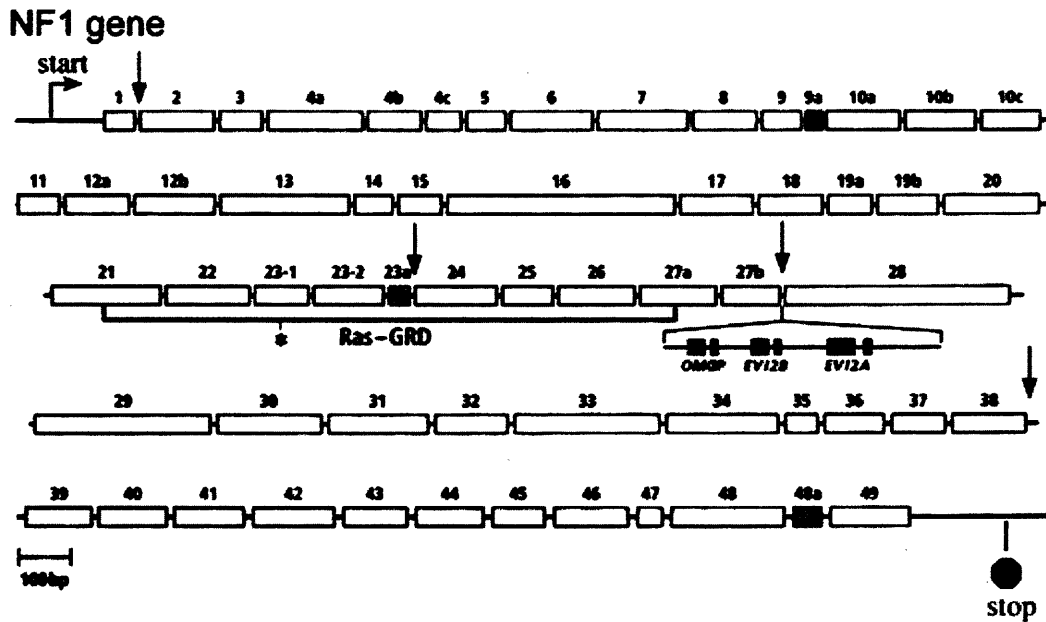
Figure 1.1. Schematic presentation of the *NF1* gene. This illustration of the *NF1* gene presents all the exons of the *NF1* gene, and the four large introns are marked with arrows. The *OMGP*, *EVI2A* and *EVI2B* genes in intron 27 are marked. The diagram is modified from Friedman & Riccardi (1999).

1.2.3 Gene expression

1.2.3.1 Tissue distribution

The *NF1* gene is ubiquitously expressed, as one would perhaps expect from the diverse clinical manifestations of the disorder (DeClue *et al.*, 1991, Gutmann *et al.*, 1991, Daston & Ratner 1992). Analysis of *NF1* gene transcription using the sensitive method of RT-PCR has shown the neurofibromin transcript to be present in many human tissues including skin fibroblasts, spleen, brain, muscle, kidney, liver and lung as well as lymphoblastoid cell lines (Wallace *et al.*, 1990; Nishi *et al.*, 1991; Suzuki *et al.*, 1991). However, the highest level of *NF1* gene expression is seen in neural tissues (Daston *et al.*, 1992).

Using antibodies generated against fusion proteins and synthetic peptides, a unique 230-320 kDa protein, which was expressed in a variety of tissues including HeLa cells,

NIH3T3 cells, spinal cord, brain, neuroblastoma, and PC12 phaeochromocytoma cell lines, was identified (Daston *et al.*, 1992; DeClue *et al.*, 1991; Gutmann *et al.*, 1991; Hattori *et al.*, 1991; The *et al.*, 1993). The difference between the predicted (327 kDa) and the observed molecular weight (~ 250 kDa) is probably due to migratory properties through denaturing polyacrylamide gels, rather than post-translational modifications (Gutmann & Collins, 1992). Immunostaining of rat tissue sections indicates that the expression of neurofibromin is most abundant in the nervous system. Subsequent analysis by Western blotting, immunoprecipitation, and immunohistochemistry demonstrated that the highest levels of neurofibromin expression were in the brain, spleen, kidney, testis, and thymus (Daston *et al.*, 1992). Neurofibromin is highly expressed in the dendritic processes of central nervous system neurons, non-myelilinating Schwann cells, oligodendrocytes, and dorsal root ganglia (Daston *et al.*, 1992; Nordlund *et al.*, 1993). It is expressed at lower levels in astrocytes, microglia, and myelinating Schwann cells. Neurons, oligodendrocytes, and nonmyelinating Schwann cells contained neurofibromin, whereas astrocytes and myelinating Schwann cells do not (Daston *et al.*, 1992).

The availability of neurofibromin antibodies has enabled the identification and subcellular localisation of the protein. Immunohistochemical staining has demonstrated the location of neurofibromin in the cytoplasm of cells from various tissues (Daston *et al.*, 1992), together with colocalisation of neurofibromin with cytoplasmic microtubules (Gregory *et al.*, 1993). The various studies of subcellular localisation of neurofibromin clearly show there are cell type-specific differences. However, how neurofibromin localisation is regulated and its functional significance is still unknown.

1.2.3.2 Alternative transcript expression

There are three major alternatively spliced exons of the *NF1* gene, but also many other splice variants with low expression levels (Gutmann *et al.*, 1995, Gutmann *et al.*, 1999, Vandenbroucke *et al.*, 2002a). Alternatively spliced *NF1* mRNA isoforms are presented in Table 1.4. The initial *NF1* transcript found is termed type I, and contains 57 exons which code for 2818 amino acids. The most commonly expressed alternative isoform is the type II which contains an additional 63-bp insertion (exon 23a) that encodes 21 amino acids in the GRD of *NF1* mRNA (Marchuk *et al.*, 1991; Nishi *et al.*, 1991). The isoform type II has been shown to display decreased Ras-GAP activity (Andersen *et al.*,

1993a). Two other common alternatively spliced exons are 9a (9br) (30bp) and 48a (TypeIII) (54bp) (Figure 1.2). The isoform containing exon 48a is highly expressed in muscle tissues (Gutmann *et al.,* 1995), and expression of the 9a-containing isoform is seen during embryonic development of the brain (Gutmann *et al.,* 1999).

The specific expression patterns of both the isoform type I and type II have been studied in several organs and cells (Bernards, 1995; Metheny & Skuse, 1996) and has provided the basis for implicating the differential expression of *NF1* type I and type II transcripts in the regulation of neuronal differentiation and development. Recent studies using *Nf1* gene targeting have reported that *Drosophila* homozygotes with *Nf1* null mutation showed significantly decreased olfactory learning performance (Guo *et al.,* 2000), *Nf1* heterozygous mice displayed spatial learning disability (Silva *et al.,* 1997; Costa *et al.,* 2002), and mice lacking alternatively spliced *Nf1* exon 23a exhibited specific learning impairment (Costa *et al.,* 2001). Furthermore, abnormal Ras activity in *Nf1* knockout mice disrupted learning and memory, indicating that functional modulation of Ras by neurofibromin is essential for learning and memory (Costa *et al.,* 2002). Yunoue *et al.,* (2003) postulated that neurofibromin plays a key role in the Ras signal-dependent pathway as a GAP in neuronal cells and that functional regulation of neurofibromin, such as alternative splicing, could be involved in the neuronal development that may be implicated in the learning disability of NF1 patients.

Table 1.4: *NF1* alternative transcripts

Transcript name	Alternative exon include	Tissues in which it is expressed	Consequence in neurofibromin	Does it affect the GRD?	Species
9br	9br	CNS only, reduced expression in brain tumours	Addition of 10 amino acids	No	Human, mouse
Type II	23a	All, increased reduced expression in brain tumours	Addition of 21 amino acids	Yes	Human, mouse, rat
Type III (rodent)	23a and 23b	Adrenal glands, kidney, ovaries	Introduction of frame shift	Yes	Mouse, rat
Type IV (rodent)	23b	Testis	Introduction of frame shift	Yes	Mouse
Type 3	48a	Fetal and adult cardiac and skeletal muscle	Addition of 18 amino acids	No	Human, mouse, rat
Type 4	23a and 48a	Fetal and adult cardiac and skeletal muscle	Addition of 21 amino acids in GRD and 18 at carboxy terminus	Yes	Human, mouse, rat
N-Isoform	Excludes exons 11-most of 49	Normal brain and brain tumours	Excludes amino acids 548-2815	No	Human

Adapted from Skuse & Cappione (1997)

1.2.3.3 mRNA editing

Intranuclear modification of pre-mRNA involves alternative splicing and alternative polyadenylation site selection that results in cells generating RNA sequence diversity. Another mechanism responsible for RNA diversification is *mRNA editing* where the sequence is altered through post-transcriptional modification of a purine or pyrimidine. *NF1* messenger RNA editing occurs at position 3916 in exon 23-1 (Skuse *et al.*, 1996). This relatively rare event changes a C to U in the messenger RNA by deamination, resulting in a nonsense codon that predicts premature truncation. This editing is performed by apobec-1, which is a peptide that edits the apoliprotein B messenger RNA in the human gastrointestinal tract (Mukhopadhyay *et al.*, 2002). *NF1* mRNA may undergo base-modification editing (Skuse *et al.* 1996, Cappione *et al.*, 1997, Skuse & Cappione 1997, Gott & Emeson 2000). The base-modification editing of NF1 mRNA changes the cytidine in the arginine coding codon (*C*GA) to uridine (*U*GA) and creates an in-frame translational stop codon within the first half of the GRD (Skuse *et al.*, 1996, Cappione *et al.*, 1997). The *NF1* mRNA editing generates a truncated *NF1* mRNA, which contains only the N-terminal part of *NF1* GRD without activity toward the Ras proteins, or possibly lead to unstable mRNA. *NF1* mRNA editing has been suggested to play a role in the tumorigenesis of NF1 patients and appears to result in the functional equivalent of biallelic inactivation. *NF1* mRNA editing has been shown to occur more frequently in tumours as compared to normal tissues (Skuse *et al.*, 1996, Cappione *et al.*, 1997). This suggests that it may play a role in the development of tumour progression.

Figure 1.2 The *NF1* MRNA and the *NF1* protein
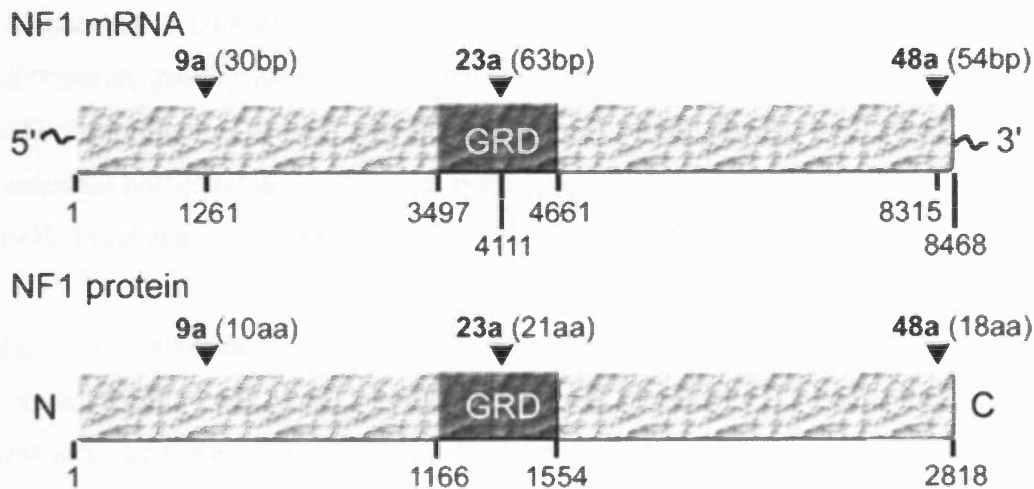
Figure 1.2. The illustrations of *NF1* mRNA and protein present the alternatively spliced exons (9a, 23a and 48a) and the GAP-related domain (Gutmann & Collins 1993, Skuse & Cappione 1997). aa, amino acid; bp, base-pair; GRD, GAP-related domain.

1.2.4 Function of neurofibromin

1.2.4.1 GTPase activating protein-related domain (GRD)

The *NF1* gene product, termed neurofibromin, is a 2818 amino acid cytosolic protein known to have an influence on guanosine nucleotide metabolism and Ras oncoprotein function (Cawthon *et al.*, 1990a). Analysis of neurofibromin revealed striking similarity between a small central portion of the protein, known as the GRD (GAP-related domain), and members of the guanosine triphosphatase (GTPase)-activating protein (GAP) family, including mammalian p120-GAP, *yeast ira1* and *ira2*, and *Drosophila* Gap1 (Ballester *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990).

GTPase activating proteins bind and inactivate Ras by enhancing the low intrinsic GTPase activity of Ras proteins (Bollag & McCormick 1992, Bassell & Singer 1993, Zang *et al.*, 1998, Sherman *et al.*, 2000, Cichowski & Jacks 2001, Gutmann 2001). The superfamily of Ras-proteins are essential components of receptor-mediated signal

24

transduction pathways (Bassell & Singer 1993, reviewed by Schlessinger 2000, Adjei 2001a). Ras proteins are linked to plasma membrane through a farnesyl residue (Prendergast & Oliff 2000, Adjei 2001, Volkert *et al.*, 2001). Upon receptor stimulation, guanine nucleotide-exchange factor (GEF) changes GDP-bound Ras in Ras-GTP form (Figure 1.3). The activated Ras utilizes several downstream effectors, of which the best characterized is the Ras-raf-MEK-ERK kinase cascade (Warne *et al.*, 1993, Friedman *et al.*, 1994, Marshall 1995).

The first mammalian GAP identified was p120GAP (Trahey and McCormick, 1987). Its primary function was to catalyse the hydrolysis of the active GTP-bound form of the Ras proteins to the inactive GDP-bound form. However, it has been shown that the interaction of neurofibromin with Ras displays kinetic and thermodynamic characteristics that are very different from that of p120GAP. This reflects different physiological requirements for the interaction, as demonstrated by the different localization of the two RasGAPs, the different phenotypes of gene knockouts in mice (Brannan *et al.*, 1994; Jacks *et al.*, 1994; Henkemeyer *et al.*, 1995) and *Drosophila* (Gaul *et al.*, 1992; The *et al.*, 1997), and the fact that neurofibromin but not p120GAP acts as a tumour suppressor in humans.

1.2.4.2 NF1-GRD/Ras

The NF1-GRD spans residues 1172-1538 of the 2818 amino acid protein, representing only 10% of the protein sequence (Figure 1.4). Neurofibromin has been shown to function as a GAP for Ras *in vitro* and *in vivo* (Ballester *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990). Ras has been implicated in the control of cell growth and differentiation, and the ability of neurofibromin to down-regulate p21-ras suggests that its absence may lead to uncontrolled cell growth or tumour formation. This has been supported by the identification of many *NF1*-deficient tumours exhibiting elevated levels of Ras-GTP (Basu *et al.*, 1992; DeClue *et al.*, 1992; Bollag *et al.*, 1996; Hiatt *et al.*, 2001). Loss of neurofibromin in a wide variety of both human tumour and *Nf1*-deficient mouse cells is associated with increased Ras activity and Ras effector activation (Bajenaru *et al.*, 2001; Sherman *et al.*, 2000). The consequences of Ras activation are well described, and mutations or amplifications of many genes encoding proteins active in the Ras pathway have been detected in a variety of human tumours. In some *NF1*-deficient cells and tumours, the inappropriate activation of the downstream

25

Ras effectors has also been reported (Mahgoub *et al.*, 1999; Hiatt *et al.*, 2000; Ingram *et al.*, 2000; Lau *et al.*, 2000). Furthermore, single point mutations, affecting Ras-GAP activity or Ras binding, have been detected in NF1 patients, indicating that inactivation of this activity results in the manifestation of the disease (Upadhyaya & Cooper, 1998). In addition, a subset of myeloid leukaemias appear to arise as a result of either *NF1* mutations or *NRAS* mutations, implying that deregulation of this pathway is a critical step in their development (Upadhyaya & Cooper, 1998). More recently, it has also been shown that learning deficiencies associated with NF1 may be caused by excessive Ras activity, which leads to impairment in the long-term potentiation caused by increased GABA-mediated inhibition (Costa *et al.*, 2002).

Figure 1.3: GTPase-activating proteins
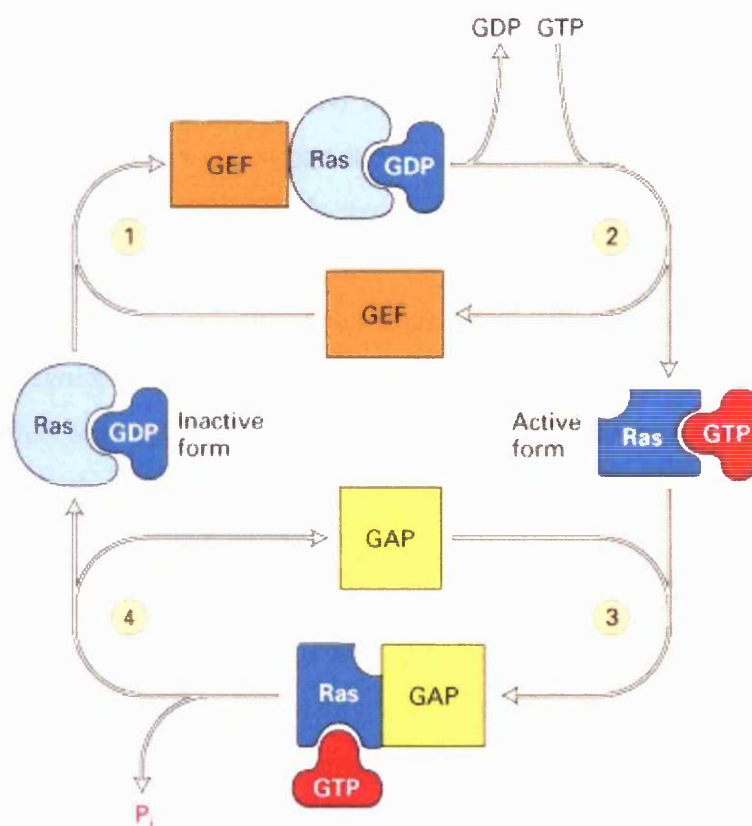
Figure1.3. Schematic representation of GTPase-activating proteins mechanism. **1**, stimulated GEF binds the inactive form GDP-Ras. **2**, the inactive form GDP- Ras to the active form GTP-Ras. **3**, GAP binds GTP-bound Ras. **4**, the active GTP-Ras to the inactive GDP-Ras.

26

1.2.4.3 Non Ras-GAP function

Neurofibromin has several biochemical functions and is expressed in a variety of different cell populations. It is involved in cell signalling and regulation of proliferation during normal development and differentiation, as well as during tumour progression. The fundamental physiological importance of neurofibromin is underscored by the observation that mice with a targeted disruption of the neurofibromin locus are embryonically lethal and show abnormalities of neural crest-derived tissues (Brannan *et al.*, 1994; Jacks *et al.*, 1994).

Based on the presence of a 300 amino acid residue Ras GTPase-activating protein (GAP)-related domain (NF1GRD), neurofibromin growth regulation has been hypothesized to reflect its ability to function as a Ras-GAP (Ballester *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990). Whereas complete loss of *NF1* has been clearly implicated in the development of some NF1-related tumours (myeloid leukaemia, pheochromocytoma), haploinsufficiency due to reduced dosage of the *NF1* gene is thought to underlie the development of other symptoms such as cognitive deficit, bone deformities, and peripheral nerve sheath tumours (Cichowski and Jacks 2001; Zhu *et al.*, 2002). Although it is tempting to attribute all of neurofibromin growth regulatory function to Ras regulation, the NF1GRD represents only 10% of the entire molecule. Neurofibromin has been also suggested to regulate the cAMP-PKA pathway, which in turn downregulates the activity of Ras-Raf-MAPK signaling cascade (Guo *et al.*, 1997; The *et al.*, 1997; Fieber 1998; Kim *et al.*, 2001).

A region comprising exons 11-17 of the *NF1* gene has been shown to contain homology to ATP binding- and cAMP-dependent protein kinase (PKA) recognition sequences (Fahsold *et al.*, 2000). This region upstream of the GRD region, is a C-terminal cysteine/serine-rich domain (CSRD) of the *NF1* gene. A cAMP-dependent protein kinase A (PKA) specifically phosphorylates the CSRD of NF1. The biological significance of the phosphorylation of neurofibromin by PKA is still unclear, but studies in *Drosophila* have suggested that neurofibromin also has non-Ras-GAP functions that involve regulation of intracellular cAMP (Guo *et al.*, 1997). There is evidence for the involvement of neurofibromin in the cAMP-mediated signalling pathway of growth

27

control in *Drosophila*. The control of cell growth through this second pathway may explain the Ras-independent tumour suppressor activity attributed to the *NF1* gene in neuroblastoma and melanoma cell lines. Furthermore, Waschek (2002) has shown that *Drosophila Nf1* is required for activation of adenylyl cyclase by the neuropeptide, pituitary adenylate cyclase activating peptide (PACAP), which potently increases cAMP levels in pituitary cells. Moreover, the small size defect of *Nf1*-deficient *Drosophila* was rescued by overexpression of activated PKA, but not by manipulating Ras signalling (The *et al.*, 1997). The rescue by PKA and not Ras supports the notion that *Drosophila* neurofibromin regulates cAMP signaling and that some of the phenotypes in *Drosophila* that result from *Nf1* inactivation are Ras-independent. Similarly, neurofibromin also regulates G-protein stimulated adenylyl cyclase activity in mammalian neurons (Tong *et al.*, 2002). In addition, Corall *et al.* (2003) showed that NF1-GRD cooperates with Ras in the anchorage-independent growth capacity of Ras-expressing fibroblasts, without affecting their ability to grow in low serum, their cellular adhesion capability, or the expression of key proteins involved in cell-cell and cell-matrix interactions. On the other hand, NF1 overexpression induces an increase in the expression levels of the focal adhesion kinase (FAK), and specific changes in the activation status of the mitogen-activated protein kinases (MAPKs). These results suggest the existence of a Ras-independent NF1-dependent pathway able to modify the levels of expression of FAK and the levels of activation of MAPKs. Since FAK, and many proteins recently found to bind *NF1*, have a role in the cytoskeleton, this pathway may involve rearrangement of cytoskeletal components that facilitate anchorage independence. Dasgupta *et al.* (2003) demonstrated that a dose-dependent impairment in cAMP generation in *Nf1* mutant astrocytes, with *Nf1*$^{-/-}$and *Nf1*$^{-/-}$ exhibited graded reductions in intracellular cAMP levels in response to PACAP.

In addition, adjacent to the GRD domain (1538-1707) lies a putative domain that shows homology to a lipid binding domain of the *S. cerevisiae* phosphatidylinositol transfer protein Sec 14p (Aravind *et al*, 1999; Bonneau *et al*, 2004) (Figure 1.4). *In vitro* studies indicate that this protein mediates the exchange of phosphatidylinositol and phosphatidylcholine between membrane bilayers. This domain has also been identified in some Rho GAPS and GEFs, suggesting therefore a possible link between the binding of lipids by these proteins and the regulation of Ras.

Structural and biochemical data have shown that the most important function of GAP is to supply an "arginine finger" inserted into the active site of Ras to stabilise the

28

transition state of the GTPase reaction (Ahmadian *et al*, 1997; Resat *et al.*, 2001; Scheffzek *et al.*, 1997). Klose *et al* (1998) identified a R1276P missense mutation in a family with classical NF1 phenotype. This mutation slightly reduced the binding affinity to Ras but resulted in an 8000 fold reduction of the GAP-stimulated GTP hydrolysis lending further evidence that R1276 represents the arginine finger for neurofibromin GRD.

There is increasing evidence that loss of *NF1* expression in neoplastic Schwann cells is associated with elevated levels of activated Ras, supporting the notion that neurofibromin acts as a growth regulator by inhibiting Ras growth-promoting activity. In addition, there is increasing evidence that other cooperating events, which may be under cytokine modulation, are important for neurofibroma development and growth.

Figure 1.4 Structure of neurofibromin
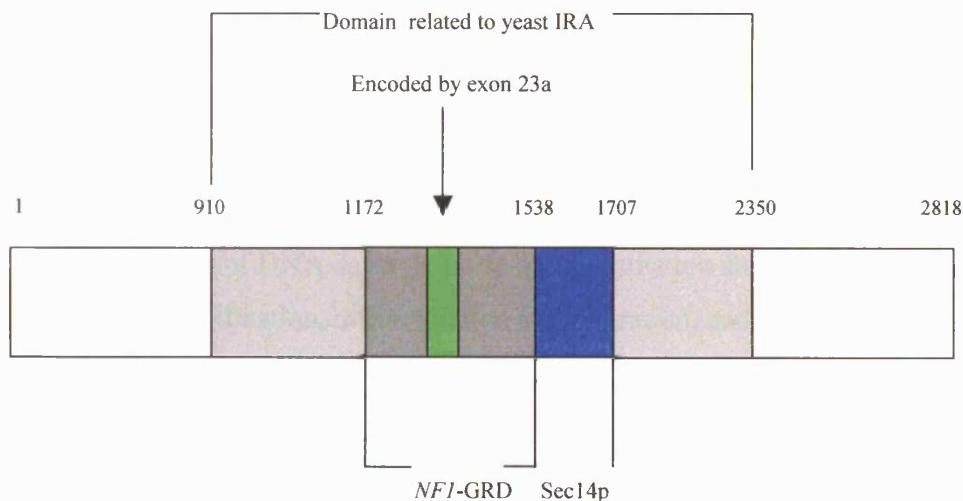
Figure 1.4: The *NF1* gene encodes a 2818 amino acid protein, neurofibromin, which contains a ~300 residue central region, termed the NF1-GRD. The region of neurofibromin containing residues 910-2350 shares sequence similarity with regions of yeast IRA proteins. The sequence encoded by the alternatively spliced exon 23a is contained within the NF1-GRD. The region of neurofibromin containing residues 1538-1707 shows homology to the yeast Sec14p lipid exchange protein. (Adapted from Dasgupta & Gutmann, 2002).

revealing similarity, albeit distant, to the G-domain fold of Ras (Milburn *et al.*, 1990; Pai *et al.*, 1990). This supports the observation that Ras and tubulin share common binding sites on their neurofibromin target. Mutations of neurofibromin critical for the interaction with Ras have indeed been shown to affect the interaction with cytoplasmic microtubules as well (Xu and Gutmann, 1997).

Neurofibromin has also been reported to be associated to be associated with syndecan (Hsueh et al., 2001).

1.2.4.5 Tumour suppression

Tumour suppressor genes are characterized by loss-of-function mutations. The protein products of tumour suppressor genes often inhibit cell proliferation or alternatively participate in the process of programmed cell death known as apoptosis that is designed to protect the organism from the consequences of proliferation of irreparably damaged cells. Thus, loss of a tumour suppressor gene may also increase the rate of cellular proliferation. Tumour suppressor genes are however involved in the regulation of a diverse array of different cellular functions including cell cycle checkpoint control, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signalling, cell specification, differentiation and migration, and tumour angiogenesis (Sherr, 2004). Mutational inactivation of both tumour suppressor alleles is required to change the phenotype of the cell (Levine, 1993). This 'two hit' hypothesis, originally proposed by Knudson (1971), provides the basis for a mechanistic understanding of tumour suppressor gene mutagenesis. Following the Knudson hypothesis, during tumorigenesis one allele of the tumour suppressor gene is inactivated by a germline mutation and the second allele by a subsequent somatic mutation in the specific tumour tissue.

Patients with NF1 develop multiple benign nerve sheath tumours and are predisposed to a number of malignancies. Since loss-of-function mutations in the *NF1* gene appear to

be responsible for the disease, *NF1* has been hypothesized to act as a tumour suppressor (Cichowski *et al.*, 1996). Genetic and biochemical data support the hypothesis that *NF1* acts as a tumour suppressor gene. In fact, molecular analysis of a number of NF1-associated peripheral nerve sheath tumours has demonstrated the inactivation of both *NF1* alleles in accordance with Knudson's 'two hit' hypothesis (Serra *et al.*, 1997). The involvement of the *NF1* gene in neuroblastoma (NB) (MIM 256700) was suggested by Weiss *et al.* (1997) who hypothesized that the loss of the *NF1* gene can have a role in the tumour development of transgenic mice overexpressing *MYCN*. Loss of *Nf1* function might therefore keep Ras in an active (GTP-bound) form resulting in a restrained mitogenic signal and subsequent cell division and proliferation. The GAP activity of neurofibromin and the identification of mutations in *NF1* alleles in malignant tumours associated with NF1 and in benign neurofibromas have led to the classification of the *NF1* gene as a tumour suppressor gene.

In keeping with Knudson's 'two-hit' hypothesis, both copies of the *NF1* gene are inactivated in benign and malignant tumours from NF1 patients (Legius *et al.*,1993; Side *et al.*, 1997; Sawada *et al.*, 1996; Eisenbarth *et al.*, 2000; John *et al.*, 2000; Serra *et al.*, 2000, 2001), while loss of heterozygosity (LOH), due to large somatic rearrangements and deletions, has been shown to be associated with the *NF1* allele that does not segregate with the disease (Serra *et al.*, 1997). In addition, *Nf1+/-* mice are predisposed to tumour formation and frequently develop malignant pheochromocytomas and myeloid leukaemia (Jacks *et al.*, 1994).

Identification of somatic mutations in the *NF1* gene, from several NF1- and non–NF1-related tumours is consistent with *NF1* being a tumour suppressor gene (Li *et al.*, 1992; Xu *et al.*, 1992; Andersen *et al.*, 1993a; Colman *et al.*, 1995; Legius *et al.*, 1993; The *et al.*, 1993; Shannon *et al.*, 1994; Sawada *et al.*, 1996; Serra *et al.*, 1997; Zhu & Parada, 2001).

Several studies have reported LOH of the *NF1* gene in both dermal and plexiform neurofibromas (Colman *et al.*, 1995; Daschner *et al.*, 1997; Serra *et al.*, 2000; John *et al.*, 2000; Rasmussen *et al.*, 2000; Upadhyaya *et al.*, 2004). In addition, somatic *NF1* point mutations have been described in neurofibromas (Sawada *et al.*, 1996; Eisenbarth *et al.*, 2000; John *et al.*, 2000).

31

Furthermore, the *NF1* gene is classified as a histogenesis control gene, since many benign dysplasias occur in the NF1 syndrome, and normal NF1 protein function is needed during tissue repair (e.g. wound healing and angiogenesis) and embryonic development (Sheela *et al.*, 1990; Brannan *et al.*, 1994; Lakkis & Epstein 1998; Atit *et al.*, 1999; Mashour *et al.*, 1999; Riccardi 2000, Riccardi 2001).

The mechanism leading to the formation of neurofibromas remain unclear although it has been presumed that the cellular defect in neurofibromas results from abnormal Schwann cell function, secondary to loss of neurofibromin function. Recently, the identification of growth hormone receptor (GHR) expression in localised neurofibromas of NF1 patients suggested that growth hormone (GH) may play some role in the development of neurofibromas (Cunha *et al.*, 2003). It is possible that neurofibroma cells have increased Ras-GTP concentrations because of the action of GH, which leads to an increase in cell number. This could also explain why during adolescence there is an increase in the size of neurofibromas.

The malignant MPNST tumours in NF1 patients often develop from benign primary plexiform neurofibromas. It has therefore been hypothesised that additional events are involved in the progression from benign tumours to malignancy. One leading candidate was the *p53* tumour suppressor (*TP53*) gene located on chromosome 17p. As soon as mutations in the *p53* tumour suppressor genes were detected in human MPNSTs, mice carrying heterozygous mutations in both *Nf1* and *p53* located *in cis* were generated. These *Nf1+/-* ; p53+/- *cis* mice specifically developed MPNST's at a high frequency. Both the wild-type *Nf1* and *p53* alleles were lost in all MPNST's examined. These results demonstrate those homozygous mutations in the *NF1* and *p53* tumour suppressor genes cooperate in the development of MPNST's.

While it is clear that neurofibromin acts as a Ras-GAP protein with tumour suppressor function, it is not known how *NF1* gene mutations cause non-tumour-related manifestations such as cognitive deficit. Several studies have focussed on the role of neurofibromin in brain function. The identification of CNS neuron-specific NF1 isoform supports the hypothesis that neurofibromin has brain-specific functions that might relate to the high incidence of cognitive deficit in NF1 individuals. The finding of

similar learning disabilities in *Nf1* heterozygous mice and humans suggest that the reduction of neurofibromin expression in brain cells could be responsible for the development of learning disabilities in humans.

Figure 1.5: <u>Function of neurofibromin</u>
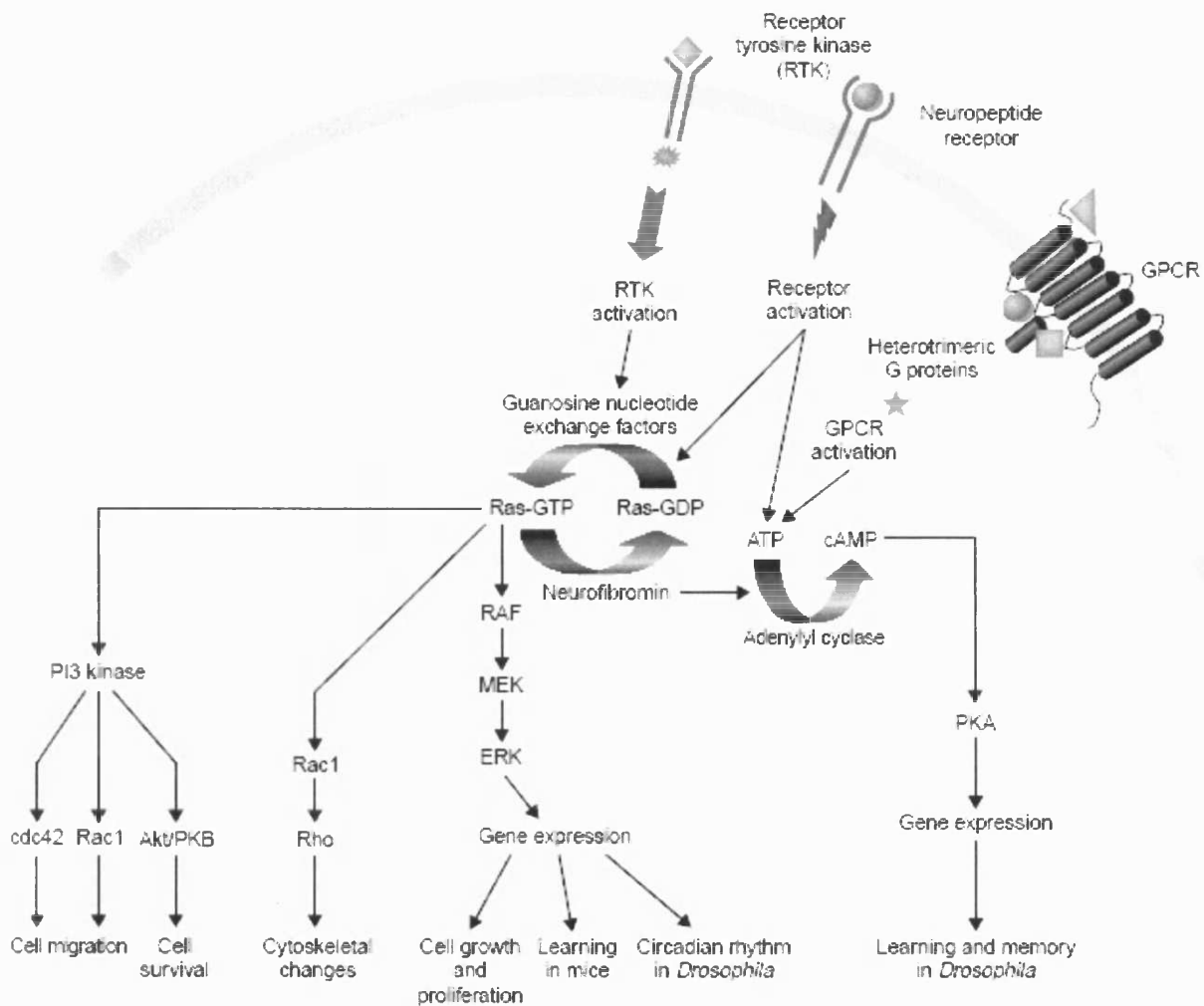
Figure 1.5: Neurofibromin accelerates inactivation of active GTP-bound RAS, such that NF1 loss in specific cells results in increased RAS activity and dysregulated cell growth. In addition, neurofibromin may also be required for cAMP-and protein kinase A (PKA)-mediated gene transcription (derived from Dasgupta & Gutmann, 2002).

neurofibromin expression, functional disruption of neurofibromin is potentially releva

to the expression of some or all of the multiple abnormalities that occur in NF1 patien

(Viskochil *et al.*, 1990).

Feng *et al.* (2004) identified a novel cellular neurofibromin-associating protein, 14-3-

which belongs to a highly conserved family of proteins that regulate intracellular sign

transduction events in all eukaryotic cells. The interaction of 14-3-3 is mainly directe

to the C-terminal domain (CTD) of neurofibromin, and the cAMP-dependent PKA-

dependent phosphorylation clustered on CTD-Ser (2576, 2578, 2580, 2813) and Thr

(2556) is required for the interaction. Interestingly, the increased phosphorylation and

association of 14-3-3 negatively regulates the function of neurofibromin. These findin

indicate that PKA phosphorylation followed by 14-3-3 protein interaction may

modulate the biochemical and biological functions of neurofibromin.

1.2.6 The NF1 gene role in benign and malignant tumours

NF1 predisposes patients to the formation of complex benign peripheral nerve tumour

called neurofibromas, in which many Schwann cells have lost contact with axons. At

least 95% of NF1 patients exhibit neurofibromas, and approximately 4% of NF1

patients develop lethal MPNSTs. Schwann cells are thought to be the primary

tumorigenic cell type, as Schwann cells in neurofibromas (Kluwe *et al.*, 1999; Serra *e

al.*, 2000) and MPNST (Legius *et al.*, 1993) lose both copies of the *NF1* gene,

consistent with the hypothesis that *NF1* acts as a tumour suppressor gene.

Although loss of *NF1* function in Schwann cells is associated with neurofibroma

formation, there is abundant evidence that additional cooperating events may be

important for neurofibroma genesis and growth. These changes include increased

expression of growth factors and growth factor receptors, including epidermal and platelet-derived growth factor receptors (DeClue *et al.*, 2000) and vascular endothelial growth factor (Arbiser *et al.*, 1998), which may promote new blood vessel formation. In addition, neurofibroma-derived Schwann cells can invade chick allantoic membranes and survive as explants in rat sciatic nerve (Sheela *et al.*, 1990).

As benign plexiform neurofibromas can transform into MPNSTs, studies have focused on identifying cooperating genetic events that might be associated with malignant transformation. Functional inactivation of several key cell cycle regulators, including p53, p27-Kip1, and p16, have been identified in MPNST as compared with their benign neurofibroma counterparts (Birindelli *et al.*, 2001; Liapis *et al.*, 1999; Nielsen *et al.*, 1999; Kourea *et al.*, 1999). Loss of the function or expression of these cell cycle regulators results in increased cell proliferation and might permit the accumulation of additional genetic mutations important for malignant transformation. In support of the notion that alterations in cell cycle growth regulators are critical for MPNST formation, two groups have demonstrated that mice with targeted mutations in the *NF1* and p53 genes develop MPNST when both the *NF1* and p53 genes are inactivated (Cichowski *et al.*, 1999; Vogel *et al.*, 1999).

1.2.7 NF1 pseudogenes

The presence of many *NF1*-related loci within the human genome has revealed a substantial problem during the *NF1* gene analysis. Such sequences represent non-functional pseudogenes, which can arise from the duplication and mutational inactivation of a gene, or by retrotransposition of the corresponding mRNA. The distribution of the *NF1* pseudogenes throughout the genome (Purandare *et al.*, 1995; Hulsebos *et al.*, 1996; Ragnier *et al.*, 1997), suggests that the centromeric region of chromosome 17 in which the *NF1* gene is located, is a target for relatively frequent interchromosomal recombination.

Sequences exhibiting >90% homology with respect to their corresponding *NF1* gene sequence have been identified on chromosomes 2, 12, 14, 15, 18, 20, 21 and 22 (Hulsebos *et al.*, 1996; Kehrer-Sawatzki *et al.*, 1997; Marchuk *et al.*, 1992; Purandare *et al.*, 1995; Suzuki *et al.*, 1994; Legius *et al.*, 1992; Gasparini *et al.*, 1993). They are

thought to have arisen >20 million years ago through partial duplication of the *NF1* gene with subsequent duplications generating new copies which were then transposed to other chromosomes (Regnier *et al.*, 1997).

1.3 Mouse Models

Animal models have shed light on the pathogenesis of neurofibromas, confirming that the Schwann cell initiates neurofibroma formation. New data suggest that individuals with neurofibromatosis type 1 have a 10% lifetime risk of developing malignant peripheral nerve sheath tumours (DeRaedt *et al.*, 2003). Such tumours associated with NF1 show a loss of NF1 expression and high levels of Ras, but malignant transformation requires additional genetic events that inactivate key cell cycle regulators.

1.3.1 Targeted disruption

Several strategies have been employed to develop a mouse model for NF1. It was found that mice with a targeted disruption of the *Nf1* gene ($Nf1^{-/-}$) have severe cardiac vessel development abnormalities and embryonic lethality between days 12.5 and 14 of gestation (Lakkis *et al.*, 1999). On the other hand, mice carrying a targeted disruption in only one allele of the *Nf1* gene ($Nf1^{+/-}$) survive and are prone to the formation of various types of tumours. They develop myeloid leukaemia and pheochromocytoma but they do not develop MPNSTs or other characteristic symptoms of human NF1 (Jacks *et al.*, 1994). The wild-type *Nf1* allele was lost in approximately half of the tumours. $Nf1^{+/-}$ mice proved to be extremely susceptible to induction of myeloid leukaemia by the alkylating agent cyclophosphamide (Mahgoub *et al.*, 1999).

Studies in heterozygous ($Nf1^{+/-}$) mice also suggest that haploinsufficiency for *Nf1* expression confers a growth advantage for astrocytes, which may account for the astrocyte growth abnormalities seen in NF1 (Bajenaru *et al.*, 2001). In addition, heterozygous $Nf1^{+/-}$ mice show spatial disabilities and memory deficits strikingly similar to the learning deficits seen in humans with NF1. However, the learning and memory deficits of $Nf1^{+/-}$ mice are not fully penetrant, they can be compensated by

36

external learning and they do not involve deficits in single associative learning (Costa *et al.*, 2002). However, experiments based on manipulations that decrease Ras function showed that the learning deficits of the $Nf1^{+/-}$ mice are due to increased Ras signaling. Deleting *Nras* gene, for example, rescued the spatial learning deficits of the $Nf1^{+/-}$ mutant mice. A K-ras heterozygous null deletion, which on its own causes spatial learning deficits, was also able to rescue the learning deficits of the $Nf1^{+/-}$ mutant mice (Costa *et al.*, 2002).

Chimeric mice partially composed of $Nf1^{-/-}$ cells by injecting $Nf1^{-/-}$ embryonic stem cells (ES) into blastocyst stage embryos were generated (Cichowski *et al.*, 1999). A large percentage of $Nf1^{-/-}$:$Nf1^{+/+}$ chimeric embryos died during gestation, due to the extensive contribution of the *Nf1*-deficient cells to the developing heart. All chimeras that survived to adulthood developed multiple neurofibromas usually in the dorsal root ganglia of peripheral nerves in the limbs. These results indicate that complete loss of *Nf1* is an obligatory step in neurofibroma development and suggest that the presence of neurofibromin-deficient cells in the developing nerves is a necessary factor in the oncogenesis of these tumours in mice.

The *NF1* gene has multiple alternatively spliced forms and this diversity has been analyzed in both learning and memory studies. For example, an isoform containing exon23a (*Nf1* type II) has increased affinity for Ras, but decreased GAP activity, thereby competing with the type I isoform and thus decreasing the conversion of Ras into its GDP-bound state. Analysis of the $Nf1$ 23a$^{-/-}$ mice showed normal life expectancy, normal levels of *Nf1* type I, seemingly normal development and no hint of increased tumour predisposition similar to the $Nf1^{+/-}$ heterozygotes. They do however display clear hippocampal-dependent learning deficits. These mice showed impairment in both the water maze and in contextual discrimination, which can be partially rescued with extended training (Silva *et al.*, 1997).

Another approach to studying NF1 tumour formation involves the generation of mice heterozygous for mutations in both *Nf1* and other relevant tumour suppressor genes. Chimeric mice composed in part of $Nf1^{-/-}$ cells did develop neurofibromas, and in a second study in double knockout mice with linked germline mutations in both *Nf1* and *p53*, the sarcomas that developed in the mice were identified as MPNSTs (Cichowski *et*

al., 1999). On the basis of genetic studies of NF1-associated MPNSTs implicating the p53 tumour suppressor, $Nf1^{+/-}$; $p53^{+/-}$ mice have been generated (Vogel *et al.*, 1999). These double heterozygote mice developed high-grade sarcomas with histopathological features of human MPNSTs, demonstrating that the loss of both neurofibromin and p53 cooperate to facilitate MPNST formation. Reilly *et al.* (2000), presented a mouse model of astrocytoma involving mutations in both the *Nf1* and *p53* genes. Humans with neurofibromatosis type 1 have an increased risk of optic gliomas, astrocytomas, and glioblastomas. The *TP53* tumour suppressor is often mutated in a subset of astrocytomas that develop at a young age and progress slowly to glioblastoma. This mouse model shows a range of astrocytoma stages, from low-grade astrocytoma to glioblastoma multiforme, and may accurately model human secondary glioblastomas involving *p53* loss.

1.3.2 Genetically engineered mice

With the generation of genetically engineered mice that bear conditional *Nf1* alleles ($Nf1^{flox/flox}$ mice), specific cell types could be rendered neurofibromin-deficient by Cre-mediated excision (Zhu *et al.*, 2001). Bajenaru *et al.* (2003), developing $Nf1^{+/-}$ (GFAPCre; $Nf1^{flox/mut}$) mice lacking neurofibromin expression in astrocytes, demonstrated that these mice develop optic nerve and chiasm low-grade fibrillary astrocytomas. However, formation of low-grade astrocytomas seems to require an interaction between $Nf1^{-/-}$ astrocytes and the surrounding $Nf1^{+/-}$ brain (Bajenaru *et al.*, 2003). Subsequent studies on (GFAPCre; $Nf1^{flox/mut}$) mice revealed that the maximal level of tumour proliferation resulted between 3 and 8 weeks of age, suggesting that these tumours arise early during a period of rapid astroglial cell proliferation (Bajenaru *et al.*, 2005). In addition, infiltrating activated microglia and new blood vessel formation were observed in those mice at 3 weeks of age, supporting a role for $Nf1^{+/-}$ cells in optic pathway glioma (OPG) tumorigenesis.

Recently, studies on murine $Nf1^{-/-}$ astrocytes have shown that in the absence of neurofibromin, KRAS is the preferential activated RAS isoform and that the activation of KRAS accounts for the proliferative advantage and abnormal actin cytoskeleton-mediated processes observed in $Nf1^{-/-}$ astrocytes *in vitro* (Dasgupta *et al.*, 2005).

A similar mechanism to the formation of low-grade astrocytomas, has been shown to operate in neurofibroma development. By crossing $Nf1^{flox/flox}$ mice lacking neurofibromin expression in Schwann cells with transgenic mice ($Nf1^{flox/flox}$ mice; KROX20-Cre) in which the Krox 20 Cre promoter induces somatic inactivation of $Nf1$ only in Schwann cells, it has been shown that mice did not develop neurofibromas. However, when $Nf1^{flox/flox}$ mice were crossed onto an $Nf1^{+/-}$ background such that all non-Schwann cell lineages were haploinsufficient for $Nf1$, the animals developed neurofibromas associated with dense mast cell infiltrations (Zhu et al., 2002). These findings indicate that tumour progression requires both the loss of NF1 in Schwann cells and the haploinsufficiency of NF1 in lineages within the tumour microenviroment. Nf1-deficient astrocytes exhibit high levels of mammalian target of rapamycin (mTOR) pathway activation, which was inhibited by blocking K-RAS or phosphatidylinositol 3-kinase activation. This mTOR pathway hyperactivation was reflected by high levels of ribosomal S6 activation in both Nf1 mutant mouse optic nerve gliomas and in human NF1-associated pilocytic astrocytoma tumors. Moreover, inhibition of mTOR signaling in $Nf1^{-/-}$ astrocytes abrogated their growth advantage in culture thereby restoring normal proliferative rates (Dasgupta et al., 2005a).

Homozygous $Nf1$ mutant ($Nf1^{-/-}$) Schwann cells have been shown to provide a potent chemotactic stimulus for $Nf1^{+/-}$ mast cells through secretion of soluble KitL and activation of a specific Ras effector-signaling pathway (Yang et al., 2003). These studies identified a novel interaction between $Nf1^{-/-}$ Schwann cells and $Nf1^{+/-}$ mast cells that is likely to be important in neurofibroma formation.

disease mechanisms in NF1, but also on the structure and function of the gene product, neurofibromin. Mutations in the *NF1* gene were first reported in 1990 (Cawthon, 1990b Viskochil, 1990; Upadhyaya, 1990). To date, 723 different mutations in the *NF1* gene have been reported to the Human Gene Mutation Database (http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/120231.html) [Table 1.5]. The majority of *NF1* mutations lead to a truncated protein product and only about 10% involve amino acid substitutions (Rasmussen & Friedman, 2000). However, it should be noted that the types of mutations identified are largely dependent on the techniques used for mutation detection. This may result in an over-representation of mutation type: that are more easily identified, such as large gene deletions, and an under-representatio of those that may be more difficult to identify, such as mutations in the 3' untranslated region. However, none of the methods used for *NF1* mutation detection are capable of identifying all mutation types. The most frequently recurring alteration is the nonsense mutation in exon 31 (R1947X) that accounts for 1-2% of the *NF1* mutations identified (Dublin *et al.*, 1995).

A significant fraction of the mutations in the *NF1* gene cause aberrant splicing and many of them are due to alterations outside the conserved AG/GT acceptor and donor sequences and even reside deep into the large introns (Messiaen *et al.*, 2000; Ars *et al.*, 2000; Perrin *et al.*, 1996). Also, a number of exonic mutations nonsense, missense and even silent mutations at the genomic level have been described that are splicing mutations and exert their effect by creating a novel splice donor or acceptor site or by affecting the function of an exonic splicing enhancer (ESE) or exonic splicing silencer (ESS) (Messiaen *et al.*, 2000; Zatkova *et al.*, 2004)

Table 1.5: Mutation types identified in the *NF1* gene (HGMD)

Mutation type	Number of mutations
Nucleotide substitutions (missense/nonsense)	194
Nucleotide substitutions (splicing)	141
Nucleotide substitutions (regulatory)	0
Small deletions	214
Small insertions	109
Small indels	9
Gross deletions	44
Gross insertions and duplications	5
Complex rearrangemements (including inversions)	7
Repeat variation	0
Total	723

1.4.2 Mosaicism in NF1

Neurofibromatosis type 1 and type 2 both occur in mosaic forms. Germline mosaicism is thought to be rare in NF1 patients (MacCollin & Kwiatkowski, 2001). By contrast, some patients have segmental or anatomically limited signs of NF1 consistent with somatic mosaicism. Early somatic mutations cause generalized disease, clinically indistinguishable from nonmosaic forms. Later somatic mutation gives rise to localized disease often described as segmental (see 1.1.2.2 section). In individuals with mosaic or localized manifestations of neurofibromatosis type 1 (segmental neurofibromatosis type 1), disease features are limited to the affected area, which varies from a narrow strip to one quadrant and occasionally to one half of the body. Distribution is usually unilateral but can be bilateral, either in a symmetrical or asymmetrical arrangement. However, most individuals who have been reported with mosaicism for an *NF1* mutation have

41

mild, but not segmental, neurofibromatosis (Wu *et al.*, 1997; Rasmussen *et al.*, 1998). Patients with localized neurofibromatosis type 2 have disease-related tumours localized to one part of the nervous system; for example a unilateral vestibular schwannoma with ipsilateral meningiomas or multiple schwannomas in one part of the peripheral nervous system. The recognition of mosaic phenotypes is important. Individuals with the mosaic form, even with a generalized phenotype, are less likely to have severe disease. They also have lower offspring recurrence risk than individuals with the nonmosaic form. The mosaic forms of neurofibromatosis provide a good example of the effects of somatic mutation.

1.4.3. Genotype-phenotype correlations

NF1 is characterized by extreme clinical variability, not only between unrelated individuals and among affected individuals within a single family, but even within a single individual with NF1 at different times in his or her life. Some investigators interpret this variability as evidence that most complications of NF1 result from the effects of additional random events in individuals (Riccardi, 1993). Evidence in support of this interpretation is provided by the occurrence of acquired "second hit" mutations and loss of heterozygosity at the *NF1* locus in some neurofibromas (Sawada *et al.*, 1996; Serra *et al.*, 1997; Eisenbarth *et al.*, 2000; John *et al.*, 2000; Rasmussen *et al.*, 2000; Rutkowski *et al.*, 2000; Perry *et al.*, 2001; Wiest *et al.*, 2003; Upadhyaya *et al.*, 2004), malignant peripheral nerve sheath tumours (Legius *et al.*, 1993; Lothe *et al.*, 1995; Rasmussen *et al.*, 2000; Perry *et al.*, 2001; Frahm *et al.*, 2004; Upadhyaya *et al.*, 2004), pheochromocytomas (Xu *et al.*, 1992), astrocytomas (Gutmann *et al.*, 2000; 2003; Tada *et al.*, 2003; Upadhyaya *et al.*, 2004), a malignant melanoma (Ishii *et al.*, 2001), and juvenile chronic myelogenous leukaemia cells (Shannon *et al.*, 1994) from individuals with NF1.

On the other hand, consistent familial transmission of NF1 variants such as the Watson syndrome (multiple café-au-lait spots, pulmonary stenosis, and low intelligence) (Allanson *et al.*, 1991) and familial spinal neurofibromatosis (Ars *et al.*, 1998; Kaufman *et al.*, 2001; Kluwe *et al.*, 2003b) indicates that allelic heterogeneity also plays a role in determining the clinical variability of NF1. The same conclusion can be drawn from the

42

observation of similar dysmorphic features in many individuals with deletions involving the entire *NF1* gene (Kayes *et al.*, 1994; Wu *et al.*, 1995; Tonsgard *et al.*, 1997; Upadhyaya *et al.*, 1998; Riva *et al.*, 2000; Venturin *et al.*, 2004). In addition, statistical analysis of clinical features in families with NF1 suggests that modifying genes at other loci influence some aspects of the NF1 phenotype (Easton *et al.*, 1993; Szudek *et al.*, 2002; 2003). It seems likely that the clinical variability of NF1 results from a combination of genetic, non-genetic and stochastic factors. Such complexity and the diversity of constitutional *NF1* mutations that occur in this disease will continue to make the study of genotype-phenotype correlations very difficult.

1.4.4 DNA methylation

Neurofibromas are believed to develop genetically in line with the Knudson two-hit hypothesis for tumour suppressor genes: one constitutional mutation and one somatic mutation, leading to protein inactivation (Knudson, 1971). The *NF1* gene is large and is located on chromosome 17q11.2, spanning 300kb of genomic DNA (Viskochil, 2002). Somatic mutations, including point changes, insertions, deletions, and loss of heterozygosity, have been found in some NF1 tumours. However, in many neurofibromas, the somatic mutations have not been identified, raising the possibility that epigenetic alterations may be the mechanism for the "second hit." One of those possible epigenetic alterations is DNA methylation, a post-synthetic modification found primarily on cytosines within CpG dinucleotides in humans. It is believed that methylation can cause transcriptional repression in two ways. First, the methyl group itself can cause direct interference with a protein binding to its target sequence (Bird, 2002). One example of this mechanism comes from studies of the CTCF protein in imprinting of the *H19/IGF2* locus (Bell & Felsenfeld, 2000; Hark *et al.*, 2000; Szabo *et al.*, 2000; Holmgren *et al.*, 2001) where methylation on the paternal allele blocks CTCF binding to its target sequence, thereby allowing enhancer activation of the promoter region and expression of *IGF2* from the paternal allele. The second method of transcriptional repression is thought to involve methylation recruiting a transcriptional silencing complex. For example, the methylated CpG dinucleotides can recruit methyl-binding proteins, which can complex with histone deacetylases and create a closed chromatin domain (Bird, 2002). Numerous examples exist of tumour suppressor genes

being transcriptionally silenced due to hypermethylation in their promoter regions in malignant and even benign tumours (Jones & Baylin, 2002).

The *NF1* gene promoter has several putative regulatory elements surrounding the transcriptional start site including a cAMP response element (CRE), SP1, and AP2 sites (Hajra *et al.*,1994). Zou *et al.* (2003) used luciferase reporter constructs to define the minimum proximal promoter region from bp -270 to +230 relative to the transcriptional start site. Through deletion constructs and EMSA studies, they also showed that within this region the putative SP1 site at bp -138 and the CRE site at bp -8 seem to play critical roles in regulating transcription. In normal tissues, including lymphocytes, fibroblasts and placenta, the *NF1* gene promoter is methylated far upstream of the transcriptional start site but is relatively unmethylated in a region from bp -286 to +165 surrounding the transcriptional start site (Mancini *et al.*, 1999). This unmethylated region contains the CRE and SP1 sites discussed above. Methylation within these putative transcription factor binding sites disrupts the binding of proteins to these regions *in vitro* (Mancini *et al.*, 1999; Zou *et al.*, 2003). In addition, two independent studies showed this region to be unmethylated in a combined group of 14 NF1-related dermal tumours and a small number (4) of NF1-related plexiform tumours (Horan *et al.*, 2000; Luijten *et al.*, 2000). Since epigenetic methylation of the *NF1* allele would be expected to occur in only a subset of neurofibromas, a larger group of tumours would need to be analyzed to rule out the possibility of methylation as a second hit. In addition, the above studies analyzed only primary tumour tissue. Owing to the heterogeneity of the neurofibromas, it is possible that methylation events are diluted by contributions from the other cell types if they occur only in the clonal element of the tumour, the Schwann cell.

1.4.5 NF1 gene mutations

Pathological lesions that occur in the *NF1* gene provide information on both disease mechanisms and the structure and function of neurofibromin. Mutation detection in NF1 has been rendered difficult by the large size of the gene, the existence of a considerable number of homologous pseudogene sequences spread throughout the genome, and the lack of defined mutational hotspots. To overcome these problems, a variety of techniques have been employed to screen the *NF1* gene. Most studies have employed

single strand conformational polymorphism, heteroduplex analysis, temperature gradient gel electrophoresis and denaturing gradient gel electrophoresis. In the largest study to date, involving 500 patients, Fahsold et al. (2000) used the protein truncation test, temperature gradient gel electrophoresis, and direct genomic sequencing to examine all of the individual exons; these authors found a total of 301 sequence variants in the patients studied. Of these variants, 278 mutations were considered pathogenic. Ars et al. (2000), using cDNA single strand conformational polymorphism and heteroduplex analysis in 80 patients, reported a detection mutation rate of 70–80%. Messiaen et al. (2000) used the protein truncation test, fluorescence in situ hybridisation, Southern blot and cytogenetic analysis to study 67 patients, and reported a detection rate of 95%, including a high frequency of unusual splicing defects. More recently, a mutation detection rate of 89%, using automated comparative sequence analysis (ACSA) was achieved (Mattocks et al., 2004). This detection rate is the highest reported for the NF1 gene for a single technique. The sensitivity of each individual technique is hard to establish, as mutation analysis reports have either concentrated on groups of exons, included small numbers in their studies, or used a combination of techniques.

In reviews of known NF1 mutations (Upadhyaya & Cooper, 1998), several mutation types are described, but only few correlations with phenotype have been documented (Castle et al., 2003). Most of the fully characterised NF1 mutations are either nonsense or frameshift mutations, which presumably lead to premature truncation of neurofibromin. Large deletions of the NF1 gene are thought to account for fewer than 10% of cases. Most cases have a de novo deletion. The deletion breakpoints of the common 1.4 Mb deletion in NF1 patients cluster in flanking duplicated sequences termed NF1-REPs. NF1 large deletions result from an unequal crossover in maternal meiosis 1, mediated by misalignment of the flanking NF1-REPs. The NF1-repeats are direct repeats that span 100-150kb and contain several pseudogenes and four expressed sequence tags (ESTs). Recently, it was demonstrated that most of the recombination events occur in a discrete 2 kb recombination hotspot within each of these flanking NF1-REPs. The development of a deletion-specific PCR assay has facilitated the identification of a recombination hotspot for NF1 large deletions (Lopez-Correa et al., 2001).

45

1.4.5.1 Gross deletions/insertions

A deletion of 12kb of the *NF1* gene that eliminates 1201 base pairs between 31 and 39 introns has been characterized (Lazaro *et al.*, 1994; 1995). This deletion is predicted to produce a truncated protein that terminates at exon 40. Multi- exon deletions have also been reported in the *NF1* gene (Ainsworth *et al.*, 1997; Cnossen *et al.*, 1997; Lazaro *et al.*, 1993, 1996; Upadhyaya *et al.*, 1995, 1998; Valero *et al.*, 1997) but the gross deletional hotspots have not been described.

About 80% of the *NF1* large deletions are of maternal origin and have a size of 1.5 Mb. An insertion of 320bp which results in deletion of the downstream exon during splicing and consequently shifts the reading frame (Wallace *et al.*, 1991). However, gross insertions seem to be rare in the *NF1* gene.

1.4.5.2 Microdeletions/insertions

The majority of small deletions identified in the *NF1* gene generates a truncated protein product (Fahsold *et al.*, 2000), however an in-frame deletion in exon 17 has been described to cause the loss of codon Met 991 (Shen *et al.*, 1993). Microinsertions also produce a truncated protein. Large deletions and insertions have recently been reported by Fahsold *et al.* (2000). These lesions generate a shift in the reading frame that results in premature protein truncation.

1.4.5.3 Missense mutations

Missense mutations result in the generation of alternative amino acids that may alter a critical function of the neurofibromin molecule. However, functional studies outside of the GRD are not available and it is therefore difficult to ascertain what function these lesions serve. A specific GRD mutation (Arg1391Ser) has been expressed *in vitro* and analysed for GAP activity using H-*Ras* as a substrate (Upadhyaya *et al.*, 1997b). This lesion was found to be subsequently 300-fold less active than the wild-type GRD.

1.4.5.4 Nonsense mutations

The majority of *NF1* lesions result in premature truncation of the protein. These lesions sometimes lead to reduced or absent mRNA (Hoffmeyer *et al.*, 1994, 1995; Xu *et al.*, 1992). Hoffmeyer *et al.*, (1994) demonstrated the reduction in the level of mRNA expressed from one allele in six NF1 patients and also observed no mRNA reduction in a patient with a termination codon in the middle of the gene. Fahsold *et al.* (2000) also

46

observed equal allelic expression in four patients that contained premature termination codons.

1.4.6 NF1 mosaicism

NF1 germline mosaics are generally thought to be uncommon due to the infrequency of reported cases. The best characterised example of NF1 germline mosaicism was the 12 kb deletion found in only 10% of spermatoza of the clinically unaffected father (Lazaro *et al.*, 1994, 1995). Germline mosaicism therefore remains a consideration when NF1 offspring are the product of unaffected parents.

The importance and frequency of somatic mosaicism in NF1 has not been fully established but a number of cases have been reported, the most notable by Tinschert *et al.* (2000), who demonstrated that segmental NF1 was a direct result of somatic mosaicism. Other reports include the identification of a child mosaic for a complete deletion of the *NF1* gene (Wu *et al.*, 1997) and the description of a patient showing a deletion stretching from exon 4 to intron 39 (Colman *et al.*, 1996).

1.5 Mutation screening methods

The ability to detect mutations or polymorphisms in human genes is of great importance in understanding gene function and the molecular basis of human disease. Numerous techniques have been developed over the years which are able to identify sequence changes in DNA or RNA. DNA sequencing, which is usually performed to confirm and characterise changes initially found with other screening techniques, is a sensitive method for small or point mutations (Maxam & Gilbert, 1977; Sanger *et al.*, 1977). A brief description of the most important screening methods is given here.

1.5.1 Single stranded conformational polymorphism (SSCP)

SSCP, first described by Orita *et al.* (1989), represents a simple, inexpensive and sensitive method for detecting DNA sequence variations. It is based on the electrophoretic separation of single-stranded DNA (ssDNA) on a non-denaturing

polyacrylamide gel. The mobility of double-stranded DNA (dsDNA) in gel electrophoresis is size-dependent but is relatively independent of the particular nucleotide sequence. However, the mobility of ssDNA is affected by very small changes in sequence. A strand of single-stranded DNA may fold differently from another if it differs by a single base, and it is believed that mutation-induced changes of tertiary structure of the DNA results in different mobilities for the two strands. Therefore, by comparing the different electrophoretic migration rate of the mutated DNA strand with the wild-type, a mutation can be detected. In brief, SSCP analysis comprises a first denaturation step of the PCR amplified products which generates the ssDNA, the electrophoretic migration of the denaturated PCR products on a non-denaturing polyacrylamide gel at a constant temperature and finally, the detection of the DNA bands on the gel by radioactive labeling or, more often, by silver staining.

SSCP is considered a highly sensitive mutation detection method, however, since the tertiary structure of single stranded DNA changes under different physical conditions, such as temperature and ionic environment, SSCP sensitivity can be affected by these factors. In addition, the fragment length affects SSCP analysis. Mutation detection for this method results of approximately 80% for fragments shorter than 300bp (Hayashi & Yandell, 1993). However, the use of glycerol in the gel, which decreases the pH, may allow the analysis of larger DNA fragments (Kukita *et al.*, 1997).

1.5.2 Heteroduplex-based analysis

A very simple method of detecting heteroduplex DNA formation is by performing electrophoresis through a non-denaturing polyacrylamide gel. The sample undergoing heteroduplex analysis (HA) is first mixed with normal DNA, then denatured and allowed to reanneal slowly. The reformed double strands of DNA, both homo- and heteroduplexes, are then separated by gel electrophoresis. The heteroduplex DNA molecules containing either mismatched or unmatched bases, migrate slower than the homoduplex molecules of the same size (Keen *et al.*, 1991).

Only a few extensive studies have been carried out to determine the detection rate for HA. However, for single base substitutions detection values of greater than 80% have been reported (White *et al.*, 1992; Ganguly *et al.*, 1993; Chen *et al.*, 1995).

There are many other methods for detecting heteroduplex DNA formation, including chemical cleavage of mismatch (CCM) (Cotton *et al.*, 1988), enzymatic cleavage

48

methods (Shenk *et al.*, 1975; Myers *et al.*, 1985; Lu & Hsu, 1992; Youil *et al.*, 1995) and denaturing high performance liquid chromatography (DHPLC) (Underhill *et al.*, 1997).

1.5.3 Denaturing high performance liquid chromatography (DHPLC)

DHPLC is a very sensitive semi-automated method for detection of single base substitutions as well as small insertions and deletions (Oefner and Underhill, 1995, 1998, 1999; Underhill *et al.*, 1997). DHPLC analysis is based on the rapid separation of homo- and heteroduplex DNA molecules by using an ion-pair reverse phase liquid chromatography system. The HPLC column is maintained at a temperature that determines partial strand denaturation in the presence of a mismatched base-pair. The DNA samples are first PCR amplified and then the PCR products are denatured and re-annealed for the heteroduplex formation prior DHPLC analysis. DNA molecules that are negatively charged cannot bind the DNASep column which is neutral and hydrophobic. However, triethylammonium acetate (TEAA) is a positively charged reagent that facilities the interaction between the DNA and the column. Subsequently, the DNA is eluted by a gradient of acetonitrile (ACN) under partially denaturing temperatures. At these temperatures, only heteroduplexes are destabilized by the mismatched base resulting in a earlier elution compared with the homoduplexes.

1.5.4 Comparative Sequence Analysis (CSA)

Comparative Sequence Analysis (CSA) (Mattocks *et al.*, 2000) is a simple and rapid technique that allows identification as well as characterization of mutations without the need of DNA sequencing. This method involves alignment of each of the four base traces separately with a control trace. The presence of a mutation is determined by directly comparing the peak heights from a reference trace with the corresponding peaks in the sample under investigation. The resulting format allows simple and rapid visualisation of any differences between the two sets of traces. The use of this method for mutation detection yielded a mutation detection rate of 89% (Mattocks *et al.*, 2004).

49

1.5.5 Protein truncation test (PTT)

The protein truncation test (PTT) is a mutation detection method that specifically detects mutations that lead to the termination of mRNA translation and subsequently protein truncation (Roest *et al.*, 1993). The region of a gene to be analysed is amplified by PCR or RT-PCR using a primer pair that incorporates into the PCR amplicons additional sequences required for efficient cell-free translation. The amplified DNA is then added to a cell-free transcription-translation extract along with radioactive amino acids. The expressed protein is analysed by SDS-PAGE and autoradiography. Chain-truncation mutations are detected by the presence of species with lower molecular weight relative to the wild type protein. Non radioactive western blot-based PTT methods using a combination of N-terminal and C-terminal epitopes have also been reported (Rowan & Bodmer, 1997; de Koning Gans *et al*, 1999). A recent study using PTT identified aberrant polypeptide bands in 23 of 40 NF1 patient samples analysed, representing a mutation detection rate of 53% (Osborn & Upadhyaya, 1999).

The advantage of PTT is the ability to screen fragments of up to 2 kb, making it an attractive alternative to SSCP and heteroduplex analysis. PTT detects the type of sequence alteration that affects the size of the encoded protein (truncating mutation), and such mutations are common in NF1 where around 80% of alterations have been shown to result in truncated proteins (Shen *et al.*, 1996). However, one limitation of the technique is its inability to detect missense mutations, as they do not affect the size of the protein.

1.5.6 Methods for large alterations

1.5.6.1 Fluorescent *in situ* hybridisation (FISH)

The development of fluorescent *in situ* hybridisation (FISH) has proved to be a very powerful and specific method for detecting large DNA rearrangements (Baurmann *et al.*, 1993). This method involves the hybridisation of a labelled DNA probe onto intact human metaphase and anaphase spread chromosomes that have been fixed onto slides. Unbound probe is removed by washing and the distribution of the bound probe is visualised by fluorescence microscopy.

1.5.6.2 Loss of heterozygosity (LOH) analysis

This method is another way for detecting large deletions. DNA from the unaffected parents of the patient is required for this method. Using suitable polymorphic markers in the particular region of interest, both parents and patient DNA can be genotyped. Any loss of heterozygosity in the patient at any of the loci examined indicates a deletion of that locus.

1.5.6.3 Multiplex amplifiable probe hybridisation (MAPH)

MAPH (Armour *et al.*, 2000) is a method for the measurement of gene copy number. This technique relies on comparative quantification of specifically bound probes that are amplified by PCR with universal primers. Reports of the application of MAPH for the detection of deletions in the *DMD* gene, subtelomeric deletions and CML tumour typing have been described (White *et al.*, 2002; Hollox *et al.*, 2002; Reid *et al.*, 2003).

1.5.6.4 Multiplex Ligation-dependent Probe Amplification (MLPA)

MPLA is a simple, fast and inexpensive new method for the detection of deletions and duplications by the analysis of the copy numbers of up to 45 nucleic acid sequences in a reaction (Schouten *et al.*, 2002). In a MPLA experiment, the DNA sample is mixed with the probe mix, which consists of two adjacent nucleotide fragments that are complementary to the target sequence. Thus, upon hybridisation to the target sequence, the two nucleotide fragments can be ligated and subsequently PCR amplified as one fragment, which ranges from 130 to 490 base pairs depending on the probe. All the probes in a probe mix have common ends and can therefore be simultaneously amplified in a multiplex PCR with a single primer pair that is fluorescently labelled. Amplified PCR products are separated by sequence type electrophoresis and comparison of the target samples with a control sample indicates which sequences show an aberrant copy number. If, for example, a target sequence is absent from one allele as a result of deletion, no probe ligation or amplification deriving from this allele will occur. MPLA has been used for the detection of chromosomal deletions or amplification in different genes such as *MLH1* and *MHL2* (Gille *et al.*, 2002; Wehner *et al.*, 2005), *TSC1* and *TSC2* (Rendtorff *et al.*, 2005), *NF2* (Kluwe *et al.*, 2005), *DMD* (Lalic *et al.*, 2005) and more recently, the MLPA kit is also available for the *NF1* gene (Wimmer *et al.*, 2006).

1.6 Aims of this study:

1) To evaluate the genetic and molecular mechanisms underlying the clinical expression of segmental neurofibromatosis (SNF1).

2) To assess the role of *NF1* gene mutations underlying the overlapping features of different disorders with some similarity or clinical overlap with NF1.

3) To investigate the methylation status of the *NF1* promoter in tumours from NF1 patients.

In addition, MLPA technique can be used as a method for the quantification of CpG methylation and also for expression profiling analysis.

1.5.6.5 Array comparative genomic hybridisation (Array CGH)

CGH has been developed to measure alterations in dosage of DNA sequences throughout the entire genome in a single experiment (Kallioniemi *et al.*, 1992). CGH employs comparative hybridisation of differentially labelled genomic DNA from two cell population (test and reference DNA).The DNAs are usually labelled with fluorochromes Cy3 and Cy5. The ratio of hybridisation intensities gives a measure of relative copy number of sequences in the test DNA. The array CGH methodology has significantly evolved over the past few years permitting different assays to be performed (Mantripragada *et al.*, 2004). An high resolution DNA chip for NF1 has been developed (Mantripragada *et al.*, 2006). The average resolution of analysis is 6kb for *NF1* gene. This will be useful and reliable tool for NF1 diagnosis.

1.5.7 Microarray analysis

DNA microarray analysis is an high throughput technique for screening human genes. This method allows the analysis of expression levels of thousands of genes simultaneously in a single experiment. Microarray technology is based on the detection of the amount of mRNA molecules in a cell at a given moment. Comparisons of the abundance of mRNA are made between two samples from, for example, normal and diseased tissues, treated and non treated cell lines, or cells at different stages of differentiation or development. The monitoring of RNA expression level can be done by either using cDNA clone microarrays or gene-specific oligonucleotide microarrays. Briefly, arrays of thousands of DNA sequences are printed on glass microscope slides and the relative abundance of each of these gene sequences in two RNA samples isolated from two different cell populations, are compared. The two samples are first labelled using different fluorescent dyes and then are mixed and hybridized with the arrayed DNA spots. After hybridization, fluorescence measurements are made with a microscope that illuminates each DNA spot and measures fluorescence for each dye separately. These measurements are used to determine the ratio, and therefore the relative abundance, of the sequence of each specific gene in the two DNA samples (See Chapter 4).

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Biochemicals and reagents

2.1.1.1 Enzymes

The restriction enzymes (*RasI, PacI, HindIII, MspI, EcoRI and TaqI*) were obtained from New England Biolabs. DNA ligase was supplied by Promega. *Taq* DNA polymerase was purchased from Qiagen and Expand Long Template PCR system were obtained from Roche.

2.1.1.2 Other biochemicals

Deoxyribonucleotides (dNTPs) were obtained from Invitrogen. Agarose was purchased from Boehringer. GelSlick was supplied by Flowgen. Ammonium persulphate, sodium bisulphite, hydroquinone, silver nitrate, sodium acetate, sodium borohydride, sodium carbonate, sodium hydroxide and Tris were purchased from Sigma. TEAA was obtained from Transgenomic and acetonitrile was purchased from BDH Merck.

2.1.1.3 Reagent kits

BigDye Terminator Cycle Sequencing Kit was obtained from Applied Biosystems. The QIAquick nucleotide removal kit, Gel extraction kit, and PCR purification kit were obtained from Qiagen.

2.1.1.4 Solvents and alcohols

Ethanol, methanol, glacial acetic acid and isopropanol were obtained from BDH. RNAzol was supplied by Biogenesis.

2.1.2 Cell cloning reagents

Tryptone, yeast extract and agar were obtained from Difco. IPTG and X-Gal were supplied by Promega, Ampicillin was purchased from Sigma. PGEM-T easy vector system I was obtained from Promega. DH5α competent cells, subcloning efficiency were purchased from Invitrogen.

2.1.3 Cell culture reagents

McCoy's 5A medium was purchased from Autogene Bioclear. Trypsin-EDTA, Dulbecco's Modified Eagle's Medium, Penicillin/streptomycin solution (5000 IU/ml penicillin, 5000 UG/ml streptomycin) and phosphate buffered saline (PBS) were obtained from Invitrogen.

2.1.4 Equipments

PCR amplification was performed either in PCR thermal cycler OmniGene (Hybaid) or in Primus 96+ (MWG-Biotech). Horizontal and vertical gel electrophersis tanks, and the well forming combs were supplied by Invitrogen. The photographic equipment was purchased from Mitsubishi and the centrifuge was supplied by Hettick Zentrifugen. The DHPLC analysis was performed in a Wave nucleic acid fragment analysis machine supplied by Transgenomic. The sequencing analysis was performed on ABI Prism 3100 Genetic Analyzer supplied by Applied Biosystems.

2.2 Patients

The study groups comprised:
- SNF1 patients.
- NF1 patients with an atypical phenotype.
- NF1 patients known to be carrying the large *NF1* gene deletion.

2.2.1 Tumour samples

A panel of 29 tumours [27 neurofibromas, one plexiform neurofibroma and one malignant peripheral nerve sheath tumour (MPNST)] from NF1 patients were analysed for the methylation status of the sense strand of a ~120-bp stretch flanking the NF1 gene transcriptional start site.

2.2.2 Normal controls

The normal controls were selected from individuals without NF1 or NF1- related syndromes

2.3 General methods

2.3.1 Nucleic acid extraction

2.3.1.1 Extraction of DNA from peripheral blood

Peripheral blood samples were collected into sterile EDTA tubes. A 10ml aliquot of sample was transferred into a 50ml tube with 30ml cold lysis buffer (8g/L NH$_4$Cl, 1g/L KHCO$_3$, 100µM EDTA [pH8.0]) mixed and placed on ice for 15 minutes before centrifugation for 10 minutes at 1000 rpm at 4°C. The supernatant was discarded and the pellet was re-suspended in a further 25ml lysis buffer and the above procedure repeated. The pellet was washed with 2ml lysis buffer to remove the remaining erythrocytes. An aliquot (3ml) of nucleus lysis buffer (10 mM M Tris-HCl, 0.4 M NaCl, 2 mM EDTA, [pH8.2]) was added and the pellet was re-suspended by pipetting. A 200µl aliquot of proteinase K and 150µl 20% SDS (10mg/ml) were added before incubation at 37°C overnight. If necessary more proteinase K was added and incubated again until the pellet was dissolved. Subsequently, the suspension was transferred into a 15ml tube containing 1ml 6M NaCl and mixed for 20 seconds before centrifugation for 15 minutes at 3000 rpm at 4°C. The supernatant was decanted into a 15ml tube and mixed gently with 2 volumes of 100% ethanol. The precipitated DNA was taken out, washed with 70% ethanol and dissolved in 100-500µl 10mM tris 1mM EDTA pH 7.5 (TE) (depending on the size of the pellet) and incubated at 4°C overnight.

The DNA concentration was measured in a spectrophotometer by diluting the sample 1 in 40 and determining the UV absorbance at 260nm. Calculation was based on a 1 OD (optical density) unit being equivalent to 50 µg/ml DNA. A ratio of absorbance at 260 to 280nm >1.8 indicated a pure (relatively protein-free) sample.

2.3.1.2 Extraction of DNA from cultured cells

A1ml aliquot of tissue extraction buffer (10mM Tris, 1mM EDTA, 150 mM NaCl), 20-50 µl proteinase K and 50 µl 10% SDS was added to the cell pellet and incubated overnight at 37°C. Once the pellet was dissolved an equal amount of phenol:chloroform was added to the mixture, shaken gently and then centrifugated at 12,000 rpm for 15 minutes. The aqueous supernatant was removed, placed in a new eppendorf tube and a second phenol-chloroform extraction was carried out. The supernatant was removed and placed in a new eppendorf tube; 0.5 volumes 7.5 M ammonium acetate and 2 volumes absolute ethanol were added for the DNA precipitation. The DNA was transferred to a tube containing 1 ml 70% ethanol to wash the pellet. After centrifugation at 13,000 for 5 minutes, the supernatant was discarded and the pellet re-suspended in 50 µl water and dissolved overnight in a rotary mixer at room temperature.
The DNA concentration was measured in a spectrophotometer as described above.

2.3.1.3 Extraction of RNA from peripheral blood

Blood samples were collected in EDTA tubes and stored at 4°C (3 days maximum) before processing. A 10ml aliquot of volume of blood was mixed with 30ml cold lysis buffer (8g/L NH$_4$Cl, 1g/L KHCO$_3$, 100µM EDTA [pH8.0]) and placed on ice for 15 minutes before centrifugation for 10 minutes at 1000 rpm at 4°C. (Smaller blood volumes were treated with an appropriately reduced volume of lysis buffer). The pellet was re-suspended in a further 25ml lysis buffer and the above procedure repeated. The pellet was homogenised with 800µl RNAzol B, transferred to a 1.5ml eppendorf tube, and placed on ice for 5 mins. 80µl chloroform was added, and the tube inverted several times before being placed on ice for 5 minutes. The mixture was centrifuged at 14,000 rpm for 15 mins at 4°C. The aqueous phase (top layer) was collected into a new eppendorf tube and an equal volume of isopropanol was added, gently mixed, and placed on ice for 45 minutes (or overnight at 4°C) before being centrifuged for a further 15 minutes at 14,000 rpm. The supernatant was discarded and the pellet washed in

800μl 75% ethanol, then centrifuged at 7500 rpm for 8 minutes. The pellet was finally re-suspended in 50μl sterile water.

The extracted RNA was diluted in 1 in 50 in water and scanned in a spectrophotometer at 260 nm to determine total RNA concentration. A ratio of absorbance at 260 and 280 nm was taken to evaluate the purity of the product.

2.3.1.4 Extraction of RNA from cultured cells

An aliquot (3ml) of RNAzol were added to the flask and the cells were collected by scraping with a sterile cell scraper, transferred to a new 12ml tube and placed on ice for 5 minutes. After the addition of 300 μl chloroform, the mixture was mixed and placed on ice for 5 minutes. After centrifugation at 14,000 rpm for 15 minutes (4°C), the aqueous phase (top layer) was collected and placed in a new eppendorf tube. An equal volume of isopropanol (400 μl) was added, the tube gently mixed, and placed on ice for 45 minutes (or overnight at 4°C). After another centrifugation at 14,000 for 15 minutes (4°C), the supernatant was discarded. The pellet was washed in 800 μl 75% ethanol, spun down at 7,500 rpm for 8 minutes and re-suspended in 50 μl RNA water with 0.5 μl RNAsin.

The purity of the extracted RNA was measured in a spectrophotometer as described above. The RNA was stored at −70°C.

2.3.2 Polymerase chain reaction (PCR)

2.3.2.1 Standard reaction

A reaction volume of 25 μl was typically used, containing 10 pmol each primer, 10 x PCR buffer (Tris-Cl, KCl, $(NH_4)_2 SO_4$, 15 mM $MgCl_2$) provided by the manufacturer with the enzyme, 10 mM dNTPs, 1U *Taq* DNA polymerase (Qiagen) and 50 ng genomic DNA. The cycling parameters consisted of an initial denaturation step of 94°C for 5 minutes, followed by 35-40 cycles of 94°C for 30 seconds, Ta °C [annealing temperature (see Table 2.1)] for 30 seconds and 72°C for 30 seconds. Each reaction was terminated with a final extension step at 72°C for 10 minutes.

Table 2.1 *NF1* primer sequences together with PCR and DHPLC conditions.

Exon	Sequence (5' - 3')	Frag Size (bp)	Ta (°C)	Tm (°C)
1	CAGACCCTCTCCTTGCCTCTT GGATGGAGGGTCGGAGGCTG	421	65	61
2	AAGCTGTTAACGTGTTTTTTTTTC AAGAAAAGAAAGCAAATTCCCC	228	58	55
3	TTTCACTTTTCAGATGTGTGTTG TGGTCCACATCTGTACTTTG	245	60	54
4a	TTAAATCTAGGTGGTGTGT AAACTCATTTCTCTGGAG	517	55	50,54,59
4b	GATGATGTCTTGCTATGTTGC TTGGTGTTCTAGTTCAGCAC	366	64	54,59
4c	TTTCCTAGCAGACAACTATCGA AGGATGCTAACAACAGCAAAT	308	58	54
5	GAAGGAAGTTAGAAGTTTGTGACA CAATCGTATCCTTACCAGCCAT	172	57	54
6	CATGTTTATCTTTTAAAAATCTTGCC ATAATGGAAATAATTTTGCCCTCC	301	58	51,56
7	ACATCTGGAATAGAAGAAACTTCA CAGTAACAACAAAAGCAAGTCC	377	64	52,57
8	GGATTTTACTGCCATTTGTGTG TAACAGCATCAGTAAATATAGTTAGATA	276	62	52,57
9	TTGAAGTTCGTTTCAAGACC ACGCAAAGAAAAGAAAGAAAA	272	56	54
10a	ACGTAATTTTGTACTTTTTCTTCC CAATAGAAAGGAGGTGAGATTC	222	58	53,58
10b	ATTATCCTGAGTCTTACGTC TAACTTAGTGTGATAATTTTGAGA	229	54	54
10c	ATTGAAGTTTCCTTTTTTTTCCTTGC GTATAGACATAAACATACCATTTCC	275	57	54,59
11	CCAAAAATGTTTGAGTGAGTCT ACCATAAAACCTTTGGAAGTG	256	60	54
12a	AAACCTTACAAGAAAACTAAGCT ATTACCATTCCAAATATTCTTCCA	303	55	50,54
12b	CTCTTGGTTGTCAGTGCTTC CAGAAAACAAACAGAGCACAT	261	58	57
13	GTCTTCCACCCTTGACTCTC GCTACTTGAATTTCCCCTGT	387	64	56,61
14	GCTCTTCCTACTCCTTTTGG TTTCTGTTGCTAAGGGCATA	191	58	60
15	ACTTGGCTGTAGCTGATTGA ACTTTACTGAGCGACTCTTGAA	247	65	56

16	TGGATAAAGCATAATTTGTCAAGT TAGAGAAAGGTGAAAAATAAGAG	549	60	54,59
17	TCTCTAGGGGGTCTGTCTTC CACCCTAGTTTGTGTGCAGT	326	56	55
18	AGAAGTTGTGTACGTTCTTTTCT CTCCTTTCTACCAATAACCGC	367	58	55
19a	TCATGTCACTTAGGTTATCTGG TAAAACCCACTAATACTTGAAGG	242	65	56
19b	TGAGGGGAAGTGAAAGAACT GGCTTTATTTGCTTTTTGCT	236	56	53,58
20	CCACCCTGGCTGATTATCG TAATTTTTGCTTCTCTTACATGC	402	64	50,55,60
21	TGGTCTCATGCACTCCATA CATCTTTCTTCTGGCTCTGA	474	58	53,58
22	TGCTACTCTTTAGCTTCCTAC CCTTAAAAGAAGACAATCAGCC	331	58	58
23-1	TTTGTATCATTCATTTTGTGTGTA AAAAACACGGTTCTATGTGAAAAG	282	57	56
23-2	CTTAATGTCTGTATAAGAGTCTC ACTTTAGATTAATAATGGTAATCTC	268	58	53,58
24	TTGAACTCTTTGTTTTCATGTCTT GGAATTTAAGATAGCTAGATTATC	266	58	53,58
25	CCTGTTTTATTGTGTAGATACTTCA TAAGTGGCAAGAAAATTACCA	134	53	56
26	AATTCTAATGACTTTGCATTTTTG ATCTAAATTTAAACGGAGAGTG	226	60	52,57
27a	GTTACAAGTTAAAGAAATGTGTAG CTAACAAGTGGCCTGTGTGCAAAC	298	60	58
27b	TTTATTTGTTTATCCAATTATAGACTT TCCTGTTAAGTCAACTGGGAAAAAC	296	53	50,54
28	TTTCCTTAGGTTCAAAACTGG CTAGGGAGGCCAGGATATAG	517	64	54,59
29	TCACCCCGTCACCACCACTTT GCAACAACCCCAAATCAAACTGA	411	57	60
30	CAACTTCATTTGTGTTTTCTCCTAG CTTTGAATTCTCTTTAGAATAATTGTTA	282	57	52,57
31	ATAATTGTTGATGTGATTTTCATTG AATTTTTGAACCAGATGAAGAG	424	64	52,57
32	ATCTAGTATTTTTGAGGCCTCAG CAGATATGCTATAGTACAGAAGG	312	58	51,56
33	TCCTGCTTCTTTACAGGTTATT AAGTAAAATGGAGAAAGGAACTGC	409	64	53,58
34	TTTTCTGTCTTTACTTGTTCCTTTA CAGTCCATGCAAGTGTTTTT	384	57	53,58
35	GCATGGACTGTGTTATTGGTA	319	65	51,56

	TGCAATTAAAAGATCCACAGA			
36	GTTCTGTGGATCTTTTAATTGC CATTGACCTCAAATTTAAACG	238	62	53,58
37	CATTCCGAGATTCAGTTTAGGAG AAGTAACATTCAACACTGATACCC	236	58	54
38	CTATGTCATGATTCATCTTACTAGC CTAAATTTGAGTAATCTAGGAACCTC	233	60	54,59
39	CTACTGTGTGAACCTCATCAACC GTAAGACATAAGGGCTAACTTACTTC	284	62	51,56
40	TCAGGGAAGAAGACCTCAGGAGATGC TGAACTTTCTGCTCTGCCACGCAACC	328	59	56
41	GTGCACATTTAACAGGTACTAT CTTCCTAGGCCATCTCTAGAT	373	65	56
42	CTTGGAAGGAGCAAACGATGGTTG CAAAAACTTTGCTACACTGACATGG	356	60	50,55,60
43	TTTTCTTTTTAGTGTATTCCCATT GATTCTAAGAAATGGCTGGAA	287	53	50,55
44	CACGTTAATTCCCTATCTTGC TGAGAAGTAGAAGACTGTATCC	268	64	53,58
45	CATGAATAGGATACAGTCTTCTAC CACATTACTGGGTAAGCATTTAAC	269	64	56
46	AAATGTTCCTCTGTTGACTT CATCAACCATCCTTCTCCAGA	211	60	56
47	CTGTTACAATTAAAAGATACCTTGC TGTGTGTTCTTAAAGCAGGCATAC	185	62	55
48	TTTTGGCTTCAGATGGGGATTTAC AAGGGAATTCCTAATGTTGGTGTC	351	66	54,59
49	CTGGGAGAAACAGGCTATAC AGCAAGCTTCACACGATCT	363	62	60

Summary of the PCR primer pair sequences used during this study. Frag size (bp), indicates the PCR product size in base-pairs. Ta (°C) indicates the annealing temperature used in the PCR reaction. Tm (°C), indicates the melting temperature used to screen the fragment by DHPLC. GenBank accession no. AC004526.

2.3.2.2 PCR from bacterial colonies

Colonies for mutation analysis were picked and PCR amplified using pGEM-T easy vector primers to allow identification of inserts prior to allele-specific PCR and sequencing. PCR was set up in microtitre plates containing 40μl volumes and one picked colony. The PCR master mix comprised 4μl 10x primers (5pm/μl), 4μl 10x buffer (15mM MgCl$_2$), 6.4μl dNTPs (5mM), 0.25μl *Taq* DNA polymerase (5U/μl) and 22.4 μl dH$_2$O. The PCR cycling parameters used were: (94°C x 3min), 35 cycles (94°C x 30sec)(58°C x 30sec)(72°C x 30sec), (72°C x 10min). Primer sequences were specific to the pGEM^{TM}-T easy vector:

pGEM-T F 5'-GCCCGACGTCGCATGCTC-3'

pGEM-T R 5'-TCCCATATGGTCGACCTGC-3'

2.3.2.3 Allele-specific PCR

PCR was set up in microtitre plates containing 40μl volumes and a single colony picked from an LB ampicillin plate (see section 2.3.22).

For the analysis of the allelic variants, two forward primers were designed, with the 3' base of each primer matching only one of the bi-allelic SNP (single nucleotide polymorphism) bases to be evaluated. Incorporation of a primer mismatch at the third base from the 3' end of the primer has been shown to enhance PCR specificity by further destabilizing the extension of the doubly mismatched primer (Papp *et al.*, 2003). A common reverse primer was used.

For analysis of exon 31 allelic variants, forward primer sequences were:

Ex31-WT F 5'-GCAAGCATAATGATGATGCCACAC-3'

Ex31-MUT F 5'-GCAAGCATAATGATGATGCCACAT-3'

and the common reverse primer was:

Ex31 R 5'CAGATAAATATGTGCACAAAGGAGA -3'

Both primer pairs amplified an exon 31-specific fragment of 184bp.

PCR cycling parameters used were: denaturation at (94°C for 5min), 25 cycles of (94°C x 30sec)(60°C x 30sec)(72°C x 1min), (72°C x 10min).

For analysis of exon 40 allelic variants, forward primer sequences were:

Ex40-WT F 5'-CAGAATTTTACATACAGTACTA-3'

Ex40-MUR F-5'-CAGAATTTTACATACAGTAACT-3'

2.3.2.4 Deletion junction-PCR

A deletion junction PCR assay (Lopez-Corrrea *et al.*, 2001) was used to detect the breakpoints of the common 1.5 Mb deletion. The previously published primers (Lopez-Corrrea *et al.*, 2001) were designed from the known sequences of the NF1 REP-P and -M paralogues so as to amplify specifically a 3.4 kb deletion-spanning fragment:

DCF 5'-TCAACCTCCCAGGCTCCCGAA-3' and

DTR 5'-AGCCCCGAGGGAATGAAAAGC-3'.

A volume of 12.5 µl PCR was performed using the Expand long template PCR system (Roche). The system utilises an enzyme mix containing thermostable *Taq* DNA and *Pwo* DNA polymerases resulting in a high yield and low error rate.

The master mix contained 0.62µl dNTP (10mM), 1.25µl 10 x buffer (22.5 mM MgCl$_2$), 1.5µl each primer (5pm/µl), 0.35µl of Expand enzyme mix and 80-100 ng DNA.

The reaction parameters were:

94°C x 3 min, 10(94°C x 40sec, 65°C x 45sec, 68°C x 3min) 26(94°C x 40sec, 65°C x 1min, 68°C x 3min), 68°C x 8min.

2.3.2.5 PCR using bisulphite-treated DNA

Polymerase chain reaction amplification of bisulphite-treated DNA was performed using primers as designed by Mirjiam Luijten (Rotterdam, Netherlands). Primers specific for both unmethylated and methylated DNA were positioned so as to amplify DNA fragments surrounding the *NF1* gene transcriptional start site.

Primers for unmethylated PCR, amplified 119 bp fragment, were:

UMSP-F (position -45→ -13)

5'-TGTTTGTTAGATGGTTTAGAGGAGTTAGATGAT-3' and

UMSP- R (position +74→ +46)

5'-AAAAACAAAAAAAAAAACAACCTACCACA-3' and

for methylated reactions were:

MSP-F (position -41→ -13)

5'-CGTTAGACGGTTTAGAGGAGTTAGATGAC-3' and

MSP-R (position +67→ +46)

5'-AAAAAAAAAACGACCTACCGCG-3'.

Amplifications were performed using Qiagen *Taq* DNA polymerase. Reaction mixtures contained either 3 µl bisulphite-treated DNA, 200µM dNTPs, 1x buffer, 1.5 mM MgCl$_2$, 0.5 units *Taq* DNA polymerase, 0.4-pmol primers in a final volume of 12.5µl.

Reactions were hot started at 94°C x 3min before the addition of 0.5 units *Taq* DNA polymerase (Qiagen). Amplification was carried out in a Hybaid Omnigene temperature cycler for 40 cycles (45secs for MSP, 1min for UMSP at 94°C, 45secs for MSP, 1min for UMSP at 53°C for MSP 56°C for UMSP and 45sec for MSP, 1min for UMSP at 72°C) followed by a final 10 min extension at 72°C.

Each PCR reaction was loaded directly onto 2% agarose gels, stained with ethidium bromide, and visualised under UV illumination.

As a positive control for methylated alleles, DNA (lymphocytes from a healthy individual) was treated with *Sss1* methyltransferase (New England Biolabs), then subjected to bisulphite treatment.

Non- converted DNA was used as a control for primer specificity.

Primers (MPR1) from Horan *et al.* (2000) were used to control the bisulphite conversion.

2.3.3 RT-PCR

2.3.3.1 First strand cDNA synthesis

Reverse transcription was performed in a 20 µl reaction volume containing 1-5 µg of total cellular RNA. The reaction mix containing RNA, 1µl random primer (0.5 µg/µl), 10 mM dNTPs and H$_2$O was heated at 65°C for 5 minutes to denature and placed on ice for 1 minute. 4 µl of 5 x First Strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM MgCl$_2$), 2 µl 0.1M DTT, 1µl RNAase inhibitor and 200 units Superscript II reverse transcriptase were added and the reaction incubated at 50°C for 60 minutes. On completion of the first strand synthesis, the enzyme was inactivated by heating at 70°C for 15 minutes.

each primer (5pm/μl), 1μl Expand buffer (17.5 mM MgCl$_2$), 2μl cDNA and 4.9μl sterile water. The second mix contained 0.25μl Expand buffer, 0.37μl Expand enzyme mix and 1.37μl sterile water. Mix 1 was overlaid with mineral oil and heated at 98°C for 2 minutes and at 94°C for 1minute before mix 2 was added.

The cycling parameters were:

98°C x 2min, 94°C x 3min, 10(94°C x 30secs, Ta °C x 30secs, 72°C x 3min) 25(94°C x 30secs, Ta °C x 30secs, 72°C x 3min + 10secs per cycle), 72°C x 10min.

2.3.3.3 Amplification of the 24 cDNA overlapping fragments

A reaction volume of 12.5 μl was used, containing 2μl dNTP (5mM), 1μl each primer (5pm/μl), 1.25μl 10 x PCR buffer (Tris-Cl, KCl, (NH$_4$)$_2$ SO$_4$, 15 mM MgCl$_2$) provided with the enzyme, 0.5 U *Taq* DNA polymerase (Qiagen), 2μl cDNA and 5.2μl sterile water. The *NF1* gene coding sequence was amplified in 24 overlapping segments using previously published primer pairs (Upadhyaya *et al.*, 2004). The primers pair which amplifies exons 1-3 was re-designed to improve the PCR amplification specificity. The PCR products were screened for mutation analysis by DHPLC. Samples manifesting mutant or ambiguous chromatographic patterns were re-amplified and sequenced (Table 2.2).

Table 2.2. cDNA primer sequences

Name	Seq 5'→3'	Size (bp)	Exons amplified	Tm (°C)
NF1-1F	AACACTGGGAGCCTGCACT			
NF1-1R	GTTGCCCAGCAAGACATTTT	425	1, 2, 3	54.5, 58, 63.5
NF1-2F	AAGCGGCCTCACTACTATTT			
NF1-2R	TATCAATGTGGATTGTGCAA	401	3, 4a, 4b	54, 57, 59.5
NF1-3F	CAGGAATTAACTGTTTGTTCAGA			
NF1-3R	ATATCCAAAGACGTGGTTGATGAAAACAA	395	4b, 5, 6	54.5, 56.5, 58
NF1-4F	CTGAAAGCACCAAACGTAAA			
NF1-4R	GCCCTCACAACAACCAACACTT	412	6, 7, 8, 9	57, 58
NF1-5F	TGATTGACTGCCTTGTTTCT			
NF1-5R	CCCCTGTTTTCTTGGATTAC	419	9, 10a, 10b, 10c	56, 58.5
NF1-6F	TCTCTTGTCCATGGTGAAAC			
NF1-6R	GGCTCCAGGAGTTACGTAGTAA	493	10c, 11, 12a, 12b	56.5, 60
NF1-7F	GGAAATACCAGTCAAATGTCC			
NF1-7R	TTAGCTTTGTTGCTTGTTCC	428	12b, 13, 14, 15	55, 60.5
NF1-8F	CACTGAGGCTTGGGAAGATA			
NF1-8R	GACCCACCAGATCCTTAACA	449	15, 16	57.5, 60
NF1-9F	GGCTGTTGTCCTTAATGGTG			
NF1-9R	CCATAACCCAGTCTGTCAGG	540	16, 17, 18, 19°	56
NF1-10F	GCCAAGAGATGAAATTTAGG			
NF1-10R	GTGCATGAGACCACTGTCTA	395	19a, 19b, 20	55, 58, 62
NF1-11F	GGTCCTTGCAATGTCAAAC			
NF1-11R	AAAGGATCCAGGAGTTTTTG	477	20, 21, 22, 23.1	55, 58.5
NF1-12F	AGACTCTCTTCCGAGGCAAC			
NF1-12R	TTCATACGGTGAGACAATGG	392	23.1, 23.2, 23a, 24	57, 59.5
NF1-13F	CAGTAGGAAGTGCCATGTTC			
NF1-13R	TATCAAAAGGTCGTCTTCCA	402	24, 25, 26, 27a	57.5
NF1-14F	CAACAGGGATCATAAAGCTG			
NF1-14R	TTTAAAGCGATTGCTAGGC	393	27a, 27b, 28	57, 59.5
NF1-15F	AAAGCCATATTATGCAAAGC			
NF1-15R	TGGACAGCAGTAGAACCAAC	396	28, 29	57.5, 59.5
NF1-16F	GCTCTCAAGCTAGCTCACAA			
NF1-16R	CTAGTAACTGGCCCTCGATT	454	29, 30	57, 60
NF1-17F	TCTGTGTGCCTTAACTTGTACC			
NF1-17R	CACTGGTTTTGATGAAACTGTC	428	30, 31, 32	57
NF1-18F	AAAATATGGGGAAGCCTTGG			
NF1-18R	CTCTAAGGGAGAGCGGACCT	376	32, 33	58, 59.5

NF1-19F	TGATGTGGCAGCTCATCTTC			
NF1-19R	ATGCCTCCATGATCTCCAAC	365	33, 34, 35	59
NF1-20F	TGACATCCTTGGAAACAGTC			
NF1-20R	AAGTGCGGTACCTGCTGAAT	416	35, 36, 37, 38	58.5
NF1-21F	CTCTTTTGGGTAGCTGTGG			
NF1-21R	CAGCGACTTCGAACTTCTT	416	38, 39, 40, 41	57.5, 58.5
NF1-22F	TGGCCTACTTAGCAGCTTTA			
NF1-22R	TGCTACTCTCCTCATTTTGG	413	41, 42, 43	57.5, 61
NF1-23F	AAAAGGCAAGAAATGGAATC			
NF1-23R	TCTGGAATTTGTGTTTGCTT	515	43, 44, 45, 46, 47	57.5
NF1-24F	TTTGGTTTTAATGGCTTGTG			
NF1-24R	AACCGGATGGGTTCATTAT	468	47, 48, 49	58, 60

Summary of the PCR primer sequences used to amplify the *NF1* cDNA. Size (bp) indicates the PCR product size. Tm (°C), indicates the melting temperature used to screen the fragment by DHPLC. GenBank accession no. NM_000267.

2.4 Other methods

2.4.1 DNA restriction digestion

DNA restriction reactions of PCR products were set up using 0.2μl bovine serum albumin (100 x BSA), 2μl 10 X buffer, provided with the enzyme, 10 units restriction endonuclease, 10μl PCR product and sterile water in a final reaction volume of 20μl. Restrictions were incubated for 1-12 hours at a temperature recommended by the enzyme supplier. Digestions were electrophoresed on 3% agarose gel for 1-2 hours (see Table 2.3).

2.4.2 Polyacrylamide gel electrophoresis

Gel plates were cleaned using soap solution, rinsed in water and then wiped with ethanol. The smaller plate was then treated with GelSlick solution (Flowgen, Staffordshire,UK) to allow for easy separation.

The gel was prepared in a glass beaker by using 60 ml Sequagel 6, 15 ml SequaGel complete and 550μl 10% ammonium persulphate. Once the gel was set (1-2 hours), it was placed in the vertical tank with 1 x TBE and heated at 60 W for 30 minutes.

The samples were prepared by adding 4μl Stop solution [Blue dye, 8 ml formamide, 2ml 10 x Tris/borate/EDTA (TBE)] to 6μl PCR product. After heating at 80°C for 3 minutes, 3-4μl of sample was loaded on the gel.

2.4.2.1 Silver staining

DNA bands in the polyacrylamide gel were detected by silver staining. The gel was first soaked for 10 minutes in 0.1% silver nitrate solution, rinsed in distilled water and then immersed in pre-chilled developer solution (1.5% sodium hydroxide, 0.01% sodium borohydride and 0.15% formaldehyde). This solution was changed regularly until bands appeared on the gel (5-10 minutes). The gel was fixed by soaking in 0.75% sodium carbonate solution for approximately 5 minutes and rinsed in water.

2.4.3 DHPLC (denaturing high performance liquid chromatography)

The PCR products were denatured at 95°C for 5 min and then allowed to reanneal over 30 mins to allow heteroduplex formation prior to DHPLC analysis. DHPLC was performed on a WAVE DNA fragment analysis system (Transgenomic) using a DNASep column (O'Donovan et al., 1998). PCR products were analysed for heteroduplexes by subjecting 5 μL PCR product to a 2% linear acetonitrile gradient at a rate of 0.9 mL/min. Eluents used for the separation were: Buffer A, 0.1 M triethylammonium acetate (TEAA), Ph 7.0 (Transgenomic) in water; Buffer B, 0.1 M TEAA and 25% acetonitrile in water pH 7.0. The temperatures for optimal heteroduplex separation were determined from the melting profiles produced for each fragment using the WAVEmaker software version 4.1. Depending on the melting domains predicted by the software, one, two or three different melting temperatures (Tm) were used to allow individual PCR fragment to be successfully analysed (Table 2.1).

Table 2.3. <u>Primer conditions for typing polymorphic markers used to analyse LOH in</u>
<u>the SNF1 patients</u>

Marker	Primer sequence	Ta (°C)	Type of analysis	Reference
UT172	F-GGTGAAAGAGCAAGACTCTGTCAC R-CCCCTTGATTGTAAGCNACAGAAAC	52	6% PAGE	Shannon *et al.*, 1994
HHH202	F-ATGAACAAGTCAAGGGACAGGCTT R-ACTTGCCAAAGGTTACAGGGCTAC	65	*RasI*-3% agarose gel	Ainsworth & Rodenhiser, 1991
NF1-X5 RFLP	F-CATGTGGTTCTTTATTTATAGGC R-TCAATCGTATCCTTACCAGCC	53	*RasI*-3% agarose gel	Hoffmeyer & Assum, 1994
NF1-EVI-20	F-CCCATACCTAGTTCTTAAAGTCTG R-TAACAATTGTGGAACTGCAGCAATTATT	55	6% PAGE	Shannon *et al.*, 1994
IVS27AC 28.4	F-GTTCTCAACTTAAATGTAAGT R-GAACATTAACAACAAGTACC	54	6% PAGE	Lazaro *et al.*, 1994
IVS38GT 53.0	F-CAGAGCAAGACCCTGTTCT R-CTCCTAACATTTTATTAACCTTA	54	6% PAGE	Lazaro *et al.*, 1994
IVS41	F-GTGCACATTTAACAGGTACTAT R-CTTCTTAGGCCATCTCTAGAT	59	*Pac*I 3% agarose gel	Lazaro *et al.*, 1994
EW207	F-AGGTATCAGTCAGGACCCTCTTTAG R-CTGACACTCTGGTTTCTGTAAGTG	60	*Hind*III *3%* agarose gel	Fain *et al.*, 1989
EW206	F TGCAGTGTGGTGCATCATTCAGTG R GACAGGGCCAGCCATATTCCTGAT	63	*Msp*I 3% agarose gel	Fain *et al.*, 1989
3'UTR C3GC	F-CATTGGCACAAATCAGAATT R-AACAGAACTTATGTCAATTA	54	*EcoR*I 3% *agarose gel*	Cowley *et al.*, 1998
3'UTR C7CT	F-GGCTGGCACTCTGTCTCCTC R-CTGACTTGTTAAAGAGGAAAC	60	*Taq*I 3% agarose gel	Cowley *et al.*, 1998

Summary of the primers, restriction enzymes and gel running conditions required for
typing the polymorphic markers used for the LOH study. Ta (°C), annealing
temperature. PAGE, polyacrylamide gel analysis.

2.4.4 Sequencing

PCR products displaying a heterozygous pattern were purified using the Montage PCR$_{96}$
Plates (Millipore), according to the manufacturer's protocol. A 5 µl aliquot of each
purified PCR product were sequenced in both forward and reverse orientations using
ABI Prism BigDye terminator cycle sequencing kit version 3.1 (Applied Biosystem).
The sequencing reaction was performed in 10µl final volume, containing 2.5µl of 5 x
buffer provided with the kit, 1.6µl primer (1pm/µl), 0.75µl BigDye terminator and 5µl

PCR product. The reaction cycling parameters were: 96°C x 2min, 25(96°C x 25sec, 55°C x10sec, 60°C x 4min), 4°C x 5min.

Sequencing reactions were also purified using the Montage SEQ$_{96}$ Plates (Millipore), with the addition of 10 μl of Hi-DI formamide and analysed on an ABI Prism 3100 genetic analyzer.

2.4.5 Loss of heterozygosity (LOH) studies

DNA samples from blood and affected skin were screened for the presence of DNA rearrangements as evidenced by LOH using a panel of 11 intragenic and extragenic RFLPs and polymorphic microsatellite markers in and around the *NF1* gene.

2.4.6 Microsatellite instability analysis (MSI)

DNA samples from blood and affected skin were screened for the presence of microsatellite instability by using 9 microsatellite markers (Sutter *et al.*, 1999; Dib *et al.*, 1996; Hazan *et al.*, 1996; Weber *et al.*, 1990; Liu *et al.*, 1995; Fujiwara *et al.*, 1995; Parson *et al.*, 1995) (Table 2.3). Labelled primers (Table 2.4) were used in a multiplex 12 μl PCR amplification reaction. PCR cycling parameters used were: denaturation at (95°C for 5min), 28 cycles of (95°C x 1min)(55°C x 1min)(72°C x 1min), (72°C x 7min). A 2μl aliquot of PCR product was diluted in 30 μl sterile water, heated at 95°C for 3 minutes, placed in ice and then analysed by using an ABI PRISM 3100 GeneScan Analyzer (PE Applied Biosystems).

Table 2.3: <u>Microsatellite instability (MSI) markers</u>

Microsatellite locus	Chromosome	Type	Min (bp)	Max (bp)	Reference
mD13S153	13q14	$(CA)_{25}$	208	228	Sutter *et al.* (1999).
mD5S406	5p15	$(AC)_3$-$(CA)_{27}$-$(GT)_2$-ATTTGC-$(AT)_4$	167	181	Sutter *et al.* (1999).
MACTC	15q11-q14	$(TG)_{25}$	71	97	Sutter *et al.* (1999).
D2S123	2p16	$(CA)_{13}$-TA-$(CA)_{15}$	207	227	Dib *et al.* (1996).
D17S250	17q12	$(TG)_{22}$ TTTGAAACCATTTGA AAGTTTATGTA-$(TG)_2$-$(TA)_7$-A-$(AC)_4$-ATA-$(T)_5$	150	166	Weber *et al.* (1990).
BAT-25	4q12	$(A)_{25}$	121	123	Liu *et al.* (1995). Parsons *et al.* (1995).
BAT-26	2p16	$(A)_{26}$	119	121	Sutter *et al.* (1999). Liu *et al.* (1995). Parsons *et al.* (1995).
BAT-40.4	1p13	$(A)_{40}$	171	187	Liu *et al.* (1995).
D5S346	5q22	$(TG)_{15}$	108	126	Joslyn *et al.* (1991). Spirio *et al.* (1991).

Table 2.4 <u>Primers sequences for the MSI study</u>

Microsatellite locus	Dye	Primer sequence
mD13S153	FAM	F-AAAGCATTGTTTCATGTTGG R-AAGGTCTAAGCCCTCGAGTT
mD5S406	FAM	F-AACCTGCCAATACTTCAAGA R-GGATGCTAACTGCTGACTAT
mACTC	FAM	F-CTTGACCTGAATGCACTGTG R-ATTCCATACCTGGGAACGAG
D2S123	HEX	F-AAACAGGATGCCTGCCTTTA R-GGACTTTCCACCTATGGGAC
D17S250	HEX	F-GGAAGAATCAAATAGACAAT R-GCTGGCCATATATATATTTAAACC
BAT-25	HEX	F-TCGCCTCCAAGAATGTAAGT R-TCTGCATTTTAACTATGGCTC
BAT-26	FAM	F-TGACTACTTTTGACTTCAGC R-AACCATTCAACATTTTTAACC
BAT-40.4	HEX	F-ACCAGTCCATTTTATATCCTCAA R-AAGATCACACCTCTGCACTCT
D5S346	FAM	F-AACCTGCCAATACTTCAAGA R-GGATGCTAACTGCTGACTAT

Summary of the PCR primer sequences used to amplify the microsatellite (MS) DNA sequences. Dye indicates the type of dye used to label the primers.

2.5 DNA cloning

2.5.1 Media

LB media was made by adding 10g tryptone, 5g yeast extract and 10g NaCl to 1L H_2O. The solution was adjusted to pH 7.0 with NaOH. To make LB agar, 7.5g agar was added to 500ml LB medium. A 2 x YT medium was made by adding 8g tryptone, 5g yeast extract and 2.5g NaCl to 500ml H_2O. All the solutions were autoclaved before use. The LB agar was cooled to 50°C before 250 µl ampicillin (100mg/ml), 400µl X-Gal (100mg/ml) and 100µl IPTG (0.1M) were added. An aliquot of 25ml was poured into each plate and allowed to set.

Specific PCR products were cloned into the pGEM-T easy vector (Promega) before being transformed into *E. coli* DH5α competent cells (Invitrogen).

2.5.2 Ligation of insert

Ligation was performed in 10µl containing 1µl vector, 3µl PCR insert, 1µl T4 DNA ligase (10U), and 5µl 2x ligase buffer. The reaction was incubated at 10°C overnight before being stored at -20°C.

2.5.3 Transformation

E.coli DH5α competent cells were removed from storage at -70°C and thawed on ice. After gentle mixing, a 50µl aliquot was placed in a 1.5ml microcentrifuge tube pre-chilled on ice. A 3µl volume of ligation reaction was added to the cells and mixed by gently tapping the tube. The cells were incubated on ice for 30 mins, followed by heat shock treatment at 37°C for 20 seconds. The tube was placed on ice for a further 2 minutes. A 950µl aliquot of LB was added, and the tubes placed in a shaking incubator at 37°C for 1 hour. After incubation, the concentrated cells were spread on a LB agar plate and incubated overnight at 37°C.

2.6 Tissue cultures

2.6.1 Culture medium

McCoy's 5A medium (Autogene Bioclear) was used to culture fibroblasts from skin biopsies. The medium was made by adding 10% of foetal bovine serum, 1% of L-glutamine and 1% of penicillin/streptomycin.

Skin biopsies of 3mm in size, brought in culture medium, were minced into small pieces covered with fresh medium and incubated at 37°C in a 95% air, 5%CO_2 incubator.

2.6.2 Subculturing

Cells were grown to full confluence using T75 flasks. The old medium was removed from the flask and the cells were washed with 5ml 1x PBS. An aliquot (2ml) of trypsin/EDTA were added, ensuring that the bottom of the flask was covered. The flask was placed in the 37°C incubator for 5 minutes, removed, and gently tapped with the palm of the hand to detach the cells. Subsequently, 10ml growth medium were added to inhibit the trypsin. The cells were transferred to a sterile vial and the flask rinsed with 5ml additional medium, which was then added to the vial. The vial was then centrifuged at 1500 rpm for 3 minutes at room temperature. The supernatant was removed and the cells re-suspended in 10ml fresh medium. Centrifugation was repeated and the cell pellets were stored at -20°C ready for DNA extraction. The cells used for RNA extraction were detached mechanically using a cell scraper and RNAzol.

2.7 Methylation analysis

2.7.1 Bisulphite conversion reaction

Sodium bisulphite chemically converts all unmethylated cytosines into thymines. Bisulphite conversion was performed as described by Horan *et al.* (2000).

Briefly, DNA was mixed with 2% low melting point agarose to a final concentration of 10ng/µl. The mix was kept denatured at 95°C and formed into a bead by adding 10µl to pre-chilled mineral oil. A total of 800µl 5M bisulphite solution [7.2M sodium bisulphite (5ml), 1M hydroquinone (1ml), 2M NaOH (1.5ml),1M urea (0.45g)] was added to

73

seven pre-formed beads and overlaid with mineral oil. The reaction was carried out at 50°C for 4 hours.

DNA converted samples were purified using Qiaquick gel purification columns (Qiagen) according to the manufacturers' instructions.

2.8 Microarray analysis

The study group comprised five SNF1 individuals of which three were sporadic and two familial cases. Both the familial cases had a parent affected by full-blown NF1 with an *NF1* gene mutation detected at the genomic level. Previous mutation analysis of the *NF1* gene of DNA samples from all those SNF individuals had revealed no mutations in the 3 sporadic cases but in one of the familial cases. However, a nonsense mutation was identified in mosaic pattern in cell lines from café-au-lait spots from the other familial case.

2.8.1 Sample processing and array hybridization

Sample labelling, hybridisation to arrays and image scanning were performed by Megan Musson (Pathology Department, Cardiff University) using a standard Affymetrix protocol (Affymetrix expression analysis technical manual). Expression levels were measured using Affymetrix U133A, that comprise >22.000 probe sets and 500,000 distinct oligonucleotide sequences, representing 14,500 characterized human genes.

2.8.2 Data normalization and statistical analysis

The statistical analysis was performed by Daniel Kirwilliam and Prof. David Kipling (Pathology Department, Cardiff University). The experiment consisted of 10 chips placed in two groups of 5; 2 for each patient.

Quantile-Quantile (QQ) normalisation is a now established method of normalisation that is very powerful when looking at datasets without major changes in the distribution of data (Bolstad *et al.*, 2003). It works in a non-linear fashion to bring the distribution of data within chips into the same shape. Here is a simplified version of the algorithm:

- Sort each chip by expression value
- Take an average (median) for each rank

- Apply this average back to the rank for each chip
- Put the probes back into their original (unsorted) order

This was performed with a small script run within the R environment for statistical computing (http://www.R-project.org). The results were imported into MS Excel and a paired t-test ran on them. A paired t-test was chosen as the most powerful standard statistical method because of the paired experimental design (Cui & Churchill, 2003). To control for multiple testing errors, the false discovery rate (FDR) method was used. The FDR method calculates the number of expected false-positives generated by the statistical test (Cui & Churchill, 2003). In other words, the FDR method calculates the proportion of false positives among the significantly over- or under-expressed genes (Tusher *et al.*, 2001). As described by Broberg (2003), the false-positive rate (FP) is an estimate of the number of false positives among the significantly over- or under-expressed genes divided by the total number of genes. Similarly, the false-negative rate (FN) may be obtained by the number of false negatives among the non-significantly over- or under-expressed genes divided by the total number of genes. In addition, dividing the number of true positives by the total number of genes, the true-positive rate (TP) can be obtained. Moreover, by calculating the number of true-negatives divided by the total number of genes, the true negatives rate (TN) may be obtained.

Chapter 3. Molecular genetic analysis of segmental neurofibromatosis type 1 (SNF1)

3.1 Introduction

The term mosaic has been used since antiquity to refer to an artistic patchwork of ornamental stones, glass, gems or other precious material. In biological systems, mosaicism implies the presence of more than one genetically distinct cell line in a single organism. Theodor Boveri (1929) first suggested that neoplasia results from a single cell that undergoes a genetic change. In the modern era, studies of chromosomes and nucleotide sequences have confirmed the correctness of his view by demonstrating the presence of somatic mutations in most cancers. In terms of inherited disease, mosaicism in human females involves the process of X inactivation (lyonization) (Hall, 1988; Happle, 2000) whilst mosaicism occurs in both sexes through mutational errors arising during either somatic or germ cell division (Hall, 1988; Zlotogora, 1998). At the level of the whole organism, it should be appreciated that the mosaic phenotype depends upon inter-tissue genetic variation that does not clearly follow Mendelian rules of inheritance. Somatic mosaicism has now been implicated in more than 30 monogenic human disorders that show variable expressivity (Gottlieb *et al.*, 2001).

The distinction between somatic and germline (also termed gonadal) mosaicism is based on the finding of genetically distinct populations of cells in the somatic and germline tissues, respectively (Hall, 1988; Youssoufian, 1996). In cases where mosaicism has arisen during embryogenesis, somatic and germline mosaicism can coexist in the same individual, depending on the specific cell affected and the developmental timing of the mosaicism–inducing event. Phenotypically normal individuals may transmit several gametes that are clonal descendants of a single progenitor cell in which a *de novo* mutation has occurred during early development (Hall, 1988; Hall and Byers, 1987). Mosaic cellular populations can in principle arise from mutations in nuclear or mitochondrial (mt) DNA in post-zygotic cells, epigenetic alterations of DNA, or even numerical or structural abnormalities of chromosomes. All of these alterations can progress from "normal" to "abnormal" or in the reverse direction.

The frequency of mosaicism in a particular disorder is critically dependent on the disorder in question, the tissue of origin, the structure of the underlying gene, the function of the gene product it encodes, and the degree of selective pressure operating at a cellular level. In some disorders, because molecular ascertainment frequently begins by the analysis of DNA in blood cells, a low level of mosaicism in these cells can remain undetected. While perhaps it should be stated that all human beings may be regarded as somatic mosaics for a multitude of gene mutations (due to the high number of cells in the body in relation to the mutation rate), this extremely low level of mosaicism is likely to be clinically important in only a relatively small proportion of cases. In a clinical context, it should be appreciated that the timing of the mutation and hence the relative numbers of the somatic cell descendents of the originally mutated cell that contribute to different lineages, can have marked effects on the clinical phenotype. During mouse and human embryogenesis, three or four cells are co-located to the germline before the emergence of somatic cell lineages. So, mutations that appear at later times in development are more likely to give rise to the tell-tale features of somatic mosaicism, such as patchy or segmental anomalies. Conversely, the mosaic character of the feature might be lost if mutations appear before the blastula stage. Mutations that arise in somatic cells at later stages *in utero* can also remain silent during the postnatal period and early development, unless unmasked by other factors or environmental events. In such cases, a somatic mosaic could also be mistaken for the *de novo* appearance of the mutation, or for gonadal mosaicism in the parental germ-line (Youssoufian & Pyeritz, 2002). Therefore, it is not surprising to note that a significant proportion of patients who have disorders characterized by high *de novo* mutation rates are increasingly being identified as somatic mosaicism (in the absence of germline mosaicism) (Colman *et al.*, 1996; Leuer *et al.*, 2001; Sippel *et al.*, 1998).

3.1.1 Mosaicism in NF1

Mosaicism has been implicated in those cases of families in which the parents appear to be phenotypically normal, but more than one of their offspring is affected with a dominant or X-linked disorder (Hall, 1988). Mutations during early embryonic development, before the determination of the germ-line, will cause gonosomal mosaicism (affecting the majority of somatic tissues and also the germline cells). Mutations that occur later can affect the somatic cells alone (somatic mosaicism). If the

mutation also affects cell types other than the gametes (somatic and gonosomal mosaicism), a mild form of the disease can also occur in one of the parents. According to Ruggieri and Huson (2001), mosaicism in NF1 can be divided into two groups: mosaic-localized NF1 (i.e. segmental NF1) and mosaic-generalized NF1 (i.e. patients cannot be distinguished clinically from classical NF1 patients with a constitutively inherited germ-line mutation).

Somatic mosaicism at the molecular level has been described in some sporadic NF1 patients with classic generalized symptoms. Up to now, all these patients (Ainsworth *et al.*, 1997; Colman *et al.*, 1996; Rasmussen *et al.*, 1998; Riva *et al.*, 2000; Streubel *et al.*, 1999; Tonsgard *et al.*, 1997; Wu *et al.*, 1997) were initially diagnosed as having classic NF1, since the signs of NF1 were not confined to a specific body region. The mutated *NF1* allele of each of these patients displayed large deletions, identified either by FISH (Riva *et al.*, 2000; Streubel *et al.*, 1999; Tonsgard *et al.*, 1997; Wu *et al.*, 1997) or by loss of heterozygosity (LOH) (Ainsworth *et al.*, 1997; Colman *et al.*, 1996; Rasmussen *et al.*, 1998). These techniques have allowed the ready detection of mosaicism in patients presenting with a large *NF1* gene deletion. Detection of mosaicism for a micro-lesion is, however, technically more challenging. This is probably why data on this type of lesion in the mosaic state have so far gone unreported in NF1 patients.

3.1.2 Segmental Neurofibromatosis type 1 (SNF1)

Historically, the first case of segmental neurofibromatosis type 1 (SNF1) was reported in 1931 by Gammel, who described a 60 year old man with multiple neurofibromas confined to a limited area of the abdomen . This condition was referred to as localized neurofibromatosis. Crowe *et al.* (1956) reported four new cases and termed the disease "sectorial neurofibromatosis", and was the first to postulate somatic mosaicism of the *NF1* gene as the likely cause. It was not until 1977 that Miller and Sparkes first suggested the term "segmental neurofibromatosis", which has since become widely used.

Patients with the typical features of NF1 limited to one or more body segments are usually referred to as having "segmental NF1" (Moss *et al.*, 1994; Viskochil and Carey, 1994; Gutman *et al.*, 1997; Huson and Ruggieri, 2000).

The disease features develop at the same time and have the same natural history as in generalized NF1; the pigmentary features and plexiform neurofibromas in childhood

and the dermal neurofibromas in adulthood (Huson and Ruggieri, 2000). The frequency of cutaneous neurofibromas in prepubertal children with NF1 is quite low (Obringer *et al.*, 1989). What is different is that not all SNF1 individuals with pigmentary changes get neurofibromas when they grow older. This presumably reflects the cell lineage in which the mutation has taken place. The disease complications in SNF1 are typically many fewer (around 5%) than in the generalized disease (Ruggieri and Huson, 2001; Ruggieri and Polizzi, 2000).

More than 150 cases of segmental neurofibromatosis have been published (Arfan-ul-Bari, Simeen-ber-Rahman (2003); Hager *et al.*, 1997; Ingordo *et al.*, 1995; Wolkenstein *et al.*, 1995; Hix *et al.*, 1998; Ruggieri *et al.*, 2004) since the first description of a localized form of NF1 by Gammel in 1931. The concept that SNF1 represents a mosaic manifestation of NF1 explains the variability of the clinical picture, since the phenotype is influenced by the point in time at which the *NF1* mutation occurred during embryogenesis, the type of cells in which the *NF1* mutation occurred and the kind of mutation. Fluorescent *in situ* hybridisation (FISH) was used to identify a heterozygous deletion of the entire *NF1* gene in ~18% of fibroblasts cultured from a CAL spot from the affected region of the patient with SNF1 (Tinschert *et al.*, 2000). This gross *NF1* gene deletion was absent from both peripheral leukocytes and fibroblasts derived from non-affected regions of the patient's body. This report indicated that SNF1 is (i) caused by *NF1* gene mutation and (ii) that the affected individual was a somatic mosaic for this lesion. Further studies are however required both to confirm the general validity of these conclusions and to elucidate the mutational spectrum and tissue-type distribution of somatic mosaicism associated with SNF1, particularly where gonadal mosaicism is also suspected.

There is a small but definite risk of passing on either full-blown NF1 or, more rarely, the localized disease, to offspring (Moss *et al.*, 1994; Viskochil and Carey, 1994; Huson and Ruggieri, 2000; Lazaro *et al.*, 1994; Huson and Hughes, 1994). Offspring of SNF1 individuals are at risk of developing the classical form of NF1 if mosaicism involves the gonadal cells of the affected parent.

Different hypotheses have been proposed regarding the inheritance of segmental neurofibromatosis. Crowe *et al.* (1956) hypothesized that a single, early embryonic, somatic mutation might be propagated by mitotic activity to involve a limited distribution of cells. A similar theory proposed by Nicholls (1969) suggests that the

condition may be the result of a somatic mutation at a single, highly mutable locus responsible for an important neural crest cell function. Such a mutation could produce aberrancy in the function of these cells or, alternatively, could cause other non-allelic genes to function abnormally. Thus, mutated genes controlling the function of neural crest cells might lead not only to neurofibromas but also to cafè au lait patches.

This study has aimed to determine: 1) The genetic and molecular mechanisms underlying the clinical expression of SNF1 and 2) The mutational spectrum and tissue/cellular distribution of somatic *NF1* gene lesions associated with SNF1.

3.2 Patients

The study groups comprised:

1) SNF1 sporadic cases: 30 unrelated SNF1-affected individuals.

2) SNF1 familial cases:

   a) 7 families in whom the NF1 affected individual has an SNF1 parent and

   b) 2 families in whom the SNF1-affected individual has an SNF1 parent.

Molecular genetic analysis was performed on all individuals. In group 1, blood samples were obtained from the 30 unrelated SNF1 patients. From 6 of these individuals, skin biopsies from pigmented (affected) and unaffected regions were obtained under local anaesthetic, and cells were cultured as described in the Method Section 2.6.

In group 2a, blood samples were obtained from 7 NF1 children and 4 SNF1 parents. From two of the 4 SNF1 patients, skin biopsies from both affected and unaffected regions were also obtained and fibroblasts were cultured. Keratinocytes from both affected and unaffected regions of the body were obtained from one of the SNF1 patients. In group 2b, blood samples from the SNF1 affected individuals in two families were available.

3.3 Results

No one technique can detect all types of *NF1* gene mutation. Therefore, DNA samples from both the SNF1 sporadic and familial cases, were screened for mutations of the *NF1* gene using several different approaches. To detect both point mutations and small deletions/insertions of the *NF1* gene, all DNA samples from SNF1 individuals were

screened by DHPLC. To detect the common 1.5Mb deletion of the entire *NF1* gene and adjacent regions, a deletion-junction PCR assay (Lopez-Correa *et al.*, 2001) was used. Moreover, LOH and MSI studies for the detection of gross gene rearrangements were applied to 9 SNF1 patients.

Group 1: In many DNA samples, variant elution profiles were observed (Table 3.1) On subsequent characterization by cycle sequencing, a previously reported nonsense mutation in exon 27a (R1513X) (Ars *et al.*, 2003; Fahsold *et al.*, 2000; Mattocks *et al.*, 2004; Side *et al.*, 1997) was found in patient N2099, a single base-pair substitution at nucleotide position +8 in intron 32 (IVS32+8 C→G) in patient N2027. Single base-pair substitutions at position +8 have been described to be disease-causing mutations in different genes, including *ATM* (Teraoka *et al.*, 2001), *NF2* (Welling *et al.*, 1996) and *VHL* (Klein *et al.*, 2001) (Table 3.3). Many silent changes and polymorphisms were also identified in this group (Table 3.1).

Group 2: Genomic DNA from 7 NF1 individuals with an SNF1 parent was screened by DHPLC for mutations in the *NF1* gene. A variant elution profile was observed in three individuals; upon subsequent characterization by cycle sequencing, a previously reported nonsense mutation in exon 31 (R1947X) (Cawthon *et al.*, 1990; Joseph *et al.*, 1996; Krkljus *et al.*, 1998; Lazaro *et al.*, 1995) was found in patient N631, whilst a novel in-frame microdeletion in exon 40 (2396delCTA) in patient N2048, and a novel single base-pair substitution in the donor splice site of intron 45 (IVS45+2 T→C) in patient N2152, were identified (Table 3.2).

In order to see whether the single base-pair substitution in intron 45 influences the splicing process, the specific sequence around the donor splice site was analysed by the ESE finder program (http://rulai.cshl.edu/tools/ESE/). This analysis revealed that the mutation caused the disruption of SRp55 motifs (Figure 3.5). In addition, using a splice site scoring program, this change is predicted to cause the loss of donor splice site recognition. Taking these results together, we surmise that IVS45ds+2 T-C may indeed be a pathological mutation affecting the 5' splice site of exon 45. This lesion may lead to the retention of the flanking intron in the mRNA. The lack of an RNA sample from this patient did not allow us to evaluate the effect of this change at the RNA level. Considering that RNA samples are not always easy to be obtained, a minigene splicing assay using genomic DNAs was recently described and the skipping of *NF1*-exon 3 was caused by a DNA variant in intron 3 (Baralle *et al.*, 2003). Therefore, this method can

be adopted in future for investigating on the role of these DNA variants on the splicing process.

The nonsense mutation in exon 31 and the microdeletion in exon 40 were not identified in DNA from blood (N2213, N299 respectively), and affected skin (N2210: keratinocytes, N2212: fibroblasts; N2314: fibroblasts) from the SNF1 parents by DHPLC analysis and sequencing. A DNA sample from the SNF1 parent of the individual with full-blown NF1 (N2152), in whom a single base-pair substitution in intron 45 was identified, was unfortunately not available..

The nonsense mutation R1947X and the microdeletion 2396delCTA were identified in lymphocyte genomic DNA from the affected child by DHPLC. Initial PCR/ direct sequencing failed to identify the two mutations in DNA derived from fibroblast or keratinocyte cell lines from affected skin of the parent. In order to detect whether both the *NF1* mutations in exon 31 (R1947X) in patient N631, and in exon 40 in patient N2048, identified by DHPLC, were present in a mosaic pattern in the affected skin from both the SNF1 parents, DNA from fibroblasts and keratinocytes was cloned and subsequently screened for the specific mutations.

The R1947X mutation was identified in 20% (29/146) of clones derived from affected keratinocytes and in 9% (12/136) of clones from affected fibroblasts of the mother using allele-specific PCR. This mutation was not detected by either technique in DNA derived from unaffected maternal fibroblasts and keratinocytes (Figure 3.3). The microdeletion in exon 40 of the *NF1* gene was not identified in 100 clones from fibroblasts derived from affected skin taken from the SNF1 parent.

In addition, the screening of DNA samples from 100 normal controls by DHPLC did not detect the mutations identified in the SNF1 patients.

Furthermore, all DNA samples without mutations were screened using a deletion junction PCR assay (Lopez-Correa *et al.*, 2001) to detect the presence of the specific *NF1* deletion breakpoint but no characteristic 3.4 kb amplified fragment was observed, indicating that no such lesions were present in the samples studied (Figure 3.1).

Figure 3.1. Analysis of the specific *NF1* deletion breakpoints by a deletion junction-PCR assay in SNF1 patients.
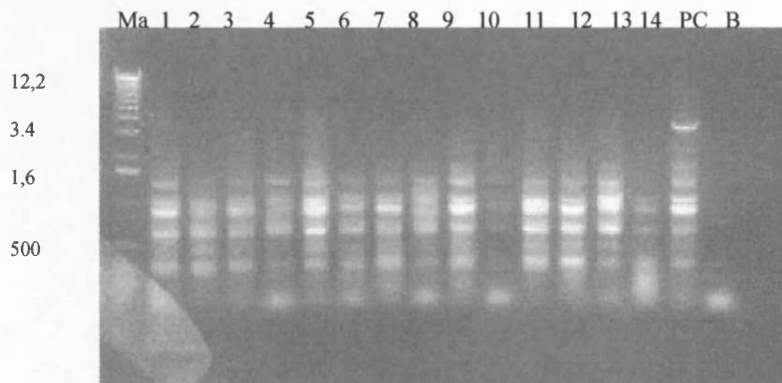
Figure 3.1: Examples of the results obtained by screening DNA samples from the SNF1 patients for the detection of the specific *NF1* deletion breakpoints by a deletion junction–specific PCR assay. The assay did not detect the specific 3.4 kb fragment in the SNF1 patients analysed. The PCR product was visualized under UV light after a time of 1 hour of electrophoretic migration into a 1% agarose gel. The DNA samples are numbered from 1-14. **Ma**: 1 kb DNA marker; **P.C.**: a positive control sample; **B**: blank.

DNA samples from 9 SNF1 patients in whom lymphocyte/fibroblast pairs were available, were screened for partial *NF1* gene deletions using a panel of 11 intragenic and extragenic RFLPs and polymorphic microsatellite markers from the *NF1* gene region. The presence of *NF1* gene large deletions in those DNA samples would be detected as a result of the loss of heterozygosity (LOH) for the specific marker which lies on the *NF1* gene deleted region. The 9 lymphocyte/fibroblast pairs of DNA samples were also screened for the presence of microsatellite instability (MSI) using 9 microsatellite markers. However, no differences were observed between the different tissues using both the LOH and the MSI analyses. This suggested that the 9 SNF1 patients did not possess large deletions of the *NF1* gene in the tissues studied.

3.3.1 RNA analysis

RNA samples from 11 SNF1 patients were screened for both multi-exon deletions and splicing defects in the *NF1* gene by long-range RT-PCR and DHPLC analysis respectively. However, these techniques did not detect either multi-exon deletions or splicing defects of the *NF1* gene in the SNF1 individuals studied.

Figure 3.2. Analysis of RNA samples from 11 SNF1 patients for multi-exon deletions and splicing defects in the *NF1* gene.
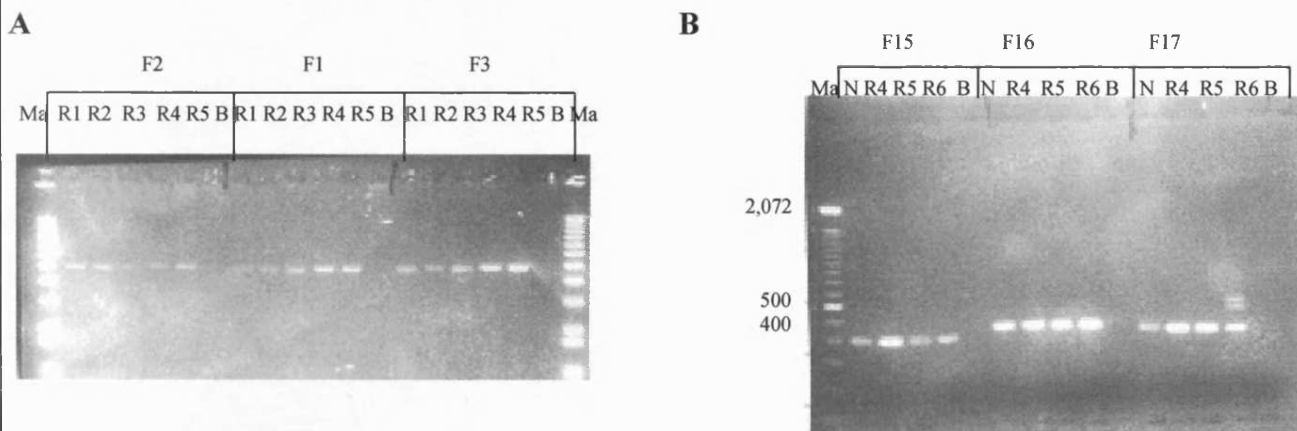
Figure 3.2.: Examples of the results obtained by screening RNA samples from 11 SNF1 patients for the detection of both multi-exon deletions and splicing defects in the *NF1* gene. A: Analysis of RNA samples by long-range RT-PCR using three primer pairs which amplify the coding region of the *NF1* gene in three overlapping fragments. The PCR product was visualized under UV light after an over night electrophoretic migration at 10w into a 0.5% agarose gel. The RNA samples are numbered from R1-R5. F1: 4.74 kb cDNA fragment (exons 1-27a). F2 : 4.8 kb cDNA fragment (exons 12b-34). F3 : 4.77 kb cDNA fragment (exons 22-49). Ma: 1 kb plus DNA marker; B: blank. **B**: Examples of the results obtained by screening the coding region of the *NF1* gene in SNF1 patients for the detection of splicing defects by RT-PCR. PCR products of three cDNA fragments (F15, F16, F17) are shown with F15 (396bp) representing exons 28-29; F16 (454bp) exons 29-30 and F17 (428bp) exons 30-31-32. R4 and R5 represent the RNA samples from 2 SNF1 patients, R6 represents the RNA samples from an NF1 patient with known mutation (5749ins171) which results in the aberrant splicing of a new exon.

Table 3.1. Group1: SNF1 sporadic cases results

DNA n°	Cell type	Location	Genomic Change	Amino acid substitution	Polymorphism previously described
N2135	Lymphocites	Ex 42	7521 A→G (Q2507Q)		
N2173	Fibroblasts from affected skin	Ex 42	7521 A→G (Q2507Q)		
N1973	Lymphocites	Ex 42	7521 A→G (Q2507Q)		
N2140	Lymphocites	IVS39			IVS39ds+37G→C
N2154	Fibroblasts from affected skin	IVS39			IVS39ds+37G→C
N1815	Lymphocites	IVS39			IVS39ds+37G→C
N1612	Lymphocites	Ex5			702 G→A (L234L)
N1759	Lymphocites	IVS39 IVS16			IVS39ds+37G→C IVS16-16 T→C
N2038	Lymphocites	IVS3 IVS10b IVS28			IVS3+41 A→G IVS10b-35delT IVS28+23 T→C
N1230	Lymphocites	Ex 12a IVS29 IVS10b IVS3	1810 T→C (L604L)		IVS29+19 T→A IVS10b-35delT IVS3+41 G→A
N2218	Fibroblasts from affected skin	Ex 12a IVS29 IVS10b IVS3	1810 T→C (L604L)		IVS29+19 T→A IVS10b-35delT IVS3+41 G→A
N2099	Lymphocites	Ex 27a	4538 C→T	R1513X	
N2027	Lymphocites	IVS32 Ex5	IVS32ds+8C→G		702 G→A (L234L)
N2027f	Fibroblasts from affected skin	IVS32	IVS32ds+8C→G		
N1974	Lymphocites	Ex 6	774 A→G (E258E)		
N1972	Lymphocites	Ex13	2034 A→G (P678P)		
N1272	Lymphocites	Ex 13 IVS 26	2034 A→G (P678P)		IVS26+50 A→G
N1857	Lymphocites	IVS11			IVS11+72 C→T

Table 3.1. The table summarizes the genomic variations identified in the *NF1* gene by DHPLC in SNF1 sporadic cases. Nucleotide numbering is based on GenBank accession no. M82814. The DNA numbers listed on the left represent the DNA samples. DNA changes detected in both lymphocytes and fibroblasts from 4 SNF1 individuals are indicated in red colour.

Table 3.2. Group2: SNF1 familial cases results

DNA n°	Cell type	Disease	Location	Genomic Mutation	Amino acid substitution	Polymorphism previously described
N2048	Lymphocytes	NF1	Ex40	7186delCTA	2396del L	
N299	Lymphocytes	SNF1		7186delCTA not identified		702 G→A, L234L
N2314	Fibroblasts from affected skin	SNF1		7186delCTA not identified in 100 clones		
N631	Lymphocytes	NF1	Ex31	C5839T	R1947X	
N2212	Fibroblasts from affected skin	SNF1	Ex31	C5839T in 9% of the cells	R1947X	
N2210	Keratynocites from affected skin	SNF1	Ex31	C5839T in 20% of the cells	R1947X	
N2152	Lymphocytes	NF1	IVS45	IVS45ds+2T→C		
N1976	Lymphocytes	NF1	Ex10b	1466 A→G	Y489C	
N1975	Lymphocytes	SNF1	Ex10b Ex5	1466 A→G	Y489C	702 G→A, L234L
N2233	Lymphocytes	SNF1	IVS3 IVS10b			IVS3+41 A→G IVS10b-35delT
N2232	Lymphocytes	SNF1	Ex10b	1466 A→G	Y489C	
N2220	Fibroblasts from affected skin	SNF1	Ex10b	1466 A→G	Y489C	

Table 3.2. The table summarizes the genomic mutations and polymorphisms identified in the *NF1* gene by DHPLC in SNF1 familial cases. Nucleotide numbering is based on GenBank accession no. M82814. The DNA numbers listed on the left represent the DNA samples. The families are designed by different colours. The family in yellow: DNA sample lymphocytes from the NF1 daughter (N2048) and DNA samples from both lymphocytes and fibroblasts from the SNF1 father (N299, N2314). The family in turquoise: DNA sample lymphocytes from the NF1 daughter (N631) and DNA samples from both fibroblasts and keratynocites from the SNF1 mother (N2212, N2210). The family in pink: DNA samples lymphocytes from both the NF1 son (1976) and the SNF1 father (1975). The family in green: DNA sample lymphocytes from the SNF1 mother (N2233) and DNA samples from both lymphocytes and fibroblasts from the SNF1 son (N2232, N2220). DNA sample from the SNF1 parent of N2152 (highlighted in red) was not available.

Table 3.3. Representative examples of disease mutations at +8

Disease	Gene	Mutation		Reference
Breast cancer, susceptibility to	*ATM*	IVS4	G-T	Teraoka *et al.* (2001)
Methaemoglobinaemia	*DIA1*	IVS5	G-C	Vieira *et al.* (1995)
Ectodermal displasia	*ED1*	IVS9	C-G	Paakkonen *et al.* (2001)
Factor VII deficiency	*F7*	IVS7	C-G	Peyvandi *et al.* (2000)
Sandhoff disease	*HEXB*	IVS10	C-T	Wakamatsu *et al.* (1992)
Neurofibromatosis 2	*NF2*	IVS2	T-G	Welling *et al.* (1996)
Von Hippel-Lindau syndrome	*VHL*	IVS2	C-T	Klein *et al.* (2001)

Figure 3.3: DHPLC and sequencing chromatograms of *NF1* mutations identified during this study

A: IVS32+8 C-G

87
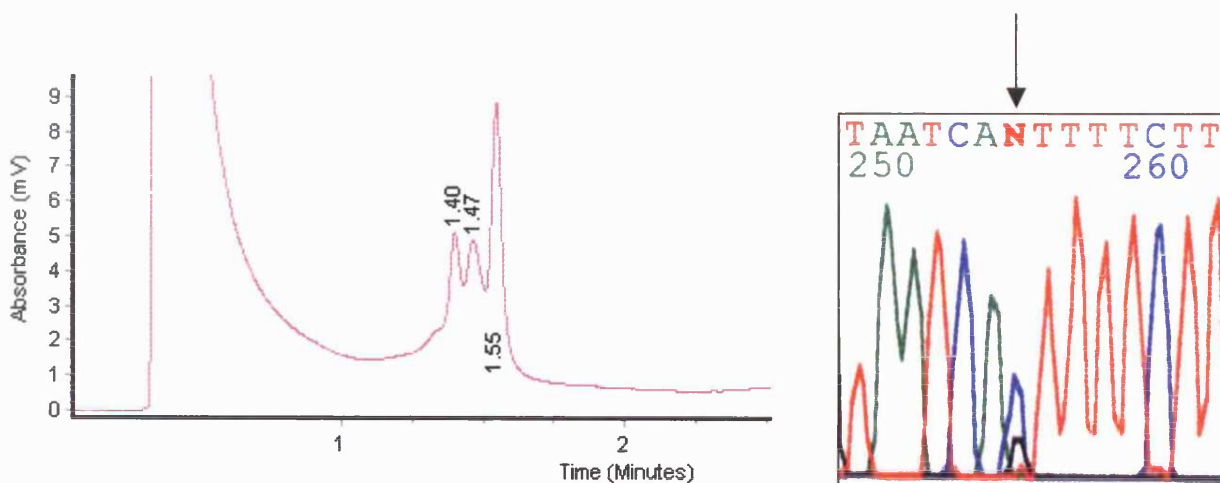
B: 4537 C-T

C: 2396delCTA

D: IVS45+2 T-C
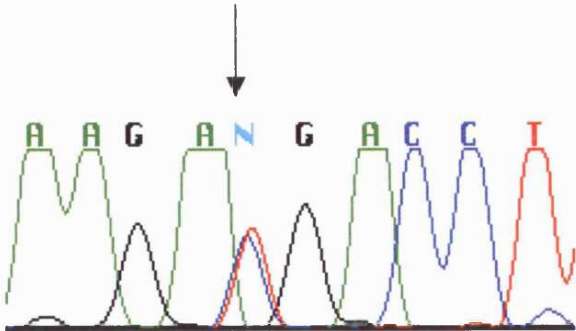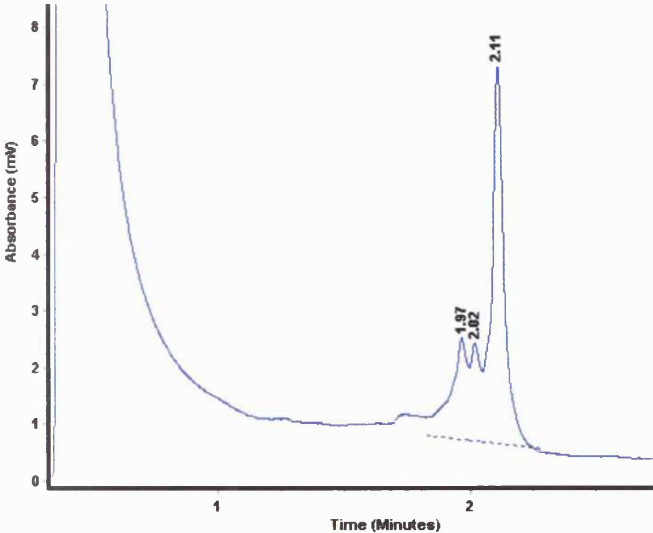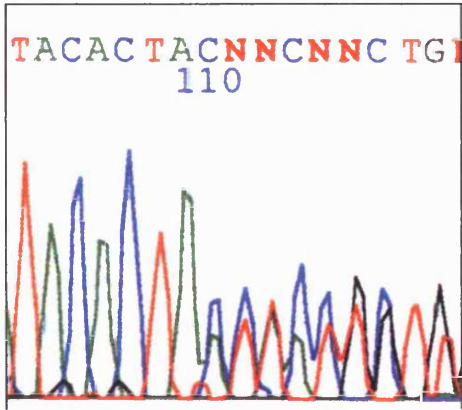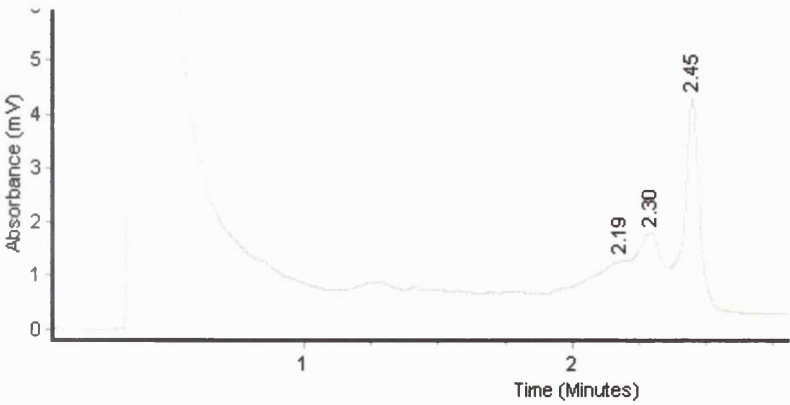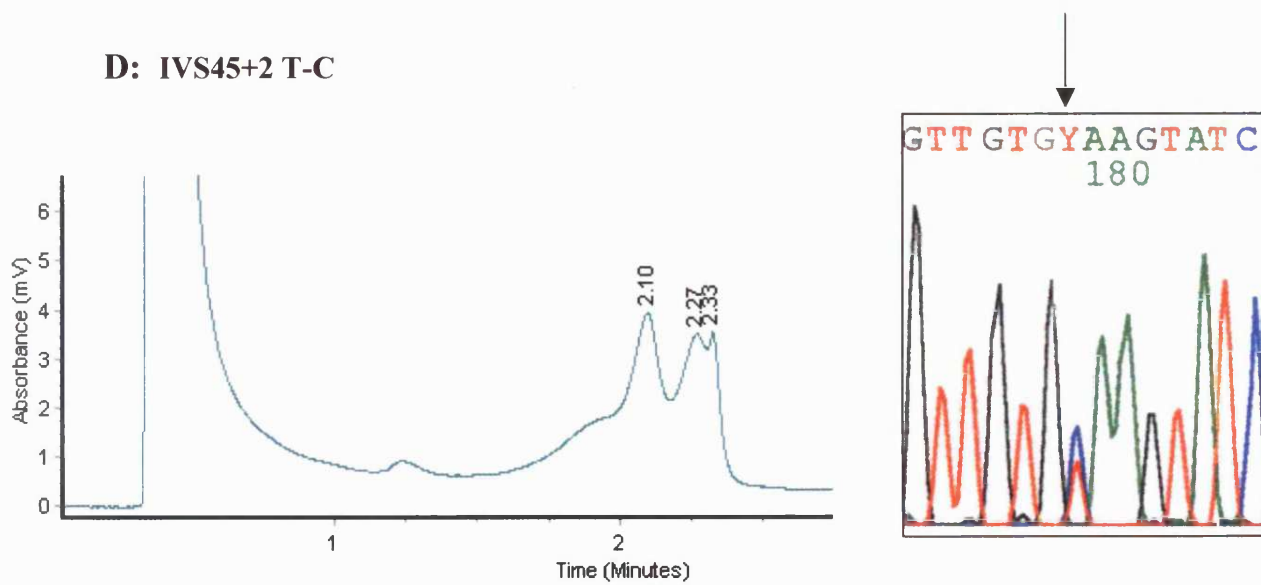
Figure 3.3: Examples of *NF1* variants identified in both SNF1 sporadic patients (A,B) and SNF1 families (C,D). DHPLC traces are shown on the left and sequencing chromatograms are illustrated on the right. Arrows indicate the position of the mutation. **A**: IVS32+8 C→G. **B** : 4537C→T in exon 27a **C**: 2396del CTA in exon 40. **D**: IVS45+2 T→C.

Figure 3.4: SNF1 family in whom the nonsense mutation (R1947X) in exon 31 of the *NF1* gene was identified
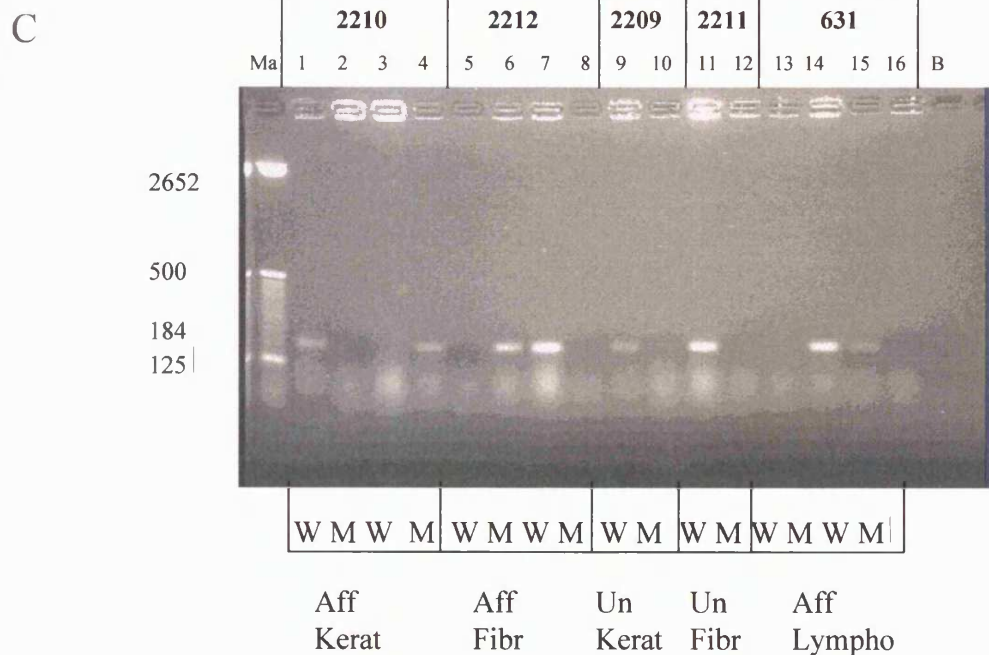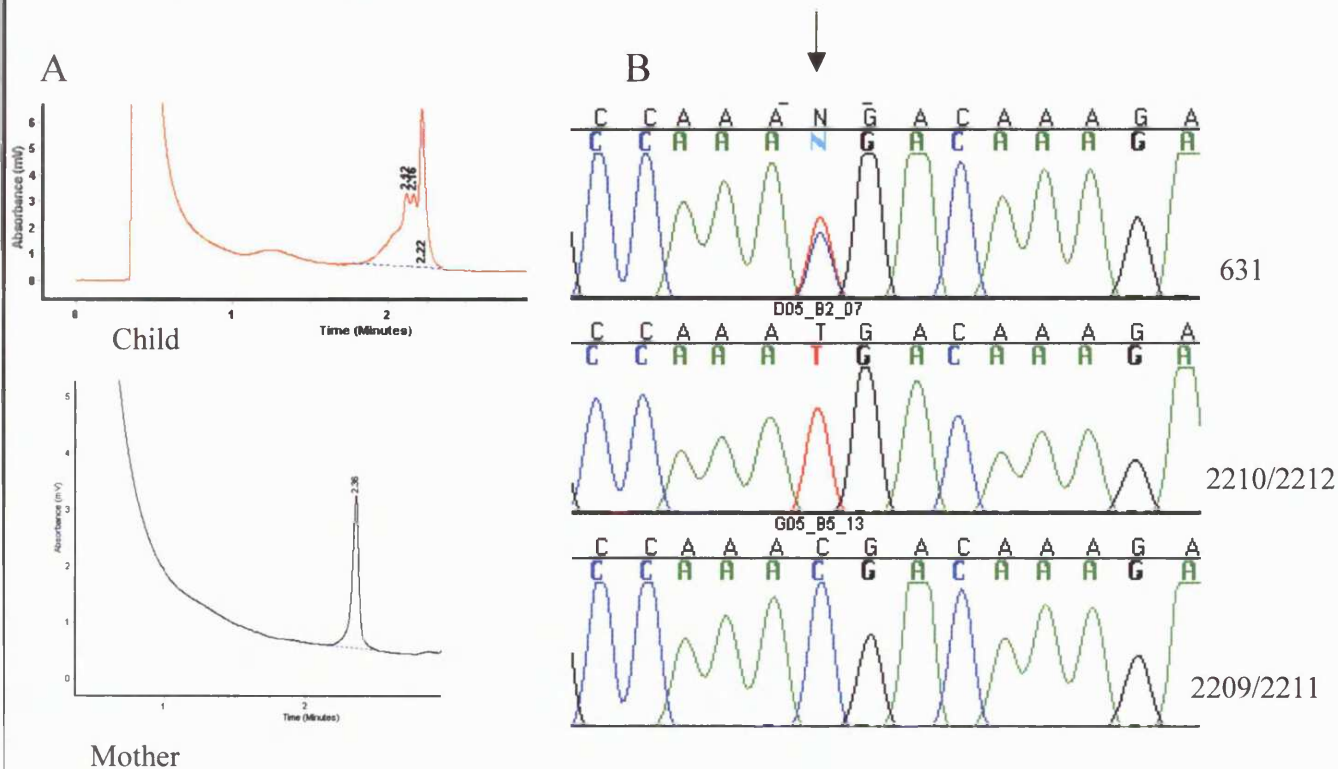
Figure 3.4: **A.** DHPLC profiles of an exon 31-containing fragment derived from the *NF1* gene from the affected child and SNF1 mother. A second lower-mobility peak is evident in the child's lymphocyte DNA sample but not in DNA of the mother's keratinocyte or the fibroblast. **B.** 631: An heterozygous 5839C→T transition (R1947X) identified in exon 31 of the child's *NF1* gene. 2210/2212: Same mutation identified in cloned PCR fragments from DNA derived from maternal affected keratinocytes and fibroblasts. 2209/2211: Wild-type *NF1* exon 31 sequence in cloned PCR fragment derived from unaffected maternal keratinocyte and fibroblast DNA. **C.** Allele-specific primers corresponding to mutant and wild-type alleles for exon 31 were designed to differentiate the 'C-containing' product (wild-type) from the 'T-containing' product (mutant). Both primer pairs amplify an exon 31-specific fragment of 184bp and the cloned PCR products were resolved on a 2% agarose gel. 2210 and 2209 represent samples of cloned DNA from keratinocytes derived from skin biopsies taken from the affected (2210) and unaffected (2209) region. Similarly, 2212 and 2211 represent cloned DNA from fibroblasts derived from the same skin biopsies from the affected (2212) and unaffected (2211) body region. 631 is cloned DNA from the lymphocytes of the affected child. The 184bp PCR product in lanes 1,7,9,11,13 and 15 represent the amplified wild-type (WT) allele while PCR products in lanes 4, 6 and 14 correspond to the mutant allele. PCR products corresponding to both the wild-type and mutant *NF1* alleles were present in cloned material from maternal affected cells (2210 and 2212) and from the child (631) but only the wild-type PCR product was evident in DNA from normal maternal cells (2209, 2211). **Ma**: 25bp DNA marker, **Kerat**: keratinocytes, **Fib**: fibroblasts, **B**: blank, **Aff**: affected; **Un**: unaffected, **Lympho**: lymphocytes, **W**: wild-type, **M**: mutant.

Figure 3.5: ESE finder results for the single base-pair substitution in intron 45 of the *NF1* gene
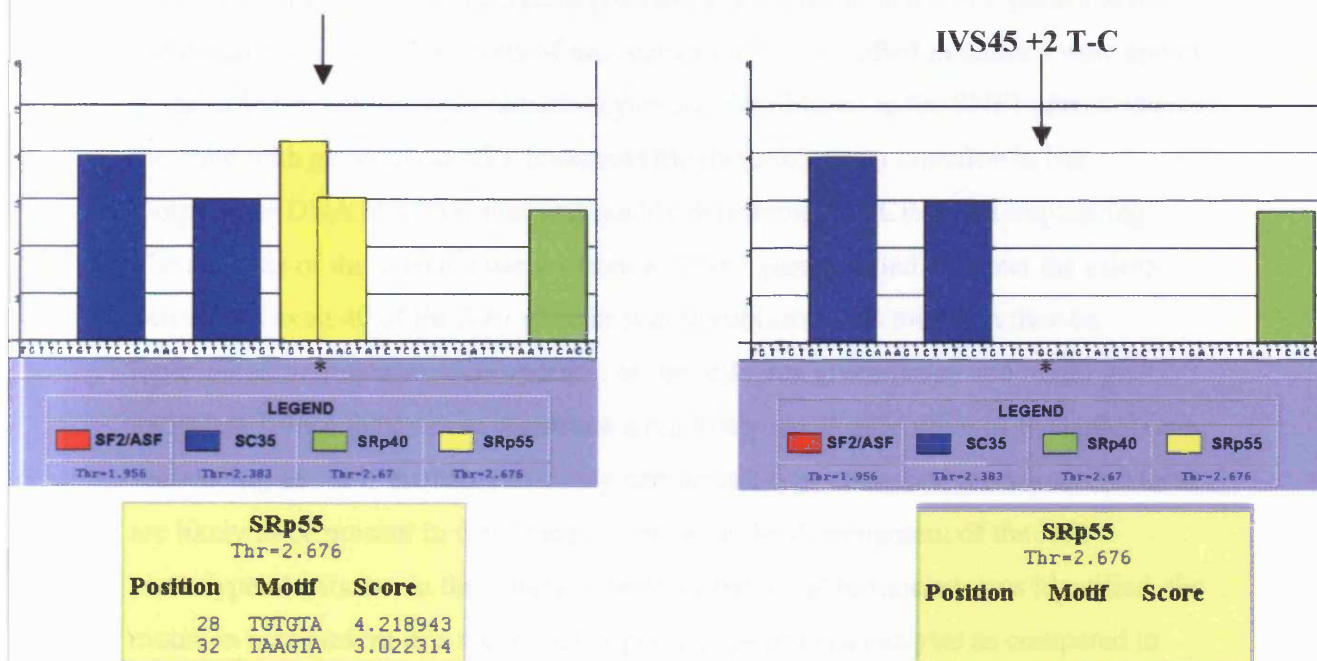
Figure 3.5 ESE finder predictions for the wild-type sequence of *NF1* exon/intron 45 (on the left) and corresponding mutant (IVS45+2 T→C) oligonucleotide (on the right). The bar heights reflect the score of the motifs. Threshold values and colour-code for each of the four motifs are indicated. The site of mutation IVS45+2 T→C is indicated by an asterisk. Arrows indicate the SRp55 motif disruption caused by the mutation.

3.4 Discussion

During this study, in order to determine the genetic and molecular mechanisms underlying the clinical expression of SNF1 and to understand the mutational spectrum and tissue/cellular distribution of somatic *NF1* gene lesions associated with SNF1, a cascade of different techniques has been used.

Mutation screening of lymphocyte cell lines from individuals with an SNF1 parent yielded three mutations that were identified in the NF1 children. The identification of *NF1* gene mutations in offspring with full-blown NF1 whose parents have SNF1 has

aided the detection of the pathological lesions in the affected tissues of the parents. No tissues were available from one SNF1 parent. The analysis of the affected tissues from an SNF1 parent detected the nonsense mutation in exon 31 of the *NF1* gene in mosaic pattern in two different cell lines.

This is the first study demonstrating gonosomal mosaicism in a SNF1 patient at the molecular level. Variable levels of mosaicism for the identified mutations were apparent in the different cell lineages (keratinocytes and fibroblasts) in the SNF1 parent whereas the child with generalised NF1 possessed the (heterozygous) mutation in her lymphocyte DNA at a level that was readily detectable by PCR/direct sequencing. The analysis of the affected tissues from an SNF1 parent failed to detect the micro-deletion in exon 40 of the *NF1* gene in skin fibroblasts. This mutation may be represented in only a small proportion of the cells in a given tissue and might go undetected since they would constitute a relatively small proportion of the signal on a sequencing gel. It is probable that only certain cell types manifest mutations and these are likely to be present in cell lineages critical to the development of the NF1 phenotype. As shown in the family in which gonosomal mosaicism was identified, the mutation was present in a much higher percentage of keratinocytes as compared to fibroblasts suggesting that somatic mosaicism is cell type-specific and that keratinocyte cell lines may be more prone to exhibit the mutation. Unfortunately, keratinocytes were not available for this SNF1 family. In addition, the somatic mosaicism might not be the only mechanism underlying the SNF1 phenotype in those families. However, mutations in other genes or *NF1* gene promoter hypermethylation might be involved in the pathogenesis of SNF1. The mutational analysis of the *NF1* gene in DNA from both keratinocytes and also melanocyte cell lines from a larger number of SNF1 families in whom a child is affected by full-blown NF1, may confirm our findings but also provide new insights into the cell lineages involved in SNF1 pathogenesis.

The results obtained by screening the 30 unrelated SNF1 patients by DHPLC for mutations in the *NF1* gene have demonstrated a nonsense mutation (R1513X) in leucocytes/DNA sample from a SNF1 affected individual. DNA from different cell lines from the patient and from the unaffected parents was unfortunately not available. Three individuals exhibited different sequence variants that were detectable in both lymphocytes and fibroblasts, but abnormalities at the RNA level were not identified.

This study has demonstrated that the analysis of the relevant cell lineages is critical to obtaining a meaningful diagnostic result. Further, molecular studies should provide new insights into the key cell lineages involved in SNF1 pathogenesis. Melanocytes, for example, would be the most relevant cell type to study in SNF1 patients. Supposing that the original embryonic mutation is transmitted to all skin cell types within a limited region and that a subsequent embryonic migration of the daughter cells leads to some scattering. Biopsy from a site defined by the melanocyte abnormality would be expected to exhibit the mutation in most melanocytes and many keratinocytes but in a smaller proportion of fibroblasts.

Identification of *NF1* gene mutations in SNF1 patients is vital for both the clinical management and genetic counselling of these individuals (Listernick *et al.*, 2003). Genetic counselling in such patients is difficult as gonadal mosaicism for NF1 has been reported (Lazaro *et al.*, 1994) and SNF1 patients have had offspring with either classical or segmental NF1 (Obringer *et al.*, 1989; Boltshauser *et al.*, 1989; Rubenstein *et al.*, 1983, Consoli *et al*, 2005). Knowing the mutation in an SNF1 parent at least makes it possible to exclude or confirm generalised NF1 in the offspring by first trimester prenatal diagnosis.

The DHPLC is a highly sensitive technique for mutation detection in the *NF1* gene and has been applied to single nucleotide polymorphism analysis and the mutational screening of numerous disease genes (Gross *et al.*, 1999; Nickerson *et al.*, 2000; Dobson-Stone *et al.*, 2000). DHPLC also has the potential to identify low level mosaicism (Emmerson *et al*, 2003). However, gross rearrangements and multi-exon deletions, which together comprise between 10% (Upadhyaya and Cooper, 1998; Fahsold *et al.*, 2000) and 15% (Kork, 1998) of all *NF1* gene mutations, are unlikely to be detected by DHPLC.

It is difficult to find mutations in mosaic individuals because one of the cell lineages is less well represented than the other. It is clear that an efficient and sensitive mutation detection technique is required for the identification of low-level mosaicism. Although DHPLC can detect low level mosaicism but in this study, DHPLC was unable to detect mosaicism.

The clinical effect of somatic mosaicism depends on the developmental stage at which the mutation occurs. A mutation that occurs very early on in embryonic development is likely to affect many somatic tissues and may therefore be expected to result in generalized NF1 (Colman *et al.*, 1996). By contrast, mutations occurring later on may give rise to a phenotype confined to a single body region or even to a single organ (Tinschert *et al*, 2000; Petek *et al*, 2003; Vandenbroucke *et al*, 2004). Somatic mosaicism arising at a very early embryonic stage can involve both somatic cells and germ cells. Such individuals (gonosomal mosaics) are at risk of having affected children.

Segmental or 'localized disease' has been mainly studied in dermatological disorders in which the pattern of affected and healthy skin follows the 'lines of Blaschko'. Moss (1999) postulated that disorders exhibiting Blaschko's lines (Moss *et al.*, 1993) are caused by mutations in genes expressed in epidermal cells (keratinocytes and melanocytes) rather than in dermal fibroblasts. This has been supported by Huson and Ruggieri (2000), who have suggested that pigmentary changes alone tend to follow Blaschko's lines whereas neurofibromas tend to have a dermatomal distribution. Moreover, there are several examples of skin disorders in which mutations of the causing gene were identified in the affected tissue but not in the unaffected. Mutations in the *ATP2A2* gene have been identified in the affected but not in the unaffected skin or leukocytes of segmental Darier's disease patients (Sakuntabhai *et al.*, 2000). A mutation in the *KRT10* gene has been identified in keratinocytes from a linear bullous congenital ichthyosiform erythroderma (BCIE) patient but not in the normal epidermis (Moss *et al.*, 1995). Three families with apparent gonosomal mosaicism for BCIE have also been described, in which a parent with linear BCIE had offspring with generalized BCIE caused by keratin gene (*KRT1 and KRT2*) mutations also present in the parents' abnormal skin (epidermal cells: keratinocytes and melanocytes) but not in the normal skin (Paller *et al.*, 1994).

The molecular mechanisms underlying disease pathogenesis in SNF1 patients are as yet unclear. SNF1 is believed to result from postzygotic *NF1* gene mutation. A spontaneous mutation would affect only the progeny of the mutant cell, thereby limiting its effects to a single unilateral dermatome. Double mutations to bilateral segments or migration of affected pluripotential cells across the midline would lead to bilateral expression of the disorder. Although the majority of cases of SNF1 are sporadic, there are reports of NF1

families in which only one parent exhibits segmental features of the disease but whose children manifest the full classical form of NF1. Mosaicism of the germ cells would explain the apparent genetic transmission of NF1 to the offspring of patients with SNF1. Mosaicism of the germ cells can also explain the cases reported by Moss and Green (1994) and Boltshauser *et al.* (1989) in which NF1 was found in the children of patients with SNF1. Tinschert *et al.* (2000) have demonstrated *NF1* gene mosaicism in a patient with segmental NF1. This patient showed CAL spots and freckles limited to the left side of the neck, upper part of the trunk, axilla and arm. An *NF1* gross deletion in a mosaic pattern was demonstrated by FISH in fibroblasts cultured from a CAL spot but not in those derived from skin or in peripheral blood leucocytes.

Further studies are needed to improve our understanding of the basic biological molecular mechanism underlying SNF1, the mutational spectrum and distribution of *NF1* gene mutations in different tissue types, and the proportion of mutations causing mosaicism in different cell lineages. With the availability of more sensitive quantitative assays capable of detecting low frequency mosaic mutations against normal backgrounds , it will become possible to elucidate the molecular basis of the pathology in the majority of SNF1 families.

Chapter 4. Gene expression analysis of fibroblast cells from SNF1 patient tissues using Affymetrix microarray chips

4.1 Introduction

Gene expression is the process by which a gene's encoded information is converted into the various structures and functional activities of a cell. The amount of protein that a cell expresses depends on the tissue, the developmental stage of the organism, and the metabolic or physiological state of the cell. The relationship between the gene expression pathway and variation in the cellular phenotype is however not well understood. Identifying a relationship between differences in gene expression and the clinical phenotype may be important in understanding the molecular basis of many complex diseases. Although genetic variation in gene expression has been associated with phenotypic variation (Schadt *et al.*, 2003; Oleksiak *et al.*, 2002; Townsend *et al.*, 2003; Brem *et al.*, 2002; Fay *et al.*, 2004), gene expression differences that correlate with a phenotype may not necessarily contribute to that phenotype. Further, changes in gene expression at the mRNA level may not be evident at the level of the translated functional protein product.

Many factors influence the control of gene expression, such as initiation of transcription, RNA processing, RNA transport, mRNA degradation, initiation of translation and post-translational modification. Moreover, a large number of environmental factors, including temperature, stress, light, and other signals, that lead to change in the level of hormones and other signalling substances may affect gene expression (Schena, 2000). In the light of all the factors mentioned above, the study of gene expression requires a complex and accurate analysis.

There are a variety of methods for detecting and measuring gene expression levels, including northern blotting (Alwine *et al.*, 1977), S1 nuclease protection (Berk & Sharp, 1977), differential display (Liang & Pardee, 1992), sequencing of cDNA libraries (Adams *et al.*, 1991; Okubo *et al.*, 1992) and serial analysis of gene expression (SAGE) (Velculescu et al., 1995). More recently, microarray technologies and real-time PCR

97

have been developed for gene expression measurement. Real-time PCR is considered to be a very quick and accurate method that allows the highly sensitive quantification of transcriptional levels of the gene of interest using a small amount of RNA sample (Kammula et al., 2000; Mandigers et al., 1998). Using the real-time PCR assay, the involvement of several molecular pathways in the genesis of plexiform neurofibroma (Levy et al., 2004) and other human diseases (Latil et al., 2003) has been described. By contrast, microarray technologies permit the parallel detection and analysis of the patterns of expression of thousands of genes in a single experiment. The sensitivity of microarrays allows the detection of subtle differences that are much harder to detect with other molecular methods.

Microarray technology is based on the ability of complementary DNA sequences to bind to each other under specific conditions. The two main types of microarrays, oligonucleotide arrays and cDNA arrays have been used to study gene expression in parallel (Schena et al., 1995; 1996; Lockhart et al., 1996). Both oligonucleotide and cDNA arrays can be used in genomic analysis, although oligonucleotide arrays are more sensitive to single nucleotide mutations. A DNA microarray experiment can be divided into several stages that comprise (i) the construction of DNA arrays, (ii) the preparation of labelled cDNA targets and (iii) hybridisation and the analysis and interpretation of the results obtained. There are differences between the two types of array construction. In a cDNA microarray, PCR products, usually generated from cDNA libraries, are used as sequences to be printed onto glass slides or nylon membranes, whereas, for oligonucleotide arrays, oligonucleotide sequences in the range of 25-80 base-pairs in length, are selected from the mRNA reference sequence of each gene or from EST (expressed sequence tag) sequences and, subsequently, synthesized in situ onto solid substrate (Figure 4.1).

Two different types of oligonucleotide arrays can be distinguished by the length of the oligonucleotide sequences on the array. Long oligonucleotide arrays that are composed by nucleotide sequences of approximately 70 bases in length, printed onto glass slides, and short oligonucleotide arrays, made by Affymetrix, that consist of small 25 base-pair oligonucleotides synthesized directly onto the chip by photolithography (Lipshutz et al., 1999; Fodor et al., 1991). In addition, on Affymetrix chips, half of the oligonucleotide sequences are designed to be a perfect match for a specific transcript, whereas the other half presents a single base mismatch in the centre of the 25 base oligonucleotide. This

allows determination of the degree of non-specific binding by comparison of target binding intensity between the two partner oligonucleotides.

Microarray was first used to study gene expression in 1997 (DeRisi *et al.*, 1997). It was initially developed to study differential gene expression using complex populations of RNA (Lipshutz *et al.*, 1999). Now, with improvements to the method, it permits the analysis of copy number imbalances and gene amplification (Pollack *et al.*, 1999). Recently, an NF1 chip encompassing 2.24 Mb of 17q11.2 has been described (Mantripragada *et al*, 2005). An array-CGH analysis of NF1 patients using this high resolution array has shown high degree of specificity and sensitivity. This array will prove to be an important tool for identification of copy number variation not only within *NF1* gene but also in the region flanking this gene. Array CGH can help to provide information on the germline and somatic polymorphisms as measured at the level of micro-deletion or micro-gains. It is also worth stressing that in addition to single nucleotide polymorphisms affecting gene expression, such microdeletion or microgains are also likely to be important. Microarrays have also been applied to the analysis of expression at the protein level (Haab, 2001).

Microarray experiments may help us to identify genes that characterise particular disease states or that permit a distinction to be made between particular subtypes of different disease states. One of the most common ways is by comparing the gene expression levels in two different samples, for example the same cells under two different conditions. Such a comparison might be for instance between knock-out and wild-type cells, tumour and normal cells, or chemically treated cells and control cells. The procedure is based on the differential labelling of the mRNA extracted from each of the samples. As the amount of fluorescence emitted upon laser excitation corresponds to the amount of nucleic acid bound to each spot, the relative expression levels of the genes in both samples can be estimated from the fluorescence intensities and colours for each spot.

The preparation of labelled cDNA targets begins with the extraction and preparation of mRNAs from two populations of cells. Then each of the two mRNAs is reverse transcribed separately with the incorporation of different fluorescently tagged nucleotides (typically Cy3 and Cy5) producing two populations of differentially labelled cDNA samples. The two complex labelled samples are combined and are then

simultaneously hybridised to the cDNAs on the microarray. After laser excitation, the resulting fluorescence of the hybridised samples on the microarray is then detected by a device called the reader. The intensity of fluorescence from the two labelled cDNAs on a particular spot is determined using the reader's confocal laser scanning microscope. The resulting data can then be put into a database and analysed. Various bioinformatics approaches have been developed to process and visualize the large quantity of data generated and further approaches are being developed to compare gene expression profiles from multiple experiments.

Comparing the amount of mRNA molecules in a cell at a given moment but under diverse conditions, microarrays can provide important information about the corresponding protein. However, the relationship between the abundance of the mRNA and the corresponding protein is not always straightforward (Gygi *et al.*, 1999). In fact, regulation of mRNA levels is only one aspect of biological control. Protein levels, as mentioned above, are also controlled at several post-transcriptional steps and protein activity is controlled by post-translational modification. The eventual level of the protein may not reflect the mRNA expression level.

Microarray analysis may provide information on disease pathology, progression, resistance to treatment, and response to cellular microenvironments and may also lead to improved early diagnosis and innovative therapeutic approaches for cancer. The identification of single gene products that are expressed in tumour cells but not in normal tissue can be of considerable interest for diagnostic purposes. Moreover, microarray analysis can be used to distinguish distinct subtypes of different tumours including leukaemia (Golup *et al.*, 1999), breast cancer (Perou *et al.*, 2000), lymphoma (Alizadeh *et al.*, 2000) and melanoma (Bittner *et al.*, 2000). In addition to the analysis of cancer, microarrays have been used to detect changes in gene expression in several other diseases, including muscular dystrophy (Chen *et al.*, 2000), Alzheimer's disease (Ginsberg *et al.*, 2000), schizophrenia (Mirnics *et al.*, 2000) and HIV infection (Geiss *et al.*, 2000).

High-quality RNA is crucial for successful microarray experiments. To evaluate both the quantity and the quality of the RNA samples, agarose gel electrophoresis or microcapillary-based devices such as the Agilent Bioanalyzer (Agilent Technologies),

can be used. The latter has a very important advantage which is the small amount of sample required. To interpret the amount of data obtained by microarray analysis and so draw conclusions, statistical and computational approaches are required (Kell & King, 2000; Gaasterland & Bekiranov, 2000; Sherlock, 2000).

Finally, gene-expression data can be combined with analysis of genomic alterations, for example by microarray analysis of comparative genomic hybridization, whereby genomic DNA is hybridized to immobilized bacterial artificial chromosome (BAC) or cDNA clones (Pollack *et al.*, 1999), to identify gene amplifications or deletions that are involved in tumour development. Moreover a correlation between copy number and expression has been reported (Ornoloft *et al*, 2002; Pollack *et al*, 2002). These findings provide evidence that widespread DNA copy number alteration can lead directly to global deregulation of gene expression.

Figure 4.1. Overview of the steps involved in DNA microarray experiments.
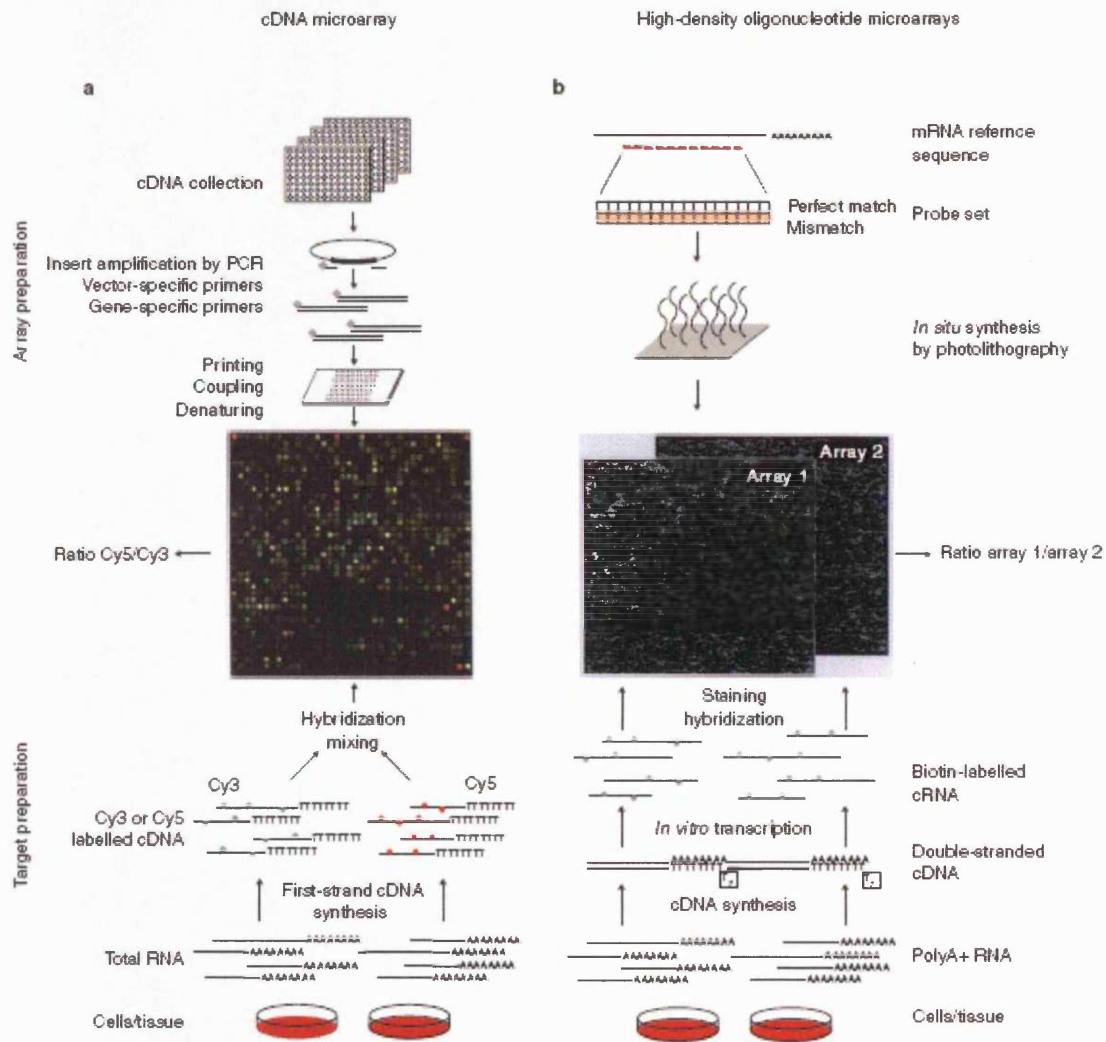
Figure 4.1. The figure shows a schematic representation of probe array and target preparation for cDNA microarrays and high-density oligonucleotide microarrays (derived from Schulze & Downward, 2001).

4.1.1 Gene expression studies in NF1

Microarray technologies have been used to characterize the expression profiles of different tumours associated with NF1. The molecular mechanisms responsible for the malignant progression of neurofibromas are unknown and only a few relevant genetic alterations have so far been identified (reviewed by Cichowski & Jacks, 2001; Dasgupta & Gutmann, 2003) (Figure 4.2).

Figure 4.2. Models of NF1-associated tumorigenesis
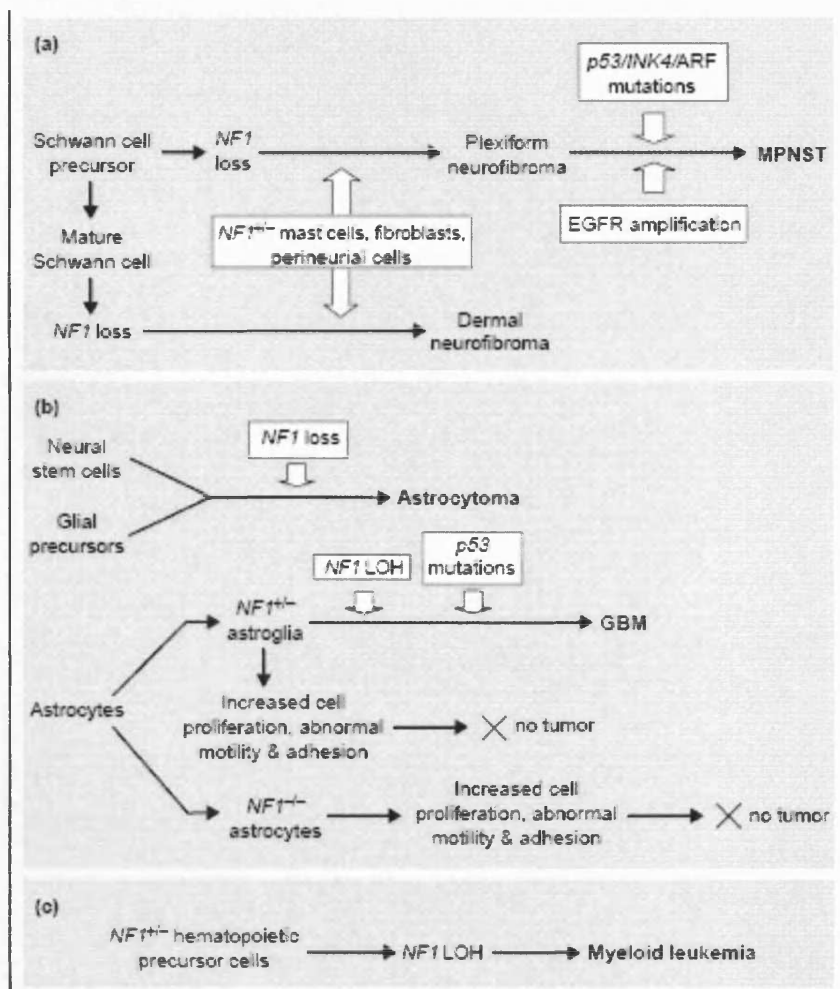
Figure 4.2. a) Formation of plexiform neurofibromas by loss of *NF1* gene in Schawnn cells in cooperation with *NF1*^{+/-}, mast cells fibroblasts and perineurial cells. Subsequent development of MPNSTs from plexiform neurofibromas caused by accumulation of other genetic alterations such as *p53*, *p16* or *p27* loss. Dermal neurofibroma formation by loss of the *NF1* gene in Schawnn cells in cooperation with *NF1*^{+/-} cell types. **b)** Astrocytoma formation caused by loss of *NF1* gene in astroglial cells. High-grade

glioblastoma multiforme (GBM) tumour development caused by *NF1* LOH in cooperation with additional genetic changes. This is very unlike in NF1 patients. **c)** Myeloid leukaemia development by *NF1* LOH (Derived from Dasgupta & Gutmann, 2003).

Different approaches have been used to characterise the gene expression profiles of neurofibromas and MPNSTs from both NF1 and non-NF1 individuals. In addition, gene expression analysis has been proven to be very useful in trying to understand the role of specific *NF1* gene mutations in the development of other NF1-related clinical features.

The use of real-time PCR assay, which is considered very accurate for analysing the expression of a small number of genes (Paradis *et al.*, 2003), revealed significant differences in the expression of genes involved in cell proliferation, senescence, apoptosis and extracellular matrix remodelling between MPNSTs and plexiform neurofibromas (Levy *et al.*, 2004). Moreover, using the same method for *NF1* gene expression analysis in sporadic pilocytic astrocytomas (PAs), Wimmer *et al.* (2002) showed a 10-20 fold increase of *NF1* expression in all the astocytoma samples studied as compared to normal brain. An increased *NF1* mRNA expression level in sporadic PAs has previously been reported (Suzuki *et al.*, 1991; Platten *et al.*, 1996). Further investigations on gene expression profiles of both sporadic and NF1-associated PAs by Western blotting and oligonucleotide array analysis identified transcripts that were only expressed in PAs as compared to other tissue and cell lines (Gutmann *et al.*, 2002), suggesting a possible unique expression pathway in PAs.

As shown for other types of tumour, including leukaemia and breast cancer, DNA microarray technologies may be used to distinguish different subtypes of tumour. Therefore, cDNA microarrays provide a new approach to classifying nerve sheath tumours based on different gene expression patterns identified between neurofibromas and MPNSTs and between dermal neurofibromas and plexiform neurofibromas (Holtkamp *et al.* 2004). Recently, Watson *et al.* (2004) performed gene expression profiling on 25 NF1-associated and 17 sporadic MPNSTs using oligonucleotide microarrays. They identified the relative over-expression of transcripts associated with neuroglial differentiation and relative down-regulation of proliferation and growth factor-associated transcripts in 9 of 42 tumours analysed.

It has been hypothesized that over-expression of the epidermal growth factor receptor (EGFR) is associated with MPNST pathogenesis (Halling et al., 1996; Kindblom et al., 1995; Kourea et al., 1999; Nielsen et al., 1999; DeClue et al., 2000). Although a recent gene expression study has confirmed that an increased EGFR expression level was present in both sporadic and NF1-associated MPNSTs as compared to dermal and plexiform neurofibromas (Holtkamp et al. 2004), Watson et al. (2004), did not identify EGFR expression in any of the MPNST samples studied.

Microarrays of mouse genes have given new insights into gene expression in embryonic development, regions of the brain and during apoptosis. Using cDNA arrays for the gene expression analysis of Nf1 mutant mouse Schwann cell cultures, the brain lipid binding protein (BLBP) gene was reported to be over-expressed in specific type of cells (Nf1 $^{-/-}$ TXF) that display characteristics of tumour cells, such as rapid proliferation and loss of interaction with axons (Miller et al., 2003). BLBP belongs to a family of fatty acid binding proteins, known to inhibit the GAP activity of neurofibromin and p120GAP (Bollag & McCormick, 1991; Golubic et al., 1998; Han et al., 1991; Sermon et al., 1996) and they have also been implicated in the pathogenesis of several malignancies (Celis et al., 1996; Custer et al., 1984; Das et al., 2001; Rasmussen et al., 2001). Elevated expression of BLBP in Nf1 $^{-/-}$ TXF cells was also shown to be related to aberrant EGFR expression (Miller et al., 2003). The hypothesis proposed by the authors was that an NF1 gene mutation might lead to EGFR over-expression leading to elevated levels of BLBP.

Considering the difficulties in obtaining tissue samples from patients, lymphocytes have also been used to explore gene expression pathways in a variety of haematological malignancies (Golub et al., 1999; Alizadeh et al., 2000; Yeoh et al., 2002), autoimmune disorders (Bennett et al., 2003; Baechler et al., 2003) and infectious diseases (Baldwin et al., 2003; Boldrick et al., 2002). Recently, it has been demonstrated that NF1 is associated with significant expression changes in lymphocyte cells (Tang et al., 2004a; 2004b). They identified a significant alteration in the expression of genes involved in protein transport and many GTPase regulator proteins. In addition, two genes (KRAS2 and GAS7) that are involved in the control of the cell cycle and/or cell proliferation, were over-expressed in NF1 patients (Tang et al., 2004a). By contrast, in the other study, a group of genes related to tissue remodelling, bone development and tumour

105

suppression have been shown to be down-regulated in NF1 lymphocyte samples (Tang *et al.*, 2004b).

As described in Chapter 1, CAL spots represent hyperpigmented areas of the body affecting approximately 99% of NF1 patients. CALs are histologically characterized by the presence of giant melanosomes within the melanocytes (Benedict *et al.*, 1968). Hypothesising a key role of these specific skin cell lines on the hyperpigmentation of CALs, the expression profiling studies of CALs from NF1 patients have been directed to the analysis of melanocyte cultured cells. Many different cytokines and cell growth factors are involved in the control of the development and function of melanocytes. Modification of this control system could lead to the altered proliferation and differentiation of the cells and also to an abnormal interaction between the other types of skin cells, such as keratinocytes and fibroblasts (Figure 4.3). One recent study shows that epidermal hyperpigmentation in NF1 CALs might be associated with the stem cell factor (SCF) and the hepatocyte growth factor (HGF) (Imokawa, 2004). Using RT-PCR in cultured fibroblasts from NF1 CALs, over-expression of SCF and HGF mRNA, was previously demonstrated (Okazaki *et al.*, 2003). These findings suggest that an increased level of SCF and HGF production by fibroblasts might affect both dermal mast cells and epidermal melanocytes and so cause the hyperpigmentation seen in CALs.

Recently, comparing the expression levels of thousands of genes by cDNA microarrays between CALs and normal skin from both a NF1 patient and normal control, a possible effect of *NF1* gene mutation on the expression pathway of melanocyte cell lines has been shown (Boucneau *et al.*, 2005). More precisely, cultured melanocyte cells from CALs exhibited differentially expressed genes involved in cell proliferation and cell adhesion control as compared with normal cultured cells. In addition, a large number of transcription factor genes, among which a specific subset important in melanocyte lineage development, were down-regulated in comparison to normal cultured cells (Boucneau *et al.*, 2005).

Comparing the gene expression in skin fibroblast lines from NF1 patients and normal skin fibroblast controls by cDNA microarrays, different expression profiles have been identified between the two groups. However, evaluation of the results obtained require

further analysis (Peltonen *et al.*, 2005). Taken together, these findings show that microarray technology is an ideal approach to analyse gene expression patterns and the underlying pathoetiology of pigmentary skin disorders.

In this work, a pilot study was therefore attempted, using Affymetrix U133A arrays, in order to understand better the mechanisms underlying the clinical phenotype seen in SNF1. Using this approach, it was aimed that the identification of over- or under-expressed genes in the affected tissue as compared to normal tissue from SNF1 patients, could lead to new insights in the pathogenesis of this disease.

Figure 4.3. Paracrine cytokine model for epidermal hyperpigmentation
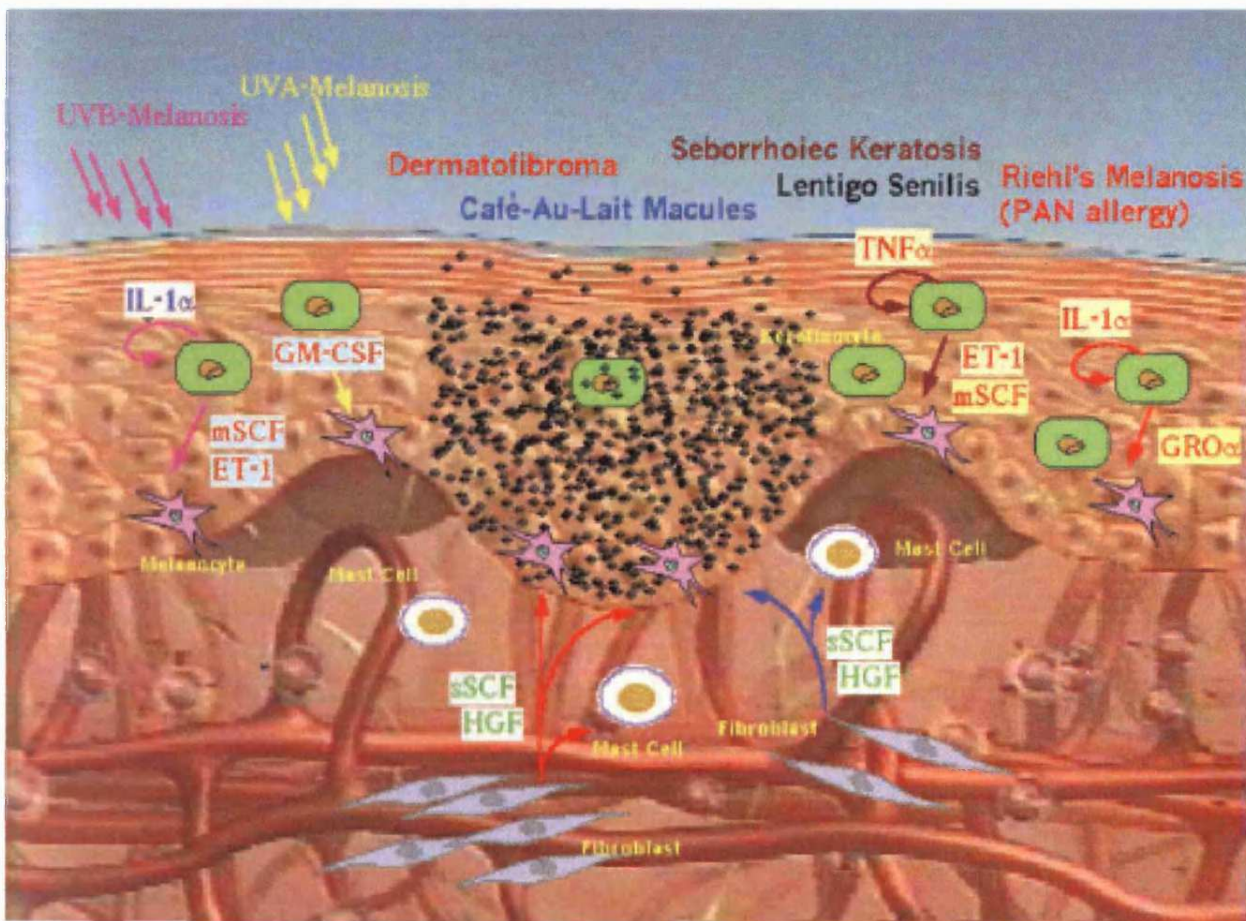
Figure 4.3. Paracrine regulation of melanocytes in UVB-melanosis, UVA-melanosis, lentigo senilis, seorrhoeic keratosis, Rielh's melanosis (PAN allergy), dermatofibroma, and café-au-lait macule. mSCF, membrane-bound type SCF; sSCF, soluble type SCF (taken from Imokawa, 2004).

4.2 Results

The gene expression analysis was performed on RNA samples derived from cultured fibroblasts from both affected and unaffected areas of the body from five SNF1 individuals of which three were sporadic and two familial cases. Both the familial cases had a parent affected by full-blown NF1 with an *NF1* gene mutation detected at the genomic level. Previous mutation analysis of the *NF1* gene of DNA samples from all those SNF1 individuals had revealed no mutations in the 3 sporadic cases but in one of the familial cases. However, a nonsense mutation was identified in mosaic pattern in cell lines from CAL spots from the other familial case.

.

The quality of all RNA samples was assessed on an agarose gel and subsequently, using Agilent 2100 Bio Sizing. Sample labelling, hybridisation to arrays and image scanning were performed by Megan Musson (Pathology Department, Cardiff University) using a standard Affymetrix protocol (Affymetrix expression analysis technical manual) as described in Figure 4.4.

Figure 4.4. GeneChip Eukaryotic target labelling assays for expression analysis
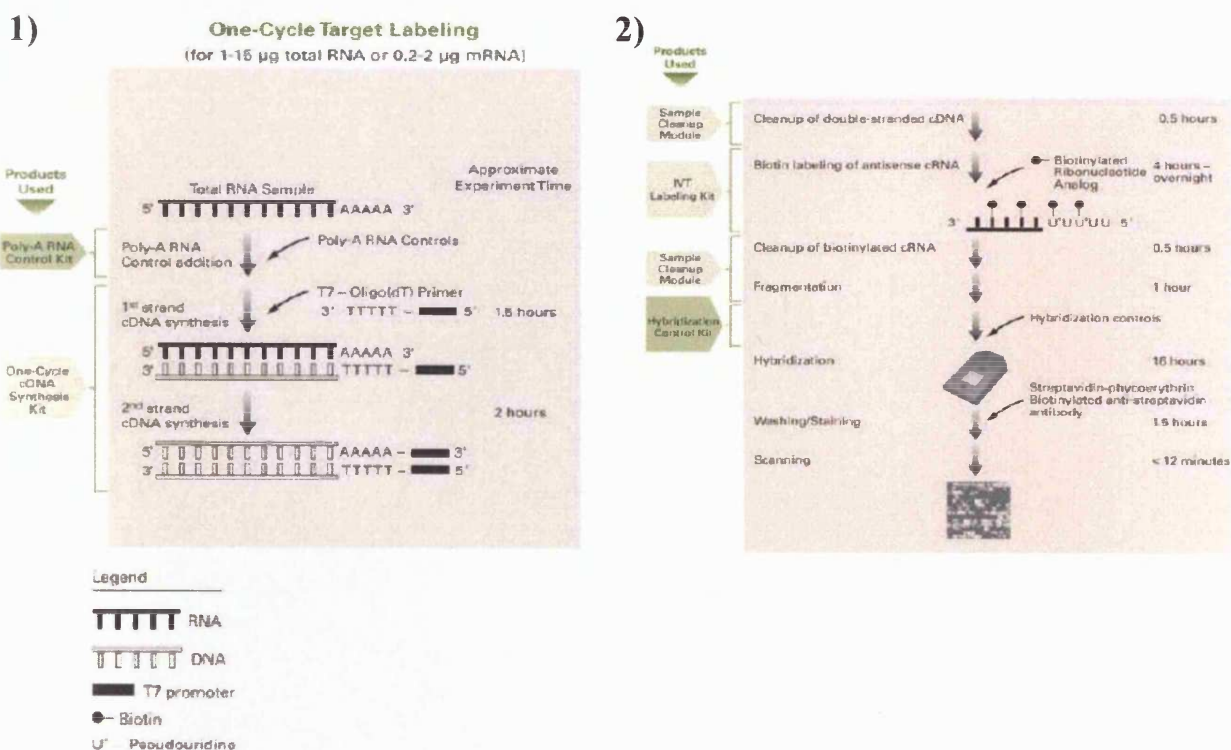
Gene expression analysis of 14,500 human genes by Affymetrix arrays on fibroblast cultured cell lines from both affected and unaffected skin from five SNF1 patients did not reveal any significant gene expression changes. The advantage of using this type of microarray is that each high-density array provides multiple, independent measurements of each transcript.

4.2.1 Statistical analysis

The aim of the pilot study was to try to obtain some idea as to which genes had significantly changed in terms of their expression in CAL fibroblasts as compared to fibroblasts from normal skin taken from five unrelated SNF1 patients. Different statistical tests were applied to the analysis of the data obtained. However, none of the analytical methods that was employed during this study succeeded in identifying any genes whose expression was significantly up- or down-regulated in CAL fibroblasts.

Initially, the standard analytical method for paired data was applied – data normalisation (Quantile-Quantile) followed by a paired t-test (using MS Excel) and FDR (False discovery rate) testing. FDR is a post-data measure of confidence that uses information available in the data to estimate the likely proportion of false positives. Although this method was used to analyse the likely presence of false positives in the expression data, differential expression call rates were still a problem. Since these methods failed to reveal any significantly differentially expressed genes, a new method called LIMMA was used. LIMMA is a package for the analysis of gene expression microarray data, that provides the ability to analyse comparisons between many RNA targets simultaneously. This method, using information across the genes, can be used even for experiments with small number of arrays. The top 100 genes identified as being differentially expressed by means of the LIMMA method are listed in Table 4.1.

Some changes were identified but they did not reach significant level, as shown in Figure 4.5 by volcano plot graph. This graph summarizes both the log odds of differential gene expression obtained from LIMMA plotted and the fold change criteria. The B-statistic (lods or B) is the log-odds that the gene is differentially expressed. A B-statistic of zero corresponds to a 50-50 chance that the gene is differentially expressed. The B-statistic is automatically adjusted for multiple testing by assuming that 1% of the genes are expected to be differentially expressed. If there are no missing

values in the data, then the moderated t and B statistics will rank the genes in exactly the same order. Data from all the genes in a replicate set of experiments are combined into estimates of parameters of a prior distribution. These parameter estimates are then combined at the gene level with means and standard deviations to form a statistic B which can be used to decide whether differential expression has occurred.

Figure 4.5. Volcano style plot graph
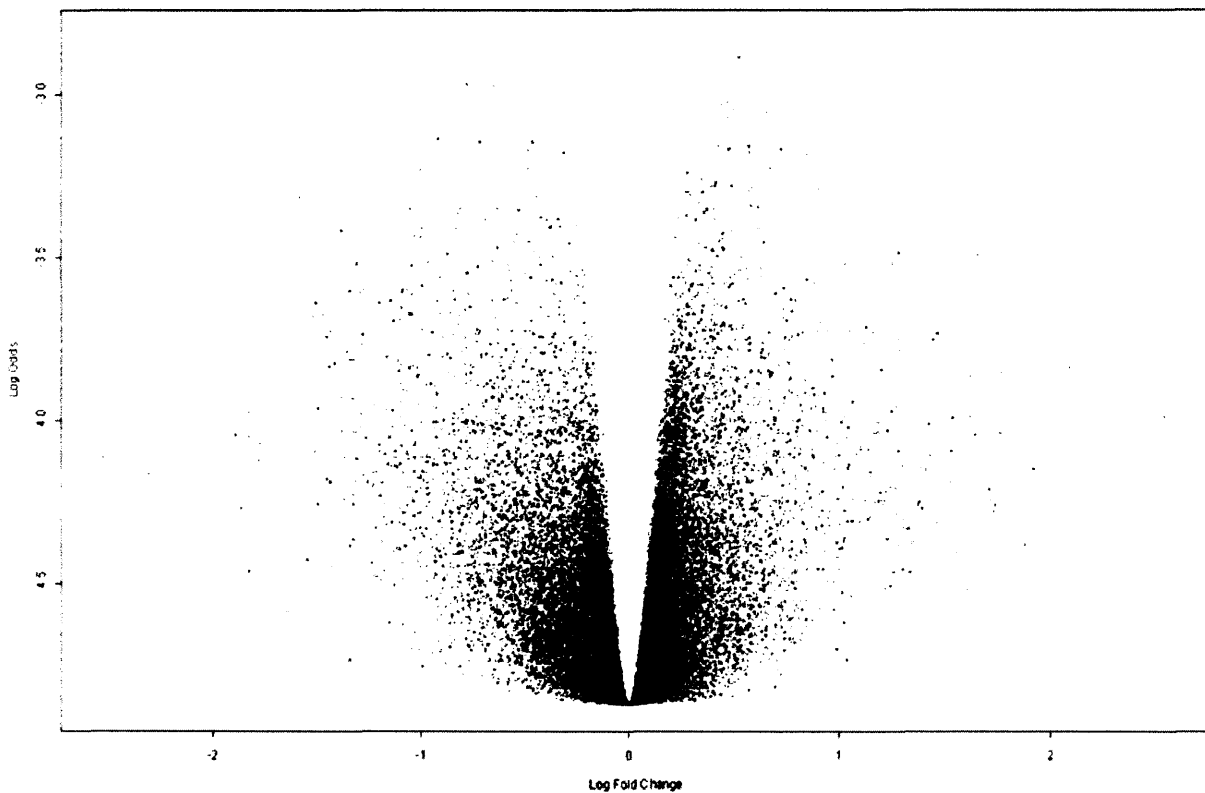
Figure 4.5. The results obtained during this study are represented by volcano plot graph. Volcano Plot is a graphical method for microarray experiments to identify genes with different expression levels between two types of samples. The Figure shows the log odds of differential gene expression obtained from LIMMA plotted against the fold-change values obtained from the average expression level within each group for each gene. Whilst it can be seen that there is a general trend for an increase of log odds with fold change, a probe set identified as significantly using the fold-change may not be similarly identified as significant in the LIMMA results.

Table 4.1: The top 100 genes identified as being differentially expressed by means of the LIMMA method

Probe Set ID	Gene Symbol	Gene Title	Chromosomal Location	Entrez Gene ID	FDR p-value	Raw p-value
228041_at	AASDH	2-aminoadipic 6-semialdehyde dehydrogenase	4q12	132949	0,825	0,059
232864_s_at	AF5Q31	ALL1 fused gene from 5q31	5q31	27125	0,983	0,865
1552306_at	ALG10	asparagine-linked glycosylation 10 homolog (yeast, alpha-1,2-glucosyltransferase)	12p11.1	84920	0,923	0,460
228445_at	AMID	apoptosis-inducing factor (AIF)-like mitochondrion-associated inducer of death	10q22.1	84883	0,864	0,202
204859_s_at	APAF1	apoptotic protease activating factor	12q23	317	0,832	0,082
221790_s_at	ARH	LDL receptor adaptor protein	1p36-p35	26119	0,884	0,280
57082_at	ARH	LDL receptor adaptor protein	1p36-p35	26119	0,870	0,225
203487_s_at	ARMC8	armadillo repeat containing 8	3q22.3	25852	0,827	0,073
205715_at	BST1	bone marrow stromal cell antigen 1	4p15	683	0,972	0,764
213410_at	C10orf137	chromosome 10 open reading frame 137	10q26.13-q26.2	26098	0,879	0,258
236952_at	C14orf105	Chromosome 14 open reading frame 105	14q22.3	55195	0,973	0,779
201303_at	DDX48	DEAD (Asp-Glu-Ala-Asp) box polypeptide 48	17q25.3	9775	0,861	0,179
203733_at	DEXI	dexamethasone-induced transcript	16p13.13	28955	0,944	0,600
212300_at	DKFZp451J0118	taxilin	1p35.1	200081	0,883	0,277
240690_at	DKFZp761P0423	Hypothetical protein DKFZp761P0423	8p23.1	157285	0,942	0,583
204602_at	DKK1	dickkopf homolog 1 (*Xenopus laevis*)	10q11.2	22943	0,836	0,105
212611_at	DTX4	deltex 4 homolog (*Drosophila*)	11q12.1	23220	0,863	0,192
208891_at	DUSP6	dual specificity phosphatase 6	12q22-q23	1848	0,983	0,859
204037_at	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	9q31.3	1902	0,825	0,065
218595_s_at	FLJ10359	protein BAP28	1q43	55127	0,836	0,092
219988_s_at	FLJ10597	hypothetical protein FLJ10597	1p34.1	55182	0,969	0,747
224721_at	FLJ12519	hypothetical protein FLJ12519	2q32.2	84128	0,904	0,371
242584_at	FLJ13305	hypothetical protein FLJ13305	2p15	84140	0,946	0,613
1553709_a_at	FLJ14936	hypothetical protein FLJ14936	1p33-p32.1	84950	0,825	0,036
206860_s_at	FLJ20323	hypothetical protein FLJ20323	7p22-p21	54468	0,888	0,298
210220_at	FZD2	frizzled homolog 2 (Drosophila)	17q21.1	2535	0,968	0,737
229936_at	GFRA3	GDNF family receptor alpha 3	5q31.1-q31.3	2676	0,987	0,905
224964_s_at	GNG2	guanine nucleotide binding protein (G protein), gamma 2	14q21	54331	0,992	0,945
202756_s_at	GPC1	glypican 1	2q35-q37	2817	0,974	0,781
225577_at	HCG18	CDNA clone IMAGE:5265581, partial cds			0,909	0,393
202708_s_at	HIST2H2BE	histone 2, H2be	1q21-q23	8349	0,836	0,097
202540_s_at	HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	5q13.3-q14	3156	0,864	0,196
201031_s_at	HNRPH1	heterogeneous nuclear ribonucleoprotein H1 (H)	5q35.3	3187	0,994	0,963
202637_s_at	ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	19p13.3-p13.2	3383	0,870	0,225
243321_at	IQGAP1	IQ motif containing GTPase activating protein 1	15q26.1	8826	0,931	0,515
229041_s_at	ITGB2	Integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	21q22.3	3689	0,911	0,399
209912_s_at	KIAA0415	KIAA0415 protein	7p22.2	9907	0,825	0,048
1570349_at	KIAA1462	KIAA1462	10p11.23	57608	0,847	0,128
227228_s_at	KIAA1509	KIAA1509	14q32.12	440193	0,983	0,861
232171_x_at	KLHDC4	kelch domain containing 4	16q24.3	54758	0,878	0,256
224980_at	LEMD2	LEM domain containing 2	6p21.31	221496	0,877	0,250
1556588_at	LOC283687	hypothetical protein LOC283687	15q25.1	283687	0,876	0,239
221826_at	LOC90806	similar to RIKEN cDNA 2610307I21	1q32.3	90806	0,855	0,154
202997_s_at	LOXL2	lysyl oxidase-like 2	8p21.3-p21.2	4017	0,880	0,270
1564906_at	MATR3	Matrin 3	5q31.2	9782	0,979	0,820
218330_s_at	NAV2	neuron navigator 2	11p15.1	89797	0,825	0,036
219231_at	NCOA6IP	nuclear receptor coactivator 6 interacting protein	8q11	96764	0,958	0,681

238346_s_at	*NCOA6IP*	nuclear receptor coactivator 6 interacting protein	8q11	96764	0,965	0,726
226974_at	*NEDD4L*	Neural precursor cell expressed, developmentally down-regulated 4-like	18q21	23327	0,924	0,465
204107_at	*NFYA*	nuclear transcription factor Y, alpha	6p21.3	4800	0,862	0,188
204108_at	*NFYA*	nuclear transcription factor Y, alpha	6p21.3	4800	0,937	0,555
215720_s_at	*NFYA*	nuclear transcription factor Y, alpha	6p21.3	4800	0,861	0,183
	NR1D1			9572		
209261_s_at	*NR2F6*	nuclear receptor subfamily 2, group F, member 6	19p13.1	2063	0,923	0,463
206550_s_at	*NUP155*	nucleoporin 155kDa	5p13.1	9631	0,908	0,391
201364_s_at	*OAZ2*	ornithine decarboxylase antizyme 2	15q22.31	4947	0,858	0,169
204853_at	*ORC2L*	origin recognition complex, subunit 2-like (yeast)	2q33	4999	0,836	0,093
208857_s_at	*PCMT1*	protein-L-isoaspartate (D-aspartate) O-methyltransferase	6q24-q25	5110	0,996	0,976
202590_s_at	*PDK2*	pyruvate dehydrogenase kinase, isoenzyme 2	17q21.33	5164	0,932	0,523
241932_at	*PHGDHL1*	Phosphoglycerate dehydrogenase like 1	13q32.3	337867	0,934	0,535
205811_at	*POLG2*	polymerase (DNA directed), gamma 2, accessory subunit	17q	11232	0,855	0,142
203783_x_at	*POLRMT*	polymerase (RNA) mitochondrial (DNA directed)	19p13.3	5442	0,985	0,880
202466_at	*POLS*	polymerase (DNA directed) sigma	5p15	11044	0,877	0,255
207725_at	*POU4F2*	POU domain, class 4, transcription factor 2	4q31.2	5458	0,980	0,838
231931_at	*PRDM15*	PR domain containing 15	21q22.3	63977	0,843	0,122
238951_at	*PRDX6*	Peroxiredoxin 6	1q25.1	9588	0,971	0,758
214545_s_at	*PROSC*	proline synthetase co-transcribed homolog (bacterial)	8p11.2	11212	0,876	0,248
211373_s_at	*PSEN2*	presenilin 2 (Alzheimer disease 4)	1q31-q42	5664	0,965	0,719
1557223_at	*RBPMS*	RNA binding protein with multiple splicing	8p12-p11	11030	0,869	0,221
203436_at	*RPP30*	ribonuclease P/MRP 30kDa subunit	10q23.31	10556	0,923	0,460
221432_s_at	*SLC25A28*	solute carrier family 25, member 28	10q23-q24	81894	0,825	0,043
205397_x_at	*SMAD3*	SMAD, mothers against DPP homolog 3 (*Drosophila*)	15q22.33	4088	0,880	0,264
205398_s_at	*SMAD3*	SMAD, mothers against DPP homolog 3 (*Drosophila*)	15q22.33	4088	0,836	0,104
208078_s_at	*SNF1LK*	SNF1-like kinase	21q22.3	150094	0,853	0,137
220140_s_at	*SNX11*	sorting nexin 11	17q21.32	29916	0,825	0,009
221489_s_at	*SPRY4*	sprouty homolog 4 (*Drosophila*)	5q31.3	81848	0,964	0,711
202440_s_at	*ST5*	suppression of tumorigenicity 5	11p15	6764	0,864	0,201
210167_s_at	*TEF*	thyrotrophic embryonic factor	22q13|22q13.2	7008	0,825	0,031
212457_at	*TFE3*	transcription factor binding to IGHM enhancer 3	Xp11.22	7030	0,869	0,214
31637_s_at	*THRA*	thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian); nuclear receptor subfamily 1, group D, member 1	17q11.2	7067	0,880	0,269
218335_x_at	*TNIP2*	TNFAIP3 interacting protein 2	4p16.3	79155	0,900	0,351
208900_s_at	*TOP1*	topoisomerase (DNA) I	20q12-q13.1	7150	0,888	0,296
203610_s_at	*TRIM38*	tripartite motif-containing 38	6p21.3	10475	0,827	0,073
224295_at	*TRIM44*	Tripartite motif-containing 44	11p13	54765	0,919	0,437
221012_s_at	*TRIM8*	tripartite motif-containing 8	10q24.3	81603	0,860	0,174
214792_x_at	*VAMP2*	Vesicle-associated membrane protein 2 (synaptobrevin 2)	17p13.1	6844	0,916	0,417
1555270_a_at	*WFS1*	Wolfram syndrome 1 (wolframin)	4p16	7466	0,825	0,060
202908_at	*WFS1*	Wolfram syndrome 1 (wolframin)	4p16	7466	0,857	0,158
1555068_at	*WNK1*	WNK lysine deficient protein kinase 1	12p13.3	65125	0,926	0,481
1554821_a_at	*ZBED1*	zinc finger, BED domain containing 1	Xp22.33;Yp11	9189	0,843	0,124
207304_at	*ZNF45*	zinc finger protein 45	19q13.2	7596	0,902	0,357
1554476_x_at		Hypothetical gene supported by BX640933; NM_198457	19q13.41	388558	0,884	0,280
1557796_at		CDNA clone IMAGE:4708811, partial cds			0,931	0,515
1568920_at		TGF-betaIIR beta			0,919	0,438
202744_at					0,921	0,449
226772_s_at					0,837	0,115

112

231699_at		0,968	0,737
235203_at	*Homo sapiens*, clone IMAGE:3866695, mRNA	0,931	0,515
235454_at		0,973	0,774
241312_at	CDNA FLJ27516 fis, clone TST08713	0,944	0,595
241726_at		0,908	0,389

Table 4.1. The genes listed comprise the top 100 genes differentially expressed between CAL fibroblasts and normal fibroblasts identified by the LIMMA method. Genes which have previously been described differently expressed in NF1-related tissues are coloured in red.

4.3 Discussion

During this study, microarray technology was adopted to analyse the gene expression profiles of SNF1. Gene expression analysis of 14,500 different genes using Affymetrix human U133A gene chips on cultured fibroblasts from both affected and unaffected areas of the body of five SNF1 patients, did not reveal significant differences in fibroblast gene expression between the two groups. Use of cultured cell lines for expression analysis is debatable owing to the potential introduction of artefacts during culture and in addition the cultured cells do not represent their true biological environment. The experiment was designed on the basis of the hypothesis that there might be transcriptional changes in fibroblasts from CALs compared with fibroblasts from normal skin.

The use of Affymetrix chips, in which 14,500 different genes can be analysed, has revealed to be optimal for this type of experiment. Our negative findings suggest that an analysis of larger panel, different cell types (melanocytes or keratinocytes) and alternative analytical techniques may provide evidence to support our hypothesis. It is possible that while culturing fibroblasts from affected skin biopsy, mutant fibroblasts have been overgrown by the wild type fibroblasts. For future studies, RNA directly derived from the biopsies should be considered. One caveat for using direct skin is not having enough RNA for analysis. An average microarray experiment requires 10-100 micrograms of RNA. However, due to their small size, most biopsies are unlikely to give this yield. Several methods have been described for RNA amplification *in vitro*. In addition it has been demonstrated that RNA amplification *in vitro* does not introduce a large systematic bias (Schneider *et al*, 2004). This experiment can be used as pilot for

future gene expression studies. Moreover, the genes listed in Table 4.1, although they are not significantly differentially expressed, might be useful for subsequent testing of differential expression based on their known biological role.

Several interesting modulated genes, cytogenetically mapped to region 17p11.2-p13, have been reported to be altered in melanocytes from CAL-associated NF1 (Boucneau *et al.*, 2005). A gene called DEAD (Asp-Glu-Ala-Asp) box polypeptide 48 (*DDX48*) (MIM 608546), located on chromosome 17q25.3, might be involved in the development of CALs in NF1 as it is present in the list of the genes changed in the samples analysed during this study and it has also previously been described as being differentially expressed in melanocytes from CALs (Boucneau *et al.*, 2005). DDX48 is a component of the exon junction complex (EJC), which assembles near exon-exon junctions of mRNAs as a result of splicing. EJC proteins play roles in post-splicing events, including mRNA export, cytoplasmic localization, and nonsense-mediated decay (Chan *et al.*, 2004).

It is interesting to note that three genes (ICAM1, intercellular adhesion molecule, ITGB2 Integrin Beta 2, and TOP1 topoisomerase 1), which were differentially expressed in this study, related genes to these have been previously reported to be dysregulated in MPNSTs (Levy *et al*, 2004a). Because of severe time constraints it was not possible to confirm the differential expression of these genes using real time PCR.

Further studies of the link between certain genetic regions or candidate genes, and specific features of NF1 might be of interest for molecular characterization of disease features.

Gene expression analysis, using microarray technologies, comprises many different steps that may influence the accuracy of the results obtained. Considering all the factors involved in a microarray experiment, interpretation of the large amount of data produced is not straightforward. A very critical point is the collection and preparation of the sample to be studied. In the present study, similar techniques were employed for obtaining the biopsies. Fibroblasts from all patients were cultured under identical conditions and RNA was immediately extracted from confluent cultures thereby, minimising the 'aging' effects which can result in the introduction of cryptic splicing (Wimmer *et al.*, 2002).

Many studies directed to the analysis of differentially expressed genes in NF1 tumour samples as compared to normal controls have recently been described (Agesen *et al.*, 2005; Gutmann *et al.*, 2002; Holtkamp *et al.*, 2004; Levy *et al.*, 2004a; 2004b; Watson *et al.*, 2004; Wimmer *et al.*, 2002). However, only a few involved the expression analysis of the characteristic hyperpigmentary defects seen in NF1 patients. Therefore, the possible mechanisms underlying these NF1 clinical features remain unclear.

Melanocytes, analogous to the Schwann cell in neurofibromas, are considered to be the primary (neoplastic) cells in the pathoetiology of CALs in NF1 (Lukacs *et al.*, 1997). Therefore, studies on the expression profiling pattern of CALs from NF1 patients have been involved in the analysis of this type of cell. Only recently has it been demonstrated that fibroblasts from CALs produced a significantly higher level of SCF and HGF as compared with fibroblasts from normal skin (Imokawa, 2004). This is analogous to reports on the SCF secretion of Schwann cells and the proliferation increase of mast cells in neurofibromas (Viskochil, 2003; Yang *et al.*, 2003). In addition, the high production of both SCF and HGF by fibroblasts from CALs related to the increased expression of mRNA encoding SCF and HGF when compared with normal fibroblasts. Thus, future studies on the possible correlation between reduced neurofibromin and mechanisms involved in the increased production of these cytokines by fibroblasts in CALs from NF1 patients might be useful to identify the mechanisms that cause the high secretion of SCF and HGF by fibroblasts in CALs.

Notably gene expression was reported to be affected by genotype (rather than lesion type) in NF1 $^{+/-}$ melanocytes compared to NF1 $^{+/+}$ melanocytes (Boucneau *et al*, 2005).

An heterozygous deletion of the entire *NF1* gene was previously identified by FISH analysis in ~18% of fibroblasts cultured from a CAL spot of a patient with SNF1 (Tinschert *et al.*, 2000). In addition, during the course of these studies, gonosomal mosaicism for a nonsense mutation in the *NF1* gene was characterized in an individual affected by SNF1 of whom a child was affected by full-blown NF1 (Consoli *et al.*, 2005). However, the mechanisms underlying the clinical phenotype characteristic of individuals affected by SNF1 are still largely unknown.

SNF1 represents an interesting model for expression studies. Patients with SNF1 (which is considered to be due to somatic mosaicism) will provide material to enable us to study three genetically distinct tissues with respect to NF1 genotype: wild type (+/+, unaffected skin), heterozygous (+/- affected skin) and mutant homozygous (-/-, tumour from affected area). The availability of all three genotypes will be useful for expression studies since it will preclude the need to obtain tissue from different individuals which may reduce variability due to inter-individual differences in gene expression.

The results obtained during this study did not reveal any significant gene expression differences between affected and unaffected tissues from five SNF1 patients. This might depend on the specific cell type analysed and therefore, expression profiling studies should in future involve melanocytes, keratinocytes or mast cell types from CALs. In addition, since individuals affected by SNF1 are thought to be mosaic for a postzygotic *NF1* gene mutation, this could lead to a very small gene expression change that might not have been detected by microarray analysis, it is, therefore, suggested that for future studies usage of affected biopsy rather than cultured cells should be considered. *In vitro* transcription (IVT) can be used to amplify low yield of RNA obtained from skin biopsies. In addition, the analysis of gene expression profiles in different cell types in a large number of SNF1 patients could help us to understand better the mechanisms underlying the clinical phenotype characteristic of individuals affected by this disorder.

Chapter 5. Mutational analysis of the *NF1* gene in atypical cases of neurofibromatosis type 1

5.1 Introduction

The relationship between pathogenic mutations and disease phenotype is a complex topic in molecular genetics. Genotype-phenotype correlations have been observed for many Mendelian diseases, including for example multiple endocrine neoplasia type 2 (MEN2) (MIM 171400) (Carlomagno *et al.*, 1997; Frank-Raue *et al.*, 1996) and adenomatous polyposis coli (APC) (MIM 175100) (Groves *et al.*, 2002; Truta *et al.*, 2003). Such observations can be extremely useful in the context of genetic counselling in the context of both antenatal and presymptomatic diagnosis. One classic example of where there is a clear, unambiguous and reliable relationship between mutant genotype and clinical phenotype is the frequent inversion in the factor VIII gene (*F8*) which causes severe haemophilia A (Bagnall *et al.*, 2002; Lakich *et al.*, 1993; Naylor *et al.*, 1996). However, well delineated clinical entities can exhibit allelic heterogeneity, and different mutations in a given gene may well be associated with very different clinical phenotypes. An example of a specific mutation in a particular gene which gives rise to different phenotypes is a missense mutation (C1409S) in the fibrillin gene (*FBN1*) identified in individuals affected by Marfan syndrome (MFS) (MIM 154700). As described by Dietz *et al.*, (1992), the phenotype of individuals carrying this missense mutation varies widely with respect to onset of disease, organ involvement, and clinical severity. The phenotypic effect exerted by a mutant allele may be modified by a variety of different factors: 1) the status of the other allele and the variable expression of the wild-type or mutant allele as a consequence either of alternative mRNA processing or the influence of genetic variation in the gene promoter region; 2) the effect of other loci and their protein products; 3) the activity state of the chromosome carrying the gene, as a consequence either of X- inactivation or genomic imprinting; and 4) the proportion of affected cells in the expressing tissues, which is clearly an important factor in determining phenotypic severity (Cooper and Krawczak, 1993; Antonarakis and Cooper, 2003).

5.1.1 Genotype-phenotype relationship in NF1

No clear genotype-phenotype relationship has yet been discerned for NF1. This may be due either to the influence of modifier loci in different individuals (Easton *et al.*, 1993) or to the variable nature, location and developmental timing of the somatic mutations which determine the rate of progression and severity of disease in different tissues. Variable expression of alternative transcripts in the different tissue types (Skuse and Cappione, 1997) or abnormal *NF1* RNA editing also account for the wide range of clinical features observed in NF1 patients (Kaufmann *et al.*, 1999; Park *et al.*, 1998; Skuse and Cappione, 1997; Vandenbroucke *et al.*, 2002). The variable manifestations of NF1 are intra-familial as well as inter-familial (Riccardi *et al.*, 1992). Affected members of a single family with typical NF1 often have quite different disease phenotypes, despite sharing an identical mutant *NF1* allele. Clearly, variation in the mutant *NF1* allele itself does not account for all of the variability seen in most disease features. This notwithstanding, a relationship between gross *NF1* gene deletions ranging from ~ 200 bp to the deletion of the entire gene and its flanking regions, and learning disability, dysmorphic features, and development delay in NF1 patients has been described (Ainsworth *et al.*, 1997; Cnossen *et al.*, 1997; Kayes *et al.*, 1994; Lazaro *et al.*, 1996; Leppig *et al.*, 1996, 1997; Lopez-Correa *et al.*, 2001; Upadhyaya *et al.*, 1996b; Wu *et al.*, 1995). However, not all patients with gross *NF1* gene deletions have this NF1 phenotype (Upadhyaya *et al.*, 1998; Tonsgard *et al.*, 1997; Kehrer-Sawatzki *et al.*, 2004), and it may be that flanking DNA sequence (or the lack of it) may influence the phenotype. Learning disabilities have however also been observed in patients with different inactivating mutations and even in *nf1* mutant flies and mice (Guo *et al.*, 2000; Costa *et al.*, 2001; Costa and Silva, 2003). Recently, Castle *et al.* (2003) showed that NF1 patients with missense mutations in the *NF1* gene had a relative risk (RR) of Lisch nodules that was less than 1, and of borderline statistical significance when compared with the NF1 patients with nonsense or frame-shift mutations and adjusted for age at examination.

The observation of similar clinical features among affected members of a few families with the NF1 variants, Watson syndrome (Allanson *et al.*, 1991), familial café-au-lait spots (Abeliovich *et al.*, 1995), or familial spinal neurofibromas (Ars *et al*, 1998; Polyhonem *et al.*, 1997; Pulst *et al.*, 1991), is consistent with a genotype-phenotype correlation, but no consistent kind of *NF1* mutation has been found in families with these or any other phenotypic variant. There are a few reports of *NF1* gene mutations in

patients who exhibit symptoms that fit other syndrome diagnoses rather better than NF1. This suggests that these different clinical disorders could be allelic: examples include two reports of families/patients with Watson syndrome (which has some overlapping features with NF1) having *NF1* gene mutations (Tassabehji *et al.*, 1993; Upadhyaya *et al.*, 1992) and a patient with LEOPARD syndrome (Wu *et al.*, 1996).

The lack of many obvious genotype-phenotype correlations in NF1 suggests that unlinked modifying genes and the normal allele may both be involved in the development of specific clinical features of NF1, and that the relative contributions vary for different features. It is also possible, however, that important *NF1* genotype-phenotype correlations exist but have not been recognized because of the complexity of the NF1 clinical phenotype (Riccardi, 1999), its strong dependence on age (DeBella *et al.*, 2000), the non-independence of many clinical features (Szudek *et al.*, 2000b; Szudek *et al.*, 2002), and the heterogeneity of pathogenic *NF1* mutations (Fahsold *et al.*, 2000, Korf, 1999; Mattocks *et al.*, 2004; Messiaen *et al.*, 2000).

There are more than 10 clinical syndromes that have been reported in NF1 patients, including Charcot-Marie-Tooth disease (CMT1), Multiple sclerosis, McCune Albright syndrome, and numerous other disorders that share at least some of the features of NF1. Some patients with classical NF1 have, in addition, features of dysmorphic syndromes, such as Noonan syndrome or Watson syndrome. The overlapping features of Watson syndrome, Noonan syndrome, and NF1, including pulmonary stenosis and lymphatic abnormalities, and perhaps also short stature and macrocephaly, may imply that a variety of genes are probably responsible for these correlations, that exert effects on the expression of other genes.

Viskochil and Carey (1992) proposed a classification of different forms of neurofibromatosis in which Watson syndrome and NF/Noonan syndrome, together with duodenal/carcinoid/phaeochromocytoma/NF1 and juvenile xanthogranuloma/NF1, were considered as *related* forms of NF based on the evidence of classic clinical features of neurofibromatosis in addition to distinctive clinical features not typically seen in either NF1 or NF2. Carey (1998) reviewed the neurofibromatosis-Noonan syndrome as a relatively rare condition that remains poorly delineated. He outlined several possible mechanisms to account for the overlap. One postulate holds that this condition is simply attributable to a subtype of *NF1* mutations that impart a Noonan phenotype. An interesting two-generation family with pulmonary stenosis and café-au-lait spots had a

3-bp deletion in exon 17 of the *NF1* gene (Carey *et al.,* 1998). Another three-generation family with café-au-lait spots, decreased cognition, and minor anomalies suggestive of Noonan syndrome (one with pulmonary stenosis) exhibited an in-frame 42-bp duplication in exon 28 (Tassabehji *et al.,*1993). It is possible that these mutations could localize to specific domains in the neurofibromatosis 1 gene product that modify the biochemical pathway involved in Noonan syndrome.

Although multiple café-au-lait spots are the diagnostic hallmark of neurofibromatosis type 1, they have been observed in families in which there have been no other changes characteristic of NF1 (Riccardi, 1980). The absence of neurofibromas and Lisch nodules of the iris suggests however that these families are expressing a trait that is genetically distinct from NF1. In support of this postulate, there are several reports of families in which café-au-lait spots are inherited as an autosomal dominant trait, without any other features of NF1 (Abeliovich *et al.,* 1995; Arnsmeier *et al.,* 1994; Brummer *et al.,* 1993; Charrow *et al.,* 1993; Korf, 1992).

A few families have been described with multiple members who have neurofibromas along the spinal cord and multiple café-au-lait spots but without either dermal neurofibromas or Lisch nodules (Pulst *et al.,* 1991; Poyhonen *et al.,* 1997; Ars *et al.,* 1998). This distinct entity maps to chromosome 17q, and one family has been shown to possess a frameshift truncating *NF1* mutation in exon 46 (Ars *et al.,* 1998).

Hereditary non-polypotic colon cancer (HNPCC) is an autosomal dominant disorder associated with early onset colon cancer. Heterozygous mutations in the mismatch repair (MMR) genes, especially *MSH2* and *MLH1*, account for most cases. In two published articles, five children from two consanguineous marriages within HNPCC families have been described (Ricciardone *et al.,* 1999; Wang *et al.,* 1999). Four of the five children had clinical features of NF1 including one patient who had only hemi-corporeal café-au-lait spots, suggestive of post-zygotic somatic mutation. All five children developed malignancies by the age of six. DNA sequence analysis documented homozygous *MLH1* mutations in affected children from both families. These cases strongly suggest that homozygous mutations in MMR genes lead to a mutator phenotype, post-zygotic somatic acquired mutations in the *NF1* gene, and subsequent manifestations of both neurofibromatosis and early onset of cancer. Wang *et al.* (2003)

identified genetic alterations in five out of ten tumour cell lines with microsatellite instability (MSI), whereas five MMR-proficient tumour cell lines expressed a wild-type *NF1* gene. Somatic *NF1* gene mutations were also detected in two primary tumours exhibiting an MSI phenotype.

MoyaMoya disease is a cerebrovascular disease of unknown cause that mainly affects Japanese children (Ikezaki *et al.*, 1997; Yamauchi *et al.*, 2000). The incidence of familial occurrence accounts for approximately 9% of cases (Yamauchi *et al.*, 1997). It is interesting to note that the characteristic lesions of MoyaMoya disease are occasionally associated with NF1. More than 50 cases of such an association have been reported so far (Barall and Summers, 1996; Edwards-Brown *et al.*, 1997; Fujimura *et al.*, 2004; Osawa *et al.*, 1992; Woody *et al.*, 1992; Yamauchi *et al.*, 2000).

For this study, in order to assess the role of *NF1* gene mutations underlying the overlapping features of different disorders with some similarity or clinical overlap with neurofibromatosis type 1, NF1 patients who exhibited atypical phenotypes were specifically selected. In order to understand the effect of *NF1* mutations on either sporadic and familial cases, 4 families in which the NF1 clinical phenotypes were inherited through one or more generations, were selected together with 10 NF1 sporadic patients. The diagnostic criteria for NF1 are based on the presence of more than 6 café-au-lait (CAL) spots, axillary and/or inguinal lentigines, 2 or more cutaneous neurofibromas of any type or a plexiform neurofibroma, 2 or more Lisch nodules (iris hamatomas), a specific bone lesion (sphenoid dysplasia, thinning of the long bone cortex and/or pseudoarthrosis), optic glioma or a first-degree relative with NF1 (*National Institutes of Health (NIH) Consensus Development Conference Statement Neurofibromatosis*, 1988). The diagnostic criteria for NF1 patients exhibiting atypical clinical phenotypes were found to be more difficult to draw up because of the lack of some of the typical or even defining clinical features of classical NF1. However, Crowe and Schull (1953) suggested that the presence of 6 CAL spots, each more than 1.5 cm in diameter, is necessary for the diagnosis of NF1.

In this chapter, the possible role of *NF1* gene mutations underlying the highly variable clinical expression manifested by atypical NF1 individuals was studied. Mutation detection in NF1 has proven laborious on account of the size of the *NF1* gene, the

The study of *NF1* mutations in NF1 patients exhibiting atypical phenotypes should help us to understand more about: 1) the mutational spectrum associated with variable expression of the clinical phenotype and 2) the possible relationship between mutant genotype and clinical phenotype.

5.2 Patients

The study group comprises:

14 NF1 affected individuals with an atypical phenotype. We can also subdivide them into two subgroups:

1) Four familial cases for which DNA samples from other members of the family were available. This subgroup comprises:

   a) An NF1 family (Family A) in which the affected members do not have neurofibromas. DNA samples from 13 family members were obtained. The clinical phenotype of the affected individuals comprised café-au-lait spots, axillary freckling and in some cases, Lisch nodules (family pedigree shown in Figure 5.1).

   b) A family (Family B) with autosomal dominant café-au-lait spots syndrome (MIM 114030). From this family, we obtained DNA samples from a 13 year old daughter who had multiple café-au-lait spots, inguinal/axillary freckling and mild pectus excavatum, from the 49 year old father who presented multiple café-au-lait spots but no other features of NF1, and also from the unaffected mother. Another two family members presented multiple café-au-lait spots as the only clinical feature of NF1, but DNA samples were not available (family pedigree shown in Figure 5.2).

   c) A family (Family C) in which two members appear to have manifestations of both NF1 (MIM 162200) and NF2 (MIM 101000).

2) 10 sporadic cases and, for some of them where possible, DNA samples from the unaffected relatives were also available. This subgroup comprises:

a) 4 cases of NF/Noonan syndrome (MIM 601321)

b) 1 individual affected by NF1 (MIM 162200) and tuberous sclerosis (TS) (MIM 191100).

c) 1 individual affected by NF1 (MIM 162200) and fascioscapulohumeral muscular dystrophy (FSHD) (MIM 158900)

d) 1 individual affected by MoyaMoya disease (MIM 252350)

e) 1 individual resulted homozygous for a mutation in the *MLH1* gene (*MLH1*$^{(-/-)}$)

f) 1 individual affected by NF1 with a particularly severe phenotype

g) 1 individual exhibiting multiple subcutaneous neurofibromas

Figure 5.1: Pedigree of the NF1 familial cases
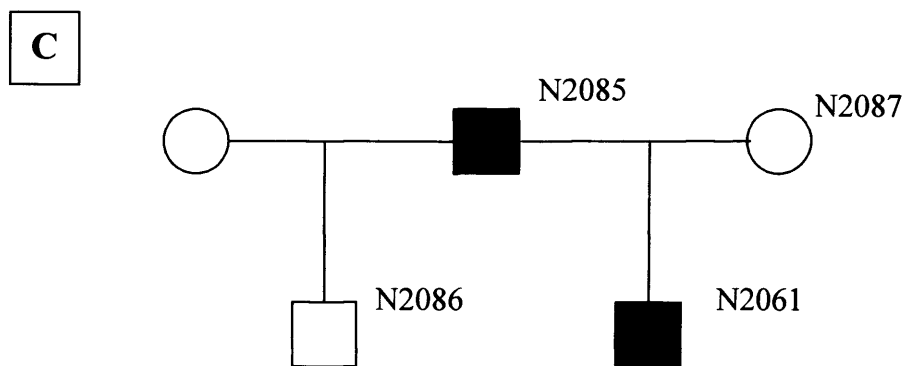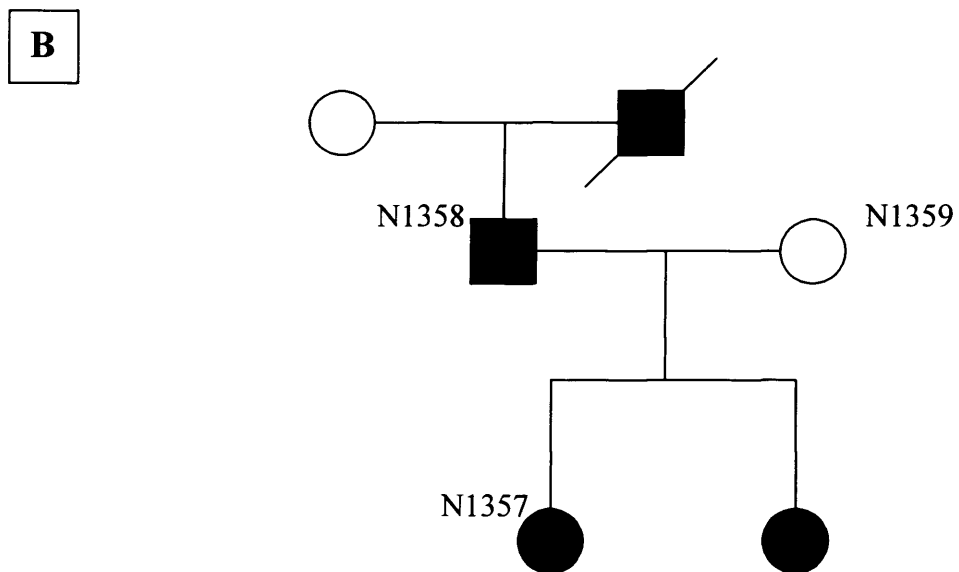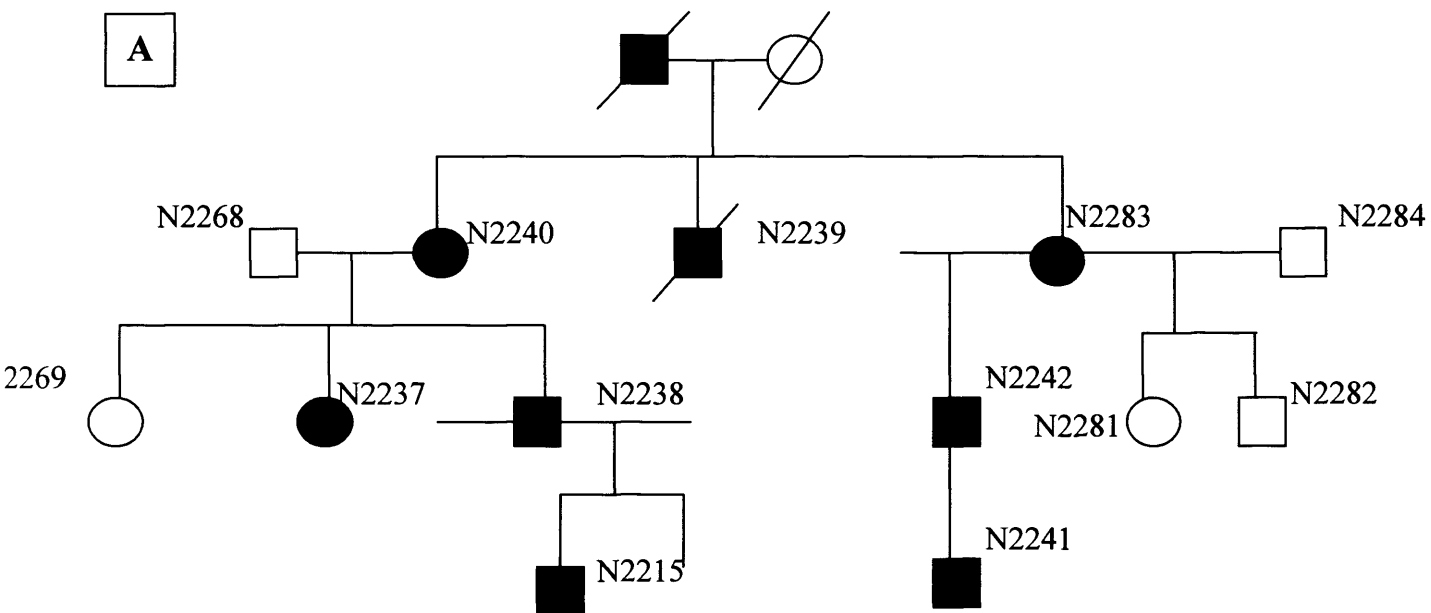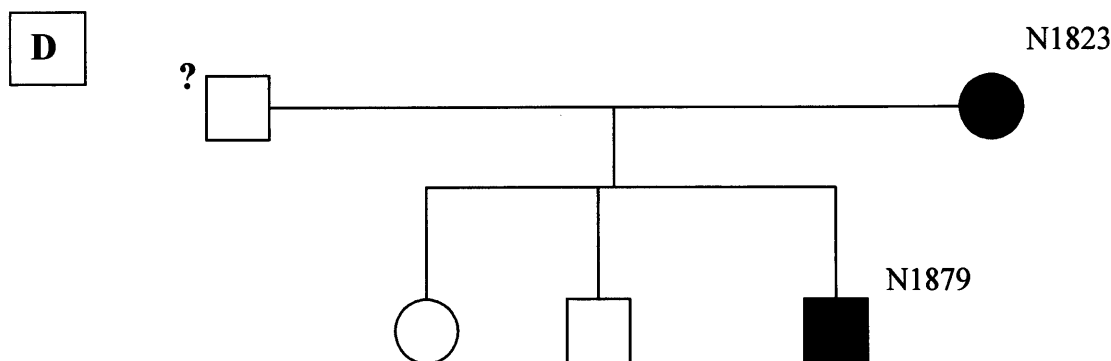
Figure 5.1: The pedigrees of the four studied NF1 families exhibiting atypical phenotypes. Both affected and unaffected individuals are represented; the numbers indicate the patient DNA number. For members lacking a number, a DNA sample was unavailable for mutation analysis. **A**: Family (Family A) in which the affected individuals do not have neurofibromas; **B**: Family (Family B) with autosomal dominant café-au-lait spots syndrome. **C**: Family (Family C) in which two members are affected by both NF1 and NF2. **D**: Family (Family D) in which two members are affected by NF1 but the child presented with a severe phenotype.

5.3 Results

Mutation analysis of the *NF1* gene in 14 NF1 affected individuals with an atypical phenotype provided results that may help us to understand better the molecular genetic mechanisms underlying expression of the clinical phenotype in NF1 patients. Genomic DNA from all 14 NF1 affected individuals was screened by DHPLC for mutations in the *NF1* gene. A variant elution profile was observed in all of them; on subsequent characterization by cycle sequencing, different mutations were identified (Table 5.1, Table 5.2).

5.3.1 Familial cases

Mutation detection by DHPLC in the *NF1* gene in two unrelated patients (N2215, N1357) in whom no neurofibromas were present, revealed a previously reported 3-bp deletion in exon 17 (2970-2972delAAT) (Ars *et al.*, 2003; Fahsold *et al.*, 2000; Mattocks *et al.*, 2004; Shen *et al.*, 1993). This mutation is predicted to lead to the loss of an amino acid (methionine) in the neurofibromin protein (Figure 5.2). For patient N2215, DNA samples from both affected and unaffected members of the family from 3

generations were obtained (family pedigree shown in Figure 5.1). Interestingly, on further screening by DHPLC of exon 17 in all 13 members of the family, the 3-bp deletion was identified in DNA from all the affected individuals, whereas the mutation was not present in any of the unaffected individuals. Patient N1357 is a member of a family with autosomal dominant café-au-lait spots. Based on the hypothesis that autosomal dominant café-au-lait spots and NF1 could be allelic, genomic DNA from the proband, her father and also the unaffected mother, was screened by DHPLC for mutations in the *NF1* gene. The 3-bp deletion was noted in exon 17 in both the affected daughter and the father but not in the unaffected mother.

Figure 5.2. Sequence of *NF1* cDNA exon 17 from codon 976 to 997

```
976   ACT GAA GGC AGC TCT GAA CAT CTA GGG CAA GCT
      Thr Glu Gly Ser Ser Glu His Leu Gly Gln Ala

      AGC ATT GAA ACA ATG ATG TTA AAT CTG GTC AGG 997
      Ser Ile Glu Thr Met Met Leu Asn Leu Val
```

Figure 5.2 The effect of the 3-bp deletion (AAT) in exon 17 of the *NF1* gene, identified in NF1 patients during this study, on the neurofibromin protein. The AAT deleted is underlined; the codon ATG (Methionine) that would be lost in the neurofibromin protein is highlighted in red; the exon 17 splice donor site (AG) is highlighted in light blue.

In patient N2061, who presented with clinical features of both NF1 and NF2, a previously reported missense mutation in exon 10b (1466 A→G;Y489C) was identified. Further mutational analysis of the entire *NF1* gene in both the affected father and unaffected mother, revealed the presence of the same change. An RNA sample from patient N2061 was also obtained and the screening by DHPLC of the *NF1* coding region using cDNA was performed. No changes were identified.

In patient N1879, who has NF1 with a severe phenotype, a previously described polymorphism in intron 25 (IVS25-46 C→G) was the only genomic change identified by DHPLC. The analysis by DHPLC for mutations in the *NF1* gene in the affected mother (N1823), revealed a different polymorphism in intron 16 (IVS16-16 T→C).

N2241	Family A	Exon 17	2970-2972delAAT	991delM	
N2242	Family A	Exon 17	2970-2972delAAT	991delM	
N2283	Family A	Exon 17	2970-2972delAAT	991delM	
N1357	Family B	Exon 17	2970-2972delAAT	991delM	
N1358	Family B	Exon 17	2970-2972delAAT	991delM	
N2061	Family C	Exon 10b	1466 A→G	Y489C	IVS28+2
N2085	Family C	Exon 10b	1466 A→G	Y489C	IVS28+2
N2087	Family C	Exon 10b	1466 A→G	Y489C	IVS28+2
N1823	Family D	IVS16	None detected		IVS16-1(
N1879	Family D	IVS25	None detected		IVS25-4(

Table 5.1: Summary of the genomic variations identified in the *NF1* gene by DHPLC the four families with an atypical phenotype. **Family A**: family in which the affected individuals do not have neurofibromas; **family B**: Family with autosomal dominant café-au-lait spots syndrome; **family C**: family in which two members are affected by both NF1 and NF2; **family D**: family in which two members are affected by NF1 but the child presented a severe phenotype. Nucleotide numbering is based on GenBank accession no. M82814.

5.3.2 Sporadic cases

Genomic DNA from 4 NF/Noonan syndrome affected individuals was screened by DHPLC for mutations in the *NF1* gene. As a result of the screening, two mutations we identified. In patient N1397, the mutation was a 2-bp frameshift insertion in exon 23-2 and in patient N1303 a previously described 3-bp in-frame deletion in exon 25 (Barall *et al.*, 2003).

In patient N2226, a previously reported 2-bp deletion in exon 21 (3525-3526delAA) (Ars *et al.*, 2003; Fahsold *et al.*, 2000) was observed. A DNA sample from the unaffected parents was obtained and mutation analysis by DHPLC in exon 21 of the *NF1* gene was performed. This was a *de novo* mutation since the screening of exon 21 of the *NF1* gene failed to identify the mutation in either of the parents. Patient N2226

In patient N2183, a previously described nonsense mutation in exon 27a (4537 C→T; R1513X) (Ars *et al.*, 2003; Side *et al.*, 1997) was identified. This patient had a very atypical phenotype as he was affected by two relatively common neurogenetic diseases, NF1 and fascioscapulohumeral muscular dystrophy (FSHD). Unfortunately, no DNA from other members of the family was available.

A novel base substitution in exon 22 (3867 C→T) was noted in patient N2201. The mutation is a silent change that, because it is located only 3-bp from the donor splice site, might be play an important role in the pathogenesis of this patient who presents clinical features of MoyaMoya syndrome (Figure 5.3). The sequence around the donor splice site was assessed for the presence of an exonic splice enhancer (ESE) alteration by the ESE finder network service (http://rulai.cshl.edu/tools/ESE/) (Figure 5.3B). The silent change resulted in the disruption of the ESE- SC35 motif and score reduction for SRp40 motif. If this mutation disrupts an ESE, however, the transcripts may be spliced incorrectly, effectively deleting an entire exon-encoded segment of the protein.. However, to assess the possible effect of this silent mutation to the splicing machinery, it would be necessary to perform analysis at the RNA level in order to confirm either exon skipping or intron retention. However, an RNA sample from this patient was not available.

Figure 5.3: Exon 22 and 23-1 sequences of the *NF1* mRNA

A

```
gatgaactagctcgagttctggttactctgtttgattctcggcatttactctaccaactg
 D   E   L   A   R   V   L   V   T   L   F   D   S   R   H   L   L   Y   Q   L
ctctggaacatgttttctaaagaagtagaattggcagactccatgcagactctcttccga
 L   W   N   M   F   S   K   E   V   E   L   A   D   S   M   Q   T   L   F   R
ggcaacagcttggccagtaaaataatgacattctgtttcaaggtatatggtgctacctat
 G   N   S   L   A   S   K   I   M   T   F   C   F   K   V   Y   G   A   T   Y
ctacaaaaactcctggatcctttattacgaattgtgatcacatcctctgattggcaacat
 L   Q   K   L   L   D   P   L   L   R   I   V   I   T   S   S   D   W   Q   H
gttagctttgaagtggatcctaccagg
 V   S   F   E   V   D   P   T   R
```

B

Ex22 3867C   Ex22 3867C>T
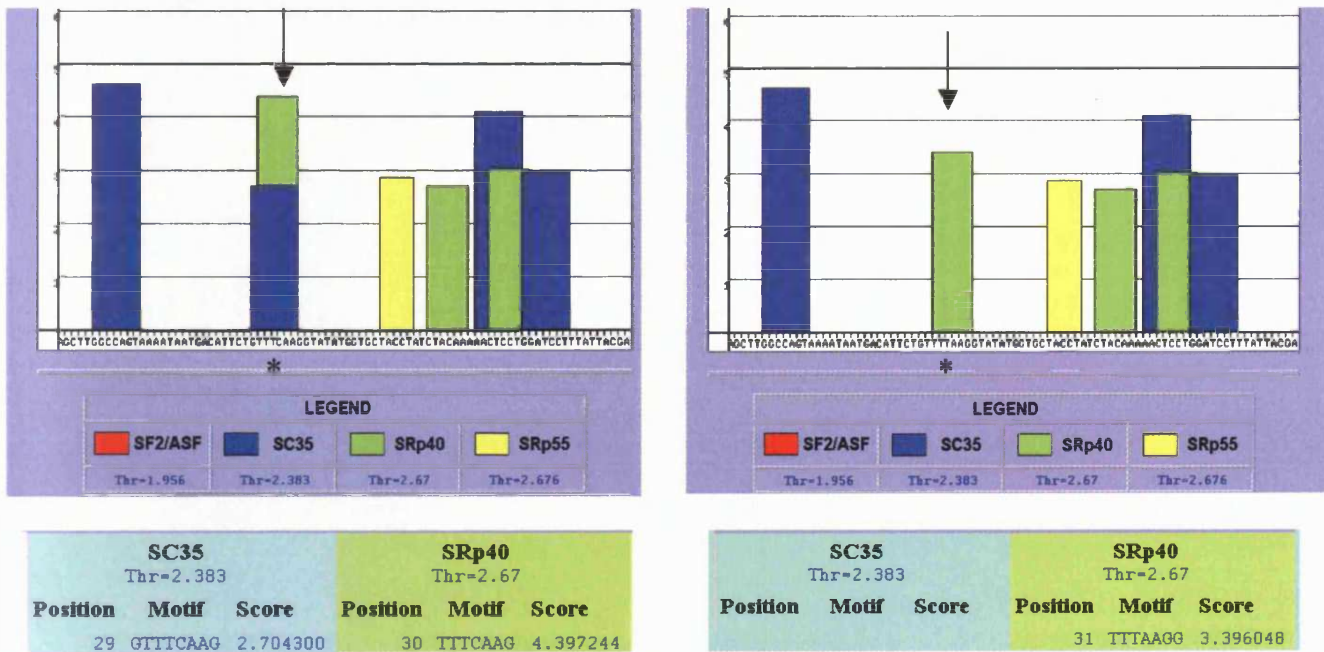
Figure 5.3: A) The site of one base substitution in exon 22 (3867 C→T) identified in patient N2201 is shown. The sequence in red represents exon 22 of the *NF1* gene and that in blue exon 23.1. The codon at position 1289 which encodes a phenylalanine in the neurofibromin is underlined. The third base (C) of that codon was changed to T in the DNA from the MoyaMoya patient. The upper case letter represents the specific amino acid in the neurofibromin protein encoded by the codon above. **B)** ESE finder predictions for the wild-type sequence of *NF1* exon 22 (on the left) and corresponding mutant (3867C>T) oligonucleotide (on the right). The bar heights reflect the score of the motifs. Threshold values and colour-code for each of the four motifs are indicated.

The site of mutation 3867C>T is indicated by an asterisk. Arrows indicate the SC35 motif disruption and the SRp 40 motif reduction caused by the mutation.

Patient N2302 presented clinical features of NF1 and early onset of haematological malignancies which led to the death of this young girl. Since only a small DNA sample was available, mutation analysis of the *NF1* gene in this patient was performed by direct sequencing. However, no mutation in the *NF1* gene was identified in patient N2302.

In patient N2290, who presented with a severe NF1 phenotype, a missense mutation in exon 11 (1658 A→G; H553R) was identified. This patient died from a cerebral glioma and an RNA sample was not available.

Patient N2244 presented with multiple subcutaneous neurofibromas as the only NF1 clinical manifestation. Mutation detection in the *NF1* gene did not reveal any *NF1* gene mutations.

Table 5.2. Mutations identified in the *NF1* gene in sporadic cases

Patient no	Phenotype	Location	Genomic mutation	Amino acid substitution	Polymorphism
N1397	NF/Noonan	Exon23-2	4095insTG	aa1366; PTC 1385	
N1303	NF/Noonan	Exon 25	4312delGAA	1438delE	
N2226	NF1-TS	Exon 21	3525-3526delAA	aa 1175-1176; PTC 1193	
N2183	NF1-FSHD	Exon27a	4538 C→T	R1513X	
N2302	MLH1$^{(-/-)}$	IVS3 IVS29			IVS3+41 A→G IVS29+19 T→C
N2201	Moya Moya	Exon 22	3867 C→T	F1289F	
N2290	Severe	IVS10b Exon 11	1658 A→G	H553R	IVS10b-35delT
N2244	Subcutaneous neurofibromas only	IVS3			IVS3+41 A→G

Table 5.2: Summary of the genomic variations identified in the *NF1* gene by DHPLC in sporadic cases with an atypical phenotype. Nucleotide numbering is based on GenBank accession no. M82814. aa: affected amino acid; PTC: premature termination codon in the predicted aberrant polypeptide.

Figure 5.4: DHPLC and sequencing chromatograms of *NF1* mutations identified during this study
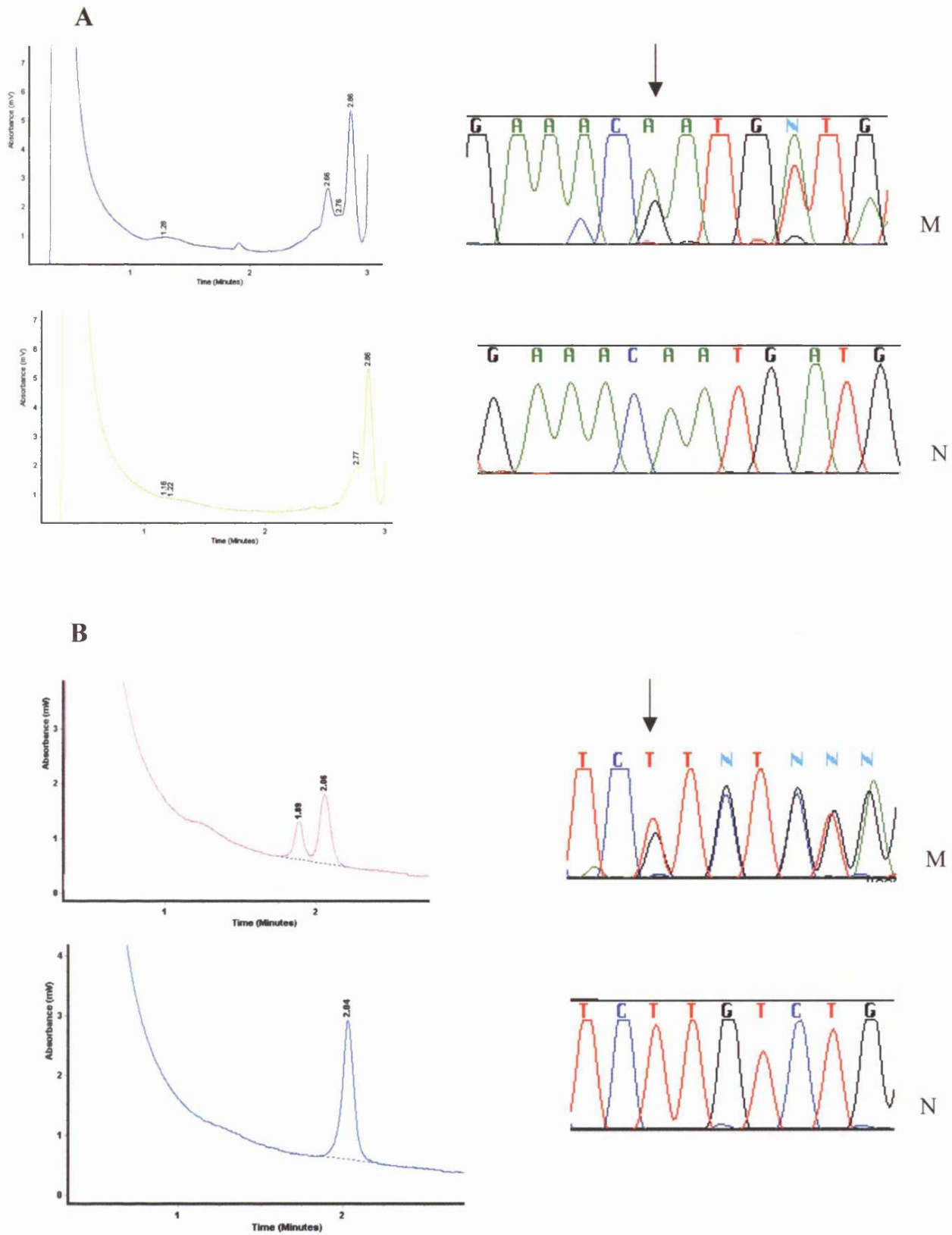
C
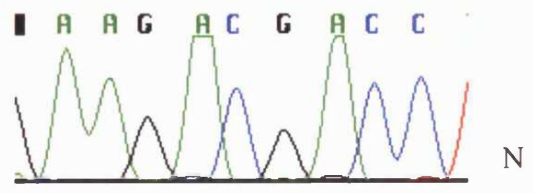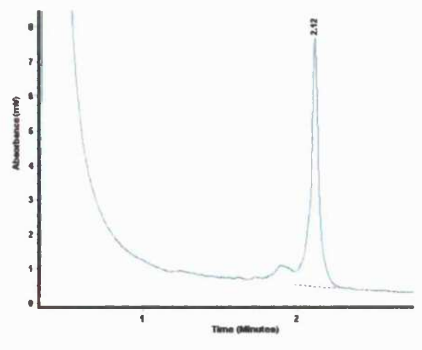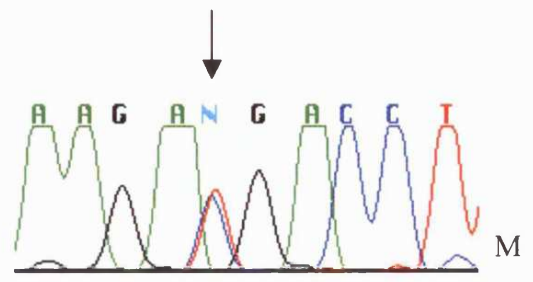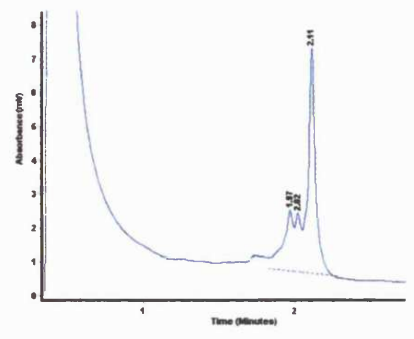
D
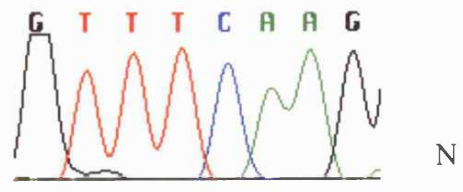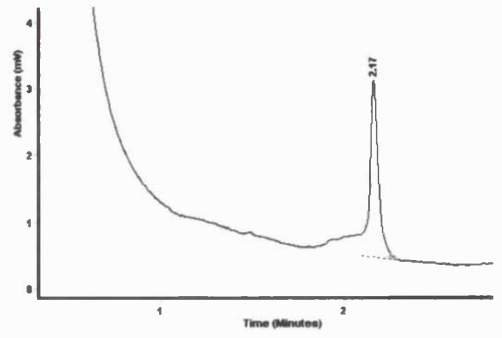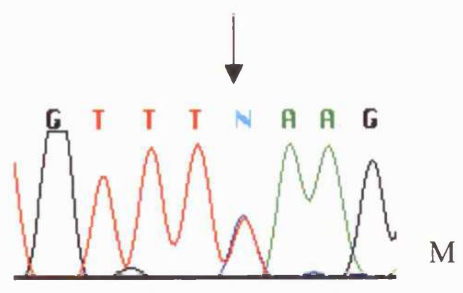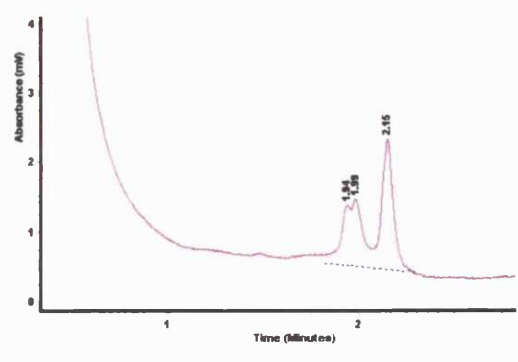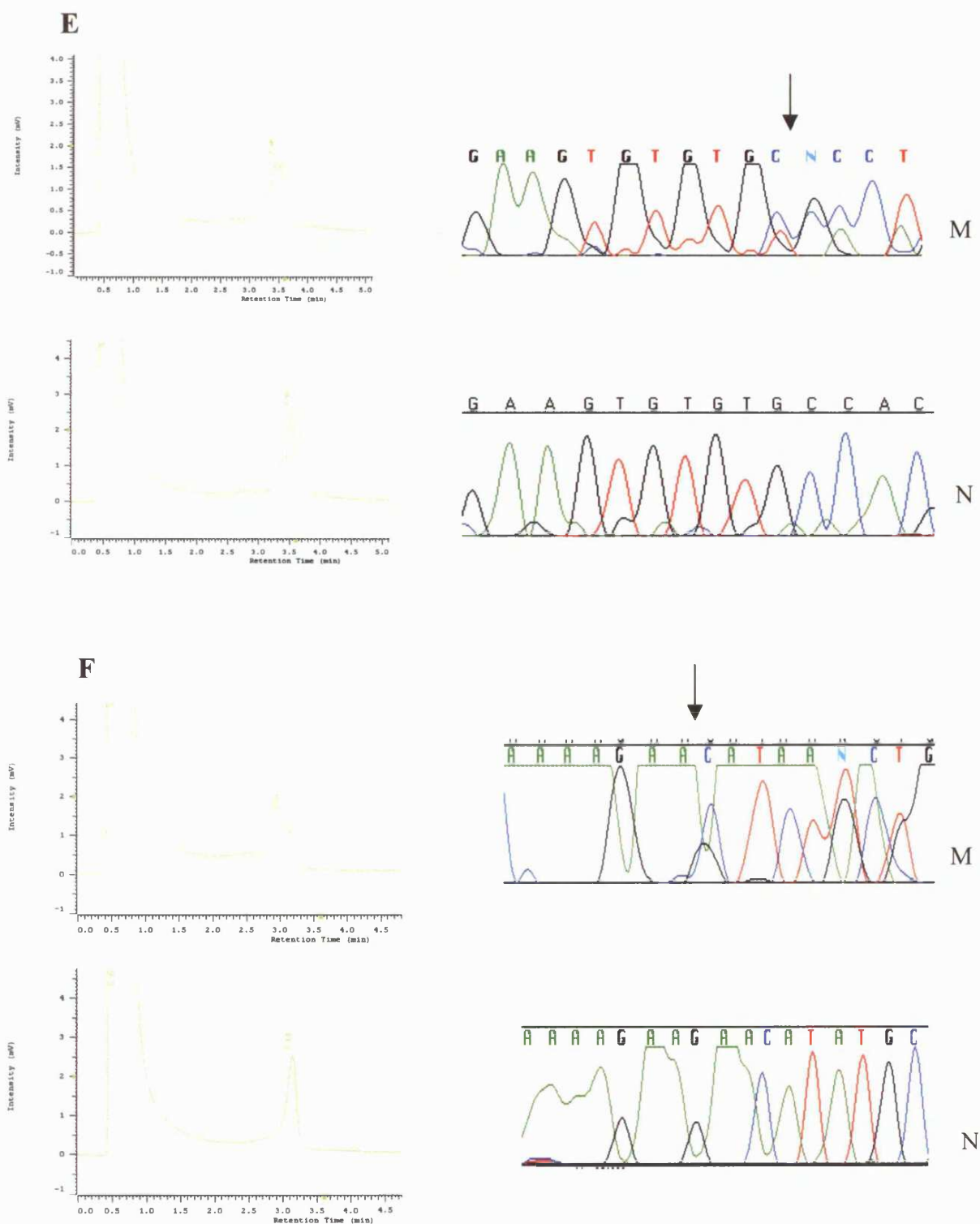
Figure 5.4: Examples of *NF1* variants identified in patients with an atypical neurofibromatosi type 1 phenotype. DHPLC traces are shown on the left and sequencing chromatograms [from the forward direction (A,C, D, E and F), from the reverse direction (B)] are illustrated on the right. Arrows indicate the position of the mutation. **A**: 2970-2972delAAT in exon 17. **B**: 3525-3526delAA in exon 21. **C**: 4538C→T in exon 27a. **D**: 3867 C→T in exon 22. **E**: 4095insTG in exon 23.2. **F**: 4312delGAA in exon 25. M, mutant; N, normal.

5.4 Discussion

During this study, which was designed to improve our knowledge of the role of either *NF1* mutation and polymorphism underlying the clinical phenotype in NF1 patients, genomic DNA samples from both sporadic and familial NF1-affected individuals with atypical phenotypes, were screened by DHPLC. The screening of the *NF1* gene in 14 atypical NF1 patients identified three small deletions (2970-2972 del AAT, 4312del GAA, 3525-3526del AA), a small frameshift insertion (4095ins TG), a nonsense mutation (R1513X), a missense mutation (H553R). and a silent change (F1289F). Interesting, the in-frame deletion (AAT) in exon 17 was identified in two families in which the affected individuals did not exhibit any neurofibromas. It has been suggested that exons 11-17 of the *NF1* gene encodes a possible functional domain due to the high number of clustered mutations identified in this region (Fahsold *et al.*, 2000). This region encodes a cysteine/serine-rich domain with three cysteine pairs suggestive of ATP binding and also three potential cAMP-dependent PKA binding sites. This suggests that the loss of a single methionine residue in neurofibromin caused by this mutation might affect cAMP signalling. However, detailed functional analysis of the effect of this mutation on the normal function of the protein is required.

In addition, the in-frame deletion (GAA) in exon 25 identified in one individual affected by NFNS during this study, has also recently described in an other NFNS patient (De Luca *et al.*, 2005). The loss of a glutamic acid at position 1438 in the neurofibromin protein caused by this mutation has been shown to promote an increased stability of the neurofibromin/Ras complex (De Luca *et al.*, 2005). These findings might give new insights about the genotype-phenotype relationship in NF1.

In conclusion, the results obtained during this study did not identify an *NF1* mutation underlying a specific clinical phenotype in the patients screened. However, the micro-deletion in exon 17 of the *NF1* gene identified only in NF1 patients lacking neurofibromas might have an important role on the formation of this type of benign tumour. Mutational analysis of the *NF1* gene in a large number of individuals exhibiting a similar phenotype and also functional studies at the RNA level, might clarify the effect of this mutation on the lack of neurofibromas. Furthermore, performing a mutational analysis on other genes that may be involved in the *NF1* gene expression

pathway, may be helpful in attaining a better understanding of the inter-individual phenotypic variation that is evident in NF1 patients.

5.4.1 Familial cases

The lack of noncutaneous abnormalities in successive generations was seen in two different families within this study group. Family B (Figure 5.1B) was diagnosed as having autosomal dominant CAL spots syndrome (MIM 114030), which is a rare syndrome as evidenced by the small number of families described so far (Abeliovich *et al.*, 1995; Arnsmeier *et al.*, 1994; Brunner *et al.*, 1993; Charrow *et al.*, 1993;). A small number of autosomal dominant CAL families are not linked to NF1. Family A (Figure 5.1A) was affected by NF1 without neurofibromas. The lack of neurofibromas is very unusual since these lesions are present in almost all adult NF1 patients. In the affected members of both families A and B, mutational analysis in the *NF1* gene revealed an in-frame 3-bp deletion (AAT) in exon 17 together with a silent codon change from ACA (threonine) to ACG (threonine). Thus, the codon ATG (methionine) at position 991 would be lost in the neurofibromin protein (Figure 5.2). The absence of that mutation in the unaffected individuals of the families, suggests that there may be a genotype-phenotype correlation. Furthermore, in our laboratory the same microdeletion in exon 17 of the *NF1* gene was identified in three other patients who did not exhibit any neurofibromas. Since these analyses were part of a diagnostic programme, they have not been included in this study group. Since no family member had neurofibromas, it is possible that Met991 in neurofibromin plays an important role in the formation of neurofibromas. To evaluate the effect of the mutational removal of Met991 on the clinical expression of NF1 in these families, a functional analysis of the *NF1* gene product, neurofibromin, is certainly worth considering, such as *in vitro* analysis using synthetic constructs. The biochemical test for GRD has been described (Gutman *et al*; 1993; Upadhyaya *et al*, 1997b). The availability of a functional or biochemical test for CSRD domain (Exon 11-17) might allow us to explore how this 3bp deletion disrupts the normal function of the gene. Furthermore the crystal structure of the mutant protein may also provide insight into the function of this protein. Fahsold *et al.* (2000) reported a mutation hotspot region in the *NF1* gene involving a possible functional domain upstream of the GAP-related domain (GRD) comprising exons 11-17. This region

encodes a cysteine/serine-rich domain with three cysteine pairs suggestive of ATP binding, as well as three potential cAMP-dependent protein kinase (PKA) recognition sites. Transfection studies have shown that overexpression of NF1 protein may lead to suppression of cell growth, transformed phenotype and decreased tumorigenicity without any change in Ras-GTP levels, suggesting that the *NF1* gene can act independently of GAP activity (Johnson *et al.* 1994, Li and White, 1996). Furthermore, in *Drosophila*, the NF1 protein seems to be a regulator of the cAMP-PKA-dependent signaling pathway instead of the Ras pathway (Guo *et al.* 1997; The *et al.* 1997; Tong *et al.* 2002). However, the GAP region of neurofibromin is only a fraction of the length of the total protein product. Consequently, it is likely that the NF1 protein has functions other than acting as a Ras-GAP. These other functions could involve regulation of the cAMP/PKA pathway or calcium-related signaling.

In 1997, Carey *et al.,* (1997) described a family in which the mother and the four children had multiple cafè-au-lait spots although one had neurofibromas, Lisch nodules or axillary freckling. Other features were short stature, molar hypoplasia, Gestalt of the Noonan syndrome face; two family members also had pulmonary stenosis. Mutational analysis of the *NF1* gene showed that the mother has the in-frame 3-bp deletion (AAT) of exon 17 that was identified during this study. However, a few families inherit typical cafè-au-lait spots seen in NF1 without other signs of NF1. By linkage analysis, some families were excluded from chromosome 17q (Brunner *et al.,* 1993; Charrow *et al.,* 1993), whereas others mapped to the *NF1* locus (Abeliovich *et al.,* 1995). The two families described by Charrow *et al.* (1993) and Abeliovich *et al,* (1995) showed genetic heterogeneity of the CALS trait. In some cases, it may be part of the clinical spectrum of NF1 while in others, it could be a distinct trait caused by different genes.

Analysis of the two generation NF1/NF2 family (Figure 5.1C), in which both the father and the child carried a frame-shift mutation in the *NF2* gene, did not reveal any mutation in the *NF1* gene. The 4-bp deletion of nucleotides 1334 to 1337 near the 3'end of exon 12 of the *NF2* gene was identified in DNA samples from the father as well as from the child and the step-sibling but not in the mother. The missense mutation in exon 10b of the *NF1* gene (Y489C) was identified in the unaffected mother as well as in the affected members. Moreover, the analysis of the cDNA in the child by both DHPLC analysis and electrophoresis on an agarose gel, did not reveal any differences as

compared with normal controls. Possible contamination of the DNA samples was eliminated as an explanation as fresh samples from all 3 individuals were obtained and the screening was repeated and the same results were obtained. The amplification by PCR of pseudogenes might be the explanation, since this particular sequence change is present in two unprocessed *NF1* pseudogenes, which have been postulated to be reservoirs of mutation for the *NF1* gene itself. The presence of the same genomic change in three different members of the same family, including an unaffected individual, may also be considered as a sequence artefact or polymorphism. A stable association of NF1 and NF2 proteins with the motor protein kinesin 1 suggests a common pathway underlying the mechanism of neurofibromatoses (Hakimi *et al*, 2002).

5.4.2 Sporadic cases

The search for mutations of the *NF1* gene in 4 sporadic NF-Noonan cases revealed the presence of two novel sequence changes (4312delGAA, 4095insTG) in two unrelated individuals. Subsequently, a different research group (Baralle *et al.*, 2003) screened these same patients for mutations in the *NF1* gene by using comparative sequence analysis (CSA), a new, rapid sequence analysis technique. The same two mutations were identified using this different mutation detection approach. Interestingly, both mutations lie within the GAP-related domain of the gene (exons 20-27a), a region previously shown to contain multiple NF1-causing mutations. The association of the Noonan like phenotype with NF1 was first noted by Allanson *et al.,* (1985) and 30 further cases have been reported (Abuelo and Meryash, 1988; Bahuan *et al.*, 1996, 1998; Baralle *et al.*, 2003; Borochowitz *et al.*, 1989; Colley *et al.*, 1996; De luca *et al.*, 2005; Meinecke, 1987; Quattrin *et al.*, 1987; Yazdizadeh *et al.*, 2004). Large studies of Noonan syndrome (NS) (Sharland *et al.,* 1992) make no reference to patients with CAL spots or neurofibromas. This suggests that NF1 features do not occur frequently in classical NS and that NF-Noonan syndrome (NFNS) is unlikely to be an unusual variant of NS. However, other studies have described cafè-au-lait macules in Noonan syndrome (Edman *et al.,* 1995). In conclusion, NFNS can represent a variant of NF1 and can be caused by different mutations, some of which cause classical NF1 in other individuals.

Mutational analysis of the *NF1* gene in an individual affected by NF1 and TS yielded a micro-deletion of two adenines, at nucleotide 3525 in exon 21, which caused a shift in the reading frame, leading to the creation of a premature stop codon at nucleotide 3580. This mutation may result in the generation of a shortened, non-functional protein of 1192 amino acids. Recently, the same mutation was reported by Fahsold *et al.* (2000), Serra *et al.* (2001) and Trovo *et al.*, (2004); the patients with the *NF1* micro-deletion in exon 21 exhibited a classical NF1 phenotype. However, the association of the two disorders in this patient together with patient N2183, who was affected by NF1 and FSHD, may be explicable as simple coincidence. Both NF1 and TSC predispose affected individuals to the development of benign tumours in a wide variety of organs and more rarely to malignant tumours. The products of the *NF1* and *TSC2* genes, both interact with and potentially regulate two different GTPases present in most cells. Neurofibromin acts as a negative regulator of Ras, while TSC2 product (tuberin) interacts with and may regulate the activity of closely related Rap1. An animal model with targeted disruption of NF1 and TSC2 has not been developed. Furthermore dysregulation of MTOR pathway has been reported in both NF1 and TSC2 (Johannessen *et al*, 2005; Inoki *et al*, 2005).

During this study, the mutation identified (3867 C→T) in exon 22 of the *NF1* gene in a patient with MoyaMoya disease, provides a very interesting result which can support the association between the two disorders. The one base-pair substitution identified in the genomic DNA results in a silent codon change in the mRNA. Since it was 3-bp distant from the donor splice site, it might produce an aberrant form of RNA splicing which could be pathogenic. To evaluate the role of this silent mutation on the splicing mechanism, RNA analysis would be necessary. Since RNA samples from this patient were not available, further studies were not possible.

MoyaMoya disease, which is characterized by the spontaneous occlusion of the circle of Willis, is a definitive clinical entity of unknown cause. Approximately 9% of cases are familial, with approximately 76% occurring in sibs and 24% in a parent and offspring (Yamauchi *et al.*, 2000). As a consequence of the advent of non-invasive diagnostic methods, the identification of familial cases has increased through the finding of asymptomatic family members. There are several lines of evidence indicating that MoyaMoya disease is related to genetic factors in familial cases; linkage studies on

families with individuals affected by MoyaMoya disease revealed that the potential gene for familial MoyaMoya disease is located on chromosome 17 and precisely at 17q25 (Yamauchi *et al.*, 2000; Fujimura *et al.*, 2004).

To evaluate the association between MMR deficiency and *NF1* gene mutation, genomic DNA from a patient identified as homozygous for a *MLH1* mutation, was screened by DHPLC for mutations in the *NF1* gene. The absence of an *NF1* mutation in this patient may depend on the cell type analysed. Since the DNA sample from this patient was received after her death, we were unable to detect a *NF1* somatic mutation in other tissues. It has been proposed that the clinical signs of NF1 in carriers of homozygous MMR gene defects are a consequence of somatic *NF1* gene inactivation (Wang *et al.*, 2003; Gutmann *et al.*, 2003). Therefore, further studies on the distribution of *NF1* mutations in different tissues from patients homozygous for a mutation in an MMR gene are required for us to understand more about the relationship between MMR deficiency and *NF1* gene mutation.

The genetic approach assumes that phenotypic characters are either determined or influenced by the genetic constitution of an organism (Wolf, 1995). Thus, either the structure of a single gene or the interaction between several genes is considered to be responsible for, or to contribute to, phenotypic variation. However, the analysis of the correlation between genotype and phenotype has to take into account various problems involved with the realization of the phenotype. There are a number of factors that can interfere with this process by modifying particular steps. The clinical phenotype can be considered to be the result of the interaction between multiplicities of factors, of which genetic variation represents only one category. To be able to interpret the genotype-phenotype relationship, the combinatorial effects of different mutations and polymorphisms, whether allelic or non-allelic, have to be considered. The study of mutations in human inherited disease should facilitate 1) the assessment of the spectrum of known genetic variation, 2) the optimisation of mutational screening strategies, 3) the search of novel gene lesions associated with different clinical phenotypes, 4) the identification of factors that influence the DNA mutagenesis and 5) understanding the different mechanisms underlying clinical expression in either inherited and somatic disease.

NF1 is a complex disorder, affecting multiple cell types and multiple systems of the body. It is characterized by a wide range of expression and unpredictable behaviour that makes clinical management a major challenge. There are however various subtypes of NF1 with distinctive phenotypes that have not yet been fully deciphered at the molecular level. Neurofibromatosis-Noonan syndrome, Watson syndrome, familial café-au-lait spots, and familial spinal neurofibromatosis are the most common conditions that exhibit significant, but distinct overlaps with NF1. These rare conditions map to the *NF1* locus in a few families (Allanson *et al.*, 1991; Stern *et al.*, 1992), and *NF1* mutations have been found in a small number of cases (Ars *et al.*, 1998; Bahuau *et al.*, 1998; Baralle *et al.*, 2003; Carey *et al.*, 1997; Kaufmann *et al.*, 2001; Messian *et al.*, 2003; Tassabehji *et al.*, 1993).

There are several possibilities to explain the combination of other disorders with neurofibromatosis 1: 1) that it is due to mutation at a locus different from that of *NF1* on chromosome 17; 2) that it is due to an allele at the *NF1* locus; 3) that it is simply the coincidence of two relatively frequent conditions; or 4) it represents associated disorders due to mutations at closely linked loci.

The high degree of variation in the expression in NF1 may be due to either allelic or non-allelic genetic factors, some aspect of the environment whether internal or external or chance or, of course, some combination of these factors. Lupski *et al.* (1993), for example, studied two unrelated patients with both CMT1 and NF1. Since both of these disorders map to the pericentric region of chromosome 17, they investigated whether this might be a contiguous gene syndrome. In both patients, however, the CMT1A was inherited from the father, who did not have NF1. Furthermore, molecular analysis showed that the CMT1A duplication was stable in the two patients. One patient transmitted both disorders to her daughter. Thus, this was a chance concurrence of two common disorders.

Although it has been shown that hypermethylation of the *NF1* gene promoter prevents binding of transcription factors *in vitro* (Mancini *et al.*, 1999), no evidence of hypermethylation has been found in the *NF1* gene in neurofibromas (Horan *et al.*, 2000). Therefore, the epigenetic inactivation of the *NF1* gene promoter does not seem to be a common event. Understanding the causes of this phenotypic variation is therefore a major challenge. At this juncture, we assume that the NF1 phenotype is determined

predominantly by the genotype including modifying genes, whereas epigenetic (at least at the *NF1* locus) and environmental factors are of minor importance.

It is hoped that further study of the underlying pathogenesis will uncover the biomedical basis for the various clinical phenotypes associated with the different types of lesions and ultimately will produce advances in both diagnosis and therapy. At the moment, detection of the specific mutation does not yet yield any additional information of clinical help in the management or prediction of disease severity/progression. *NF1* mutation analysis will certainly become much more sensitive and straightforward in the near future, which will permit the implementation of newer methods and the use of new equipment designed for large gene/multiple mutation analysis. Therefore, the pathogenetic mechanism underlying NF1 should continue to be investigated for all mutations, on the hypothesis that genotype-phenotype correlations may be based on the RNA/protein-level effects, which would lead to clinically useful information. Understanding the molecular basis for this variable expression will be of diagnostic and prognostic importance and should allow improved clinical management for the medical complications associated with this inherited condition. Analysis of somatic mutations in a variety of human tumours should help us to understand the possible mechanistic pathway(s) through which inherited and somatic mutations in the *NF1* gene eventually lead to tumorigenesis. Structural studies of the domains of neurofibromin, by X-ray crystallography and nuclear magnetic resonance spectroscopy, should provide explanations of both the structural and functional contributions of mutational lesions, whether somatic or inherited.

In conclusion, our difficulty in correlating specific *NF1* mutations with a particular clinical phenotype suggests that modifying genes are involved in the clinical variability of NF1. This conclusion is strongly supported by the observation that identical *NF1* gene lesions can cause very different phenotypes in unrelated NF1 patients (Ainsworth *et al.*, 1993; Cawthon *et al.*, 1994; Shen *et al.*, 1993; Upadhayaya *et al.*, 1997a; Wiest *et al.*, 2003). Identifying such modifying genes is an important challenge not only because it could assist with counselling of NF1 families but also because such genes may be important determinants of the degree of disease severity.

Chapter 6. Evaluation of the 17q11.2 deletion breakpoint boundaries in patients with neurofibromatosis type 1

6.1 Introduction

An increasing number of human diseases have been recognized to result from recurrent DNA rearrangements involving unstable genomic regions. In 1998, Lupski both proposed and defined a new concept of the *genomic disorder,* a term he applied to those conditions that result from DNA rearrangements whose nature and frequency are influenced by the regional genomic architecture. These rearrangements lead to the gain or loss of a dosage-sensitive gene (or genes) or to the disruption of a gene (Lupski, 1998). Non-allelic homologous recombination (NAHR) is usually the mechanism responsible for such rearrangements, with breakpoints clustering in low-copy repeat (LCR) regions; these are also known as segmental duplications (Mazzarella & Schlessinger, 1998; Lupski, 1998; Ji *et al.*, 2000). The process of NAHR between LCRs in the same orientation on the same chromosome results in reciprocal deletions and duplications, whereas NAHR between LCRs in the opposite orientation on the same chromosome results in inversions. NAHR can also occur between LCRs located on different chromosomes, resulting in reciprocal translocations. Other mechanisms such as non-homologous end joining (NHEJ) have been observed (Inoue and Kupski, 2002; Roth and Wilson, 1986; Shaw and Lupski, 2004), particularly with rearrangements that exhibit scattered breakpoints (Figure 6.1).

Regardless of the precise recombination mechanism, a variety of different genomic architectural features have been associated with the known rearrangement breakpoints. This suggests that chromosomal rearrangements are not random events, but rather result from a predisposition to genetic rearrangements conferred by complex genomic architectural features that have the potential to create instability in the genome. Genes residing within the recombined unique genomic segments may be responsible for the specific clinical features of the associated genomic disorders. Furthermore, identifying those genes specifically involved in the recombination events might help us to

understand more about the relationship between the genomic rearrangements and the consequent clinical phenotypes in genomic disorders. When the LCRs lie within a functional gene, this gene may either be interrupted or fused with another gene by the recombination event, resulting in the abrogation of its function. This is generally the case when the recombined genomic fragment is small or when the LCRs flank a functional gene and its pseudogene. When the recombined genomic region is large enough to encompass multiple genes, the recombinant allele may involve the loss or gain of one or more dosage-sensitive genes located between the LCRs (Inoue and Lupski, 2002; Lupski, 1998). In contrast to nucleotide substitutions and other point mutations in which alteration of the protein sequence is the major cause of the disease mechanism, large genomic rearrangements alter gene copy number. In such cases, the disease mechanism does not involve a change in the conformation of the encoded protein, but rather results from a gene (and protein) dosage effect. Patients in whom this type of genomic change occurs usually exhibit a more severe clinical phenotype.

LCRs that originate from the duplication of large segments of genomic DNA typically share ~97% sequence identity and represent 5-10% of the genome (Bailey et al., 2001; Shaikh et al., 2001; Stankiewicz and Lupski, 2002). Chromosomal rearrangement breakpoints have been located throughout the genome. However, intervals containing LCRs or AT-rich palindromes predominate in the pericentromeric and subtelomeric regions of human chromosomes (Eichler et al., 1996, 1997; Regnier et al., 1997; Trask et al., 1998a). This has led to the suggestion that the sequence of pericentromeric and subtelomeric regions of chromosomes promote both the generation and expansion of segmental duplications (Ji et al., 2000).

In recent years, LCRs have been increasingly implicated as mediators of NAHR, resulting in a number of congenital genomic disorders. Misalignment, followed by recombination between non-allelic segmental duplications on homologous chromosomes, has been proposed to give rise to many genomic disorders. These disorders, include NF1 on 17q11.2 (Dorschner et al., 2000), Charcot-Marie Tooth disease type 1A (CMT1A)/hereditary neuropathy with liability to pressure palsies (HNPP) on chromosome 17p11.2 (Chance et al., 1994; Lupski, 1998), Angelman/Prader-Willi syndromes (PWS/AS) (MIM 1058/MIM 176270) on 15q11-q13 (Christian et al., 1999; Amos-Landgraf et al., 1999), Williams-Beuren syndrome (WBS)

(MIM 194050) on 7q11.23 (Perez-Jurado *et al.*, 1998; Peoples *et al.*, 2000), Smith-Magenis syndrome (SMS)/duplication 17p11.2 (MIM 182290) on 17p11.2 (Chen *et al.*, 1997; Potocki *et al.*, 2000) and several rearrangements associated with 22q11 such as DiGeorge syndrome and velocardiofacial syndrome (DGS/VCFS) (MIM 188400) (Edelmann *et al.*, 1999; Shaikh *et al.*, 2000).

Figure 6.1: Mechanisms of genomic rearrangements.
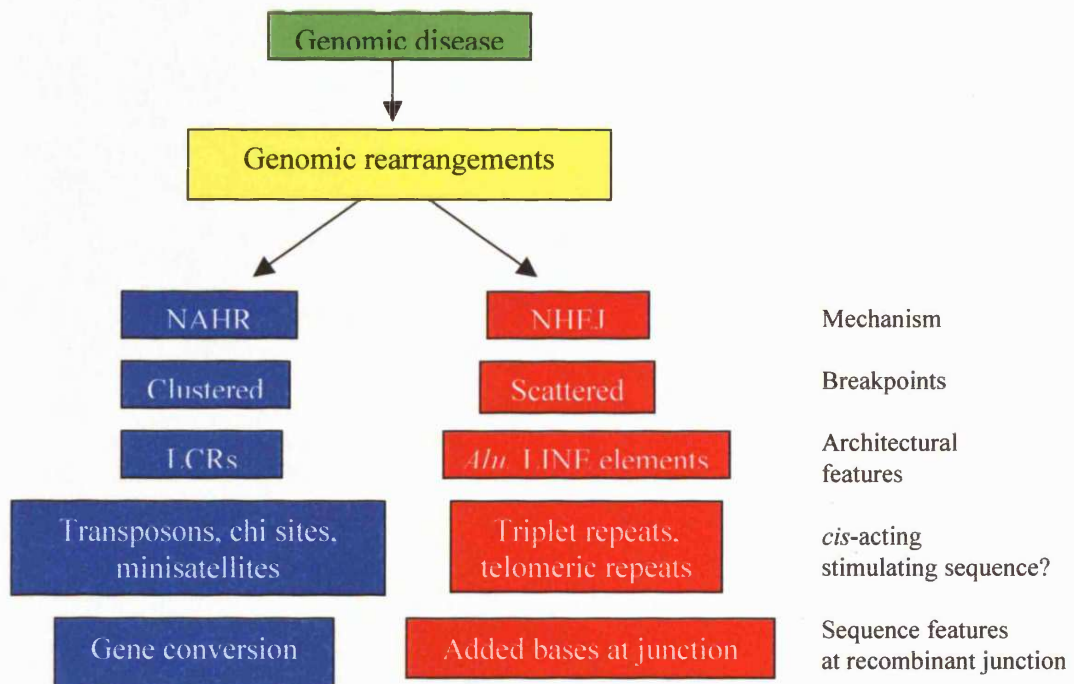
Figure 6.1: Two primary recombinational mechanisms, non-allelic homologous recombination (NAHR; blue) and non-homologous end joining (NHEJ; red), are shown. Features associated with NAHR or NHEJ are shown in blue and red, respectively (Shaw and Lupski, 2004).

6.1.1 NF1 deletion syndrome

Over 70% of the germline mutations identified in NF1 patients are intragenic mutations of the *NF1* gene that are predicted to cause either truncation or loss of the encoded protein (Ars *et al.*, 2000; Fahsold *et al.*, 2000; Messiaen *et al.*, 2000). Between 5 and 20% of all NF1 patients carry a heterozygous deletion involving the *NF1* gene and the contiguous genes lying in its flanking regions (Riva *et al.*, 2000; Jenne *et al.*, 2001). In

the majority of cases, the deletions at 17q11.2 span 1.5Mb and are caused by recombination between highly homologous low copy repeats (LCRs) that flank the *NF1* gene at distances of ~400 kb on the proximal side and 700 kb on the distal side (Dorschner *et al.*, 2000; Jenne *et al.*, 2000, 2001; Lopez-Correa *et al.*, 1999, 2001) (Figure 6.2). These highly homologous low copy repeats, which are termed NF1-REP-P (proximal) and NF1-REP-M (medial), are ~85kb in length and lie in the same orientation (Dorschner *et al.*, 2000). 46% of the microdeletion breakpoints are located within a 2 kb recombination hotspot located in these flanking paralogous sequences (Lopez-Correa *et al.*, 2001). A third copy, termed distal NF1-REP maps to 17q24. The REP-mediated deletions probably occur as a result of interchromosomal recombination of misaligned NF1-REP elements or intrachromosomal looping-out (Dorschner *et al.*, 2000; Jenne *et al.*, 2001). A second type of recurrent *NF1* deletion, mediated by intrachromosomal recombination between the *JJAZ1* (MIM 606245) gene and its closely linked pseudogene, has been described (Venturin *et al.*, 2004; Petek *et al.*, 2003; Kehrer-Sawatzki *et al.*, 2004). Most of the sequenced deletion breakpoints, localized within the NF1-REP-P and –M or in the *JJAZ1* gene/pseudogene, are mediated by nonallelic homologous recombination (NAHR) (Kehrer-Sawatzki *et al.*, 2004; Inoue *et al.*, 2002; Stankiewicz *et al.*, 2003). One case of a non-LCR mediated deletion with a sequenced breakpoint junction has been reported (Venturin *et al.*, 2004), and the underlying mechanism has been proposed to be nonhomologous end-joining (Inoue *et al.*, 2002; Stankiewicz *et al.*, 2003) (Figure 6.3).

The LCRs mediating intrachromosomal deletions at 17q11.2 are derived from the *WI-12393* gene and contain sequences with homology to 19p13 (Dorschner *et al.*, 2000; Jenne *et al.*, 2001; Lopez-Correa *et al.*, 2001; Jenne *et al.*, 2003). The structure of the *NF1* region at 17q11.2 is further complicated by other duplicated sequences, such as the pseudogene fragments of the *SMURF2* and the *KIAA0160* genes located at 17q (Dorschner *et al.*, 2000; Jenne *et al.*, 2003). These sequences may also represent templates for unequal recombination and subsequent deletions of the intervening chromosomal regions at 17q11.2.

"NF1 microdeletion syndrome" is often characterized by the presence of a more severe phenotype with variable facial dysmorphism, learning disabilities, mental retardation, cardiovascular abnormalities, developmental delay and a number of neurofibromas that

would be expected from patient's age. (Cnossen *et al.*, 1997; Leppig *et al.*, 1997; Tonsgard *et al.*, 1997; Rasmussen *et al.*, 1998; Upadhyaya *et al.*, 1998; Riva *et al.*, 2000; Venturin *et al.*, 2004). Patients with an *NF1* microdeletion have a higher lifetime risk for the development of MPNSTs (Kluwe *et al.*, 2003a; De Raedt *et al.*, 2003). The severe phenotype of patients carrying gross deletions encompassing more than just the *NF1* gene may be explained by variation in the expression of the additional genes involved in the rearrangement caused by different mechanisms, such as gene interruption, position effect and decreased gene dosage. As with the other genomic disorders, such as DiGeorge/velocardiofacial (Shaikh *et al.*, 2000), Smith Magenis (Shaw *et al.*, 2002), Angelman/Prader-Willi (Amos-Landgraf *et al.*, 1999) and Williams-Beuren (Valero *et al.*, 2000) syndromes, *NF1* deletions are mediated by specific architectural genomic features that explain the common occurrence of standard-sized recurring deletions (Dorschner *et al.*, 2000), even although different deletion endpoints and sizes have also been identified in a subset of patients (Cnossen *et al.*, 1997; Dorschner *et al.*, 2000; Riva *et al.*, 2000; Jenne *et al.*, 2001; Kayes *et al.*, 1992; Kehrer-Sawatzki *et al.*, 2003; Petek *et al.*, 2003; Upadhayaya *et al.*, 1996b; Venturin *et al.*, 2004).

About 80% of gross *de novo NF1* deletions occur on maternally derived chromosomes (Upadhayaya *et al.*, 1998; Lopez-Correa *et al.*, 2001). This is consistent with the results of haplotype analyses of healthy parents of the *de novo NF1* microdeletion individuals, which suggested that paralogous recombination occurred during maternal meiosis II (Lopez-Correa *et al.*, 2000). However, those *NF1* deletions that are not bordered by LCRs were found to be of paternal origin and to have arisen by an intrachromosomal mechanism during spermatogenesis (Kehrer-Sawatzki *et al.*, 2003). To explain these observations, Jenne *et al.* (2000) suggested that haploinsufficiency for the genes involved in the deletion may confer some disadvantage during the maturation of spermatocytes or a competitive disadvantage in the fertilization process, whereas oocytes acquiring gross deletions during maturation may not be eliminated by selective mechanisms.

By combining clinical and genetic evidence from 92 patients with the 1.5 Mb *NF1* gene deletion, Venturin *et al.* (2004) reviewed specific clinical signs associated with the NF1 deletion syndrome. They found that the most common NF1 clinical signs in patients

with the deletion were learning disability, cardiovascular malformations, and dysmorphism. From the gene content of the deleted region, Venturin *et al.* (2004) proposed that haploinsufficiency of the *OMG* (MIM 164345) and/or *CDK5R1* (MIM 603460) genes (located in an *NF1* intron) may be implicated in learning disability. In relation to cardiovascular malformations, only *JJAZ1* (MIM 606245) and *CENTA2* (MIM 608635) were considered possible candidate genes by hint of their being significantly expressed in the heart.

Lopez-Correa *et al.* (2001) mapped and sequenced the deletion breakpoints in 54 NF1 patients. Using a deletion junction-specific PCR assay, they also demonstrated that the recombination event between the NF1-REP-P and -M paralogues occurred within a 3.4 kb fragment in 46% of cases of deletions that span the *NF1* gene.

Kehrer-Sawatzki *et al.* (2004) identified a high frequency of mosaicism among patients with NF1 caused by gross deletions resulting from somatic recombination of the *JJAZ1* gene. The observed deletions fell into two distinct groups. The classic 1.5-Mb deletion was found in some patients. These "type I deletions" encompass a total of 14 genes and have breakpoints within the *NF1* low-copy repeats (LCRs). However, they also identified a second major type of *NF1* deletion which spanned 1.2 Mb and affected 13 genes. This "type II deletion" was mediated by recombination between the *JJAZ1* gene and its pseudogene. The *JJAZ1* gene, which was completely deleted in patients with type I *NF1* deletions and disrupted in deletions of type II, is highly expressed in brain structures associated with learning and memory. Thus, its haploinsufficiency could contribute to mental impairment in patients with constitutional *NF1* deletions. Conspicuously, 7 of the 8 mosaic deletions were of type II, whereas only one was a classic type I deletion. Therefore, the *JJAZ1* gene is a preferred target of strand exchange during mitotic nonallelic homologous recombination. Although type I *NF1* deletions occur by interchromosomal recombination during meiosis, the findings of Kehrer-Sawatzki *et al.* (2004) imply that type II deletions are mediated by intrachromosomal recombination during mitosis at an early stage in development.

In this current study, we have aimed to identify the breakpoint boundaries present in 46% of NF1 patients with deletion of the entire *NF1* gene and flanking regions (Lopez-Correa *et al.*, 2001). In all the patients studied, the *NF1* deletion has been previously identified by FISH analysis performed by Dr Peter Thompson and colleagues in

Molecular Cytogenetics Section of the Institute of Medical Genetics in Cardiff. Such patients were then referred to us in order to confirm the incidence of the specific breakpoint in the *NF1* microdeletion patients with a specific clinical phenotype. Correlations between the clinical phenotype and the presence or absence of the typical *NF1* deletion breakpoints in 21 NF1 patients with the *NF1* deletion have been evaluated.

Figure 6.2: Arrangement and structure of the NF1REP paralogs
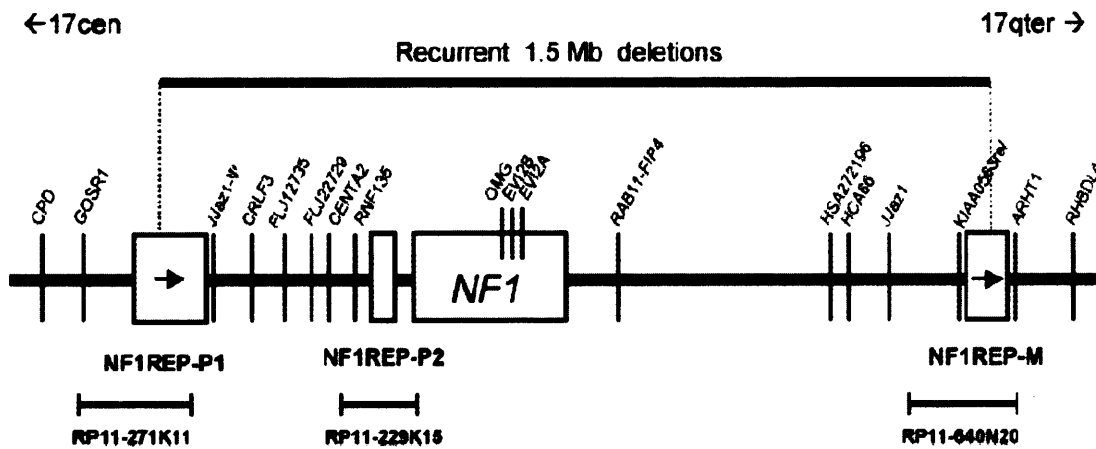
Figure 6.2: The diagram shows the *NF1* gene and the flanking paralogous elements NF1REP-P1 and –M that occur in the same orientation. The extent of the recurrent 1.5Mb deletion is shown above the map. The vertical bars represent functional genes, with the exception of *JJAZ1-Ψ*, which is a pseudogene of *JJAZ1*. The reference BACs for NF1REPs are indicated. A forward primer specific to NF1REP-P1 and a reverse primer specific to NF1REP-M were used in this study to amplify a 3.4 kb junction fragment in DNAs from NF1 patients with the 1.5Mb deletion. (Forbes *et al.*, 2004).

Figure 6.3: Generation of deletion rearrangement by NAHR and NHEJ.
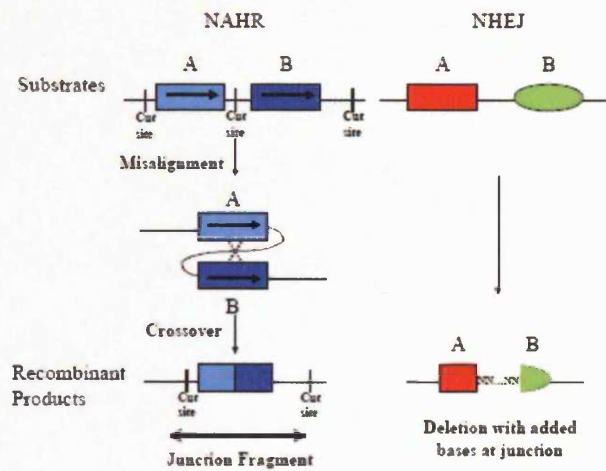
Figure 6.3: Generation of deletion rearrangement by NAHR and NHEJ. The substrates and products of recombination are shown. NAHR (left) utilizes two non-allelic LCRs (A and B) as substrates for recombination. The LCRs are shown as blue rectangles. LCRs A and B, similarly oriented, (shown by arrows) then misalign, and the subsequent homologous recombination results in a deletion with a single recombinant LCR, shown as a two-tone blue rectangle. NHEJ (right) utilizes two nonhomologous sequences (red rectangle (A) and green oval (B)) as substrates for recombination. The two sequences are joined via NHEJ, leading to the deletion of the intervening fragment. Additional bases (NN…NN) are frequently added at the deletion junction (Shaw and Lupski, 2004).

6.2 Patients

Genomic DNA samples were obtained from 21 *NF1* deletion patients, comprising 16 sporadic and 2 familial cases. Previous diagnostic reports had documented the deletion in all 21 patients (Dr Peter Thompson, Molecular Cytogenetics). The deletion had been shown to extend beyond the borders of the *NF1* gene in all these cases as evidenced by fluorescence *in situ* hybridisation (FISH) studies.

6.3 Results

A common breakpoint interval of ~3 kb was previously identified in 46% of NF1 patients with a deletion of the entire *NF1* gene (Lopez-Correa *et al.*, 2001). In this present study, a deletion-specific PCR assay described by Lopez-Correa *et al.* (2001) was performed on DNA samples from 21 NF1 patients known to be carrying the large gene deletion. In order to minimize the amount of DNA sample used for each reaction, the deletion-specific PCR assay conditions were optimised. The 3.4 kb deletion junction fragment was detected in 10 of 21 patients. DNA samples from 30 control subjects were also screened by the deletion-specific PCR assay for the common breakpoint interval, but no characteristic 3.4 kb amplified fragment was observed. These results served not only to document the specificity of the assay in detecting only chimeric NF1 REP sequences that arose from this specific deletion event, but also confirmed the previously reported 46% detection frequency (Lopez-Correa *et al.*, 2001) of the specific deletion hotspot in *NF1* deletion patients.

All of the 21 NF1 patients included in this study were NF1 patients diagnosed by conventional clinical criteria. Indeed, all of the patients had a severe NF1 phenotype with a number of other clinical features (Table 6.1). As shown in Table 6.1, nine of ten NF1 patients with the common recombination hotspot presented dysmorphic features, five had development delay and three had learning disabilities. Considering the clinical phenotype of the *NF1* deletion patients in whom the 3.4 kb deletion junction fragment was detected in this study, dysmorphic features and multiple neurofibromas seem to be the most common clinical problems related to haploinsufficiency for genes involved in that specific region on 17q11.2. The same recombination hotspot was detected in DNA samples from 3 NF1 affected individuals from the same family. Patient N1666, who presented a relatively mild NF1 phenotype, had two affected children (N1869, N1668) with a severe NF1 phenotype (both presented with dysmorphic features and development delay). Whereas N1668 also had speech problems and scoliosis, N1869 had cardiovascular involvement and malignancy. An NF1 patient with the common 1.5 Mb deletion and skeletal development problems was previously described by Oktenli *et al.* (2003); this suggests that such large deletions could well also involve flanking gene loci that encode proteins with functions in the skeletal system. The other family in

which two affected individuals (N2217, N2266) exhibited a deletion of the entire *NF1* gene, proved negative for the 3.4 kb deletion breakpoint.

Table 6.1: Clinical features of the 21 NF1 patients

DNA n°	DOB	Mult Neurofi	Dysmor features	Devel delay	Learn disab	Age at onset	Lish nodules	Others	Malignan	Comment
N2179	22/08/1975	Yes	Yes	Yes	Yes		Yes			
N2178	11/10/1966	Yes	Yes			Relatively young age				her dad died of malignancy
N2286	11/07/2002		Yes		mother's brother	At birth		Noisy breathing		New mutation
N2287	10/11/1996		Yes	Yes						
N1666	12/10/1971	Yes					Yes			
N1869	15/10/1997		Yes	Yes				Cardiovascular problems	Prostate cancer	
N1668	01/10/1993		Yes	Yes	Yes			Scoliosis		
N2164	13/09/1944	Yes	Yes							
N2165	21/07/1996		Yes	Yes						
N2180	03/02/1992		Yes		Yes					
N2131	11/03/1986	Yes								
N2168	29/06/1957	Spinal neurofibromas	Yes					Cervical kyphosis		
N2285	19/02/2002									
N2289										
N2217	07/08/1957	Yes+ plexiform		Low IQ	Yes		Probable optic chiasm	Hypotonia in infancy		
N2266	28/08/1992									
N2288		Yes	Yes							New mutation
N1350	26/04/1980	Cervical neurofibroma	Yes			5 years	Yes	Scoliosis	Sarcoma	
N1918	25/04/1983	Yes+plexiform			Yes			Epilepsy		
N2167	11/12/1970	Yes		Yes						
N2166										

Table 6.1: Clinical features of the 21 NF1 patients from the study group. The patients in whom the 3.4 kb PCR fragment was detected are highlighted in red. The patients in whom the 3.4 kb PCR fragment was not detected are highlighted in blue. CAL: café-au-lait spots; Freck: frecklings; Dysmor features: dysmorphic features; Devel delay: development delay; Learn disab: learning disabilities; Others: other clinical features; Malignan: malignancy.

151

Figure 6.4: Detection of the *NF1* deletion junction fragments

Ma 2164 2165 2166 2178 2179 2180 P.C. B


```
12.2
 3.4
 1.6
 500
```
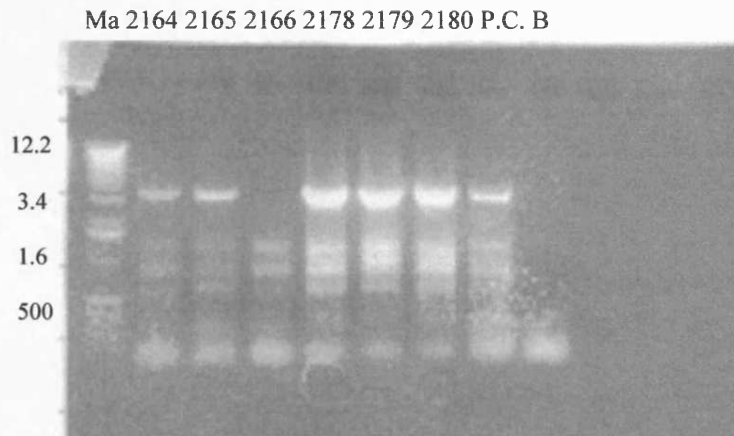
Figure 6.4: The 3.4 kb fragment identified in 10 *NF1* deleted patients by a deletion junction–specific PCR. The PCR product was visualized under UV light after a time of 1 hour of electrophoretic migration into a 1% agarose gel. The numbers at the top indicate the patient DNA numbers. **Ma**: 1 kb DNA marker; **P.C.**: a positive control sample; **B**: blank.

6.4 Discussion

During this study, 21 NF1 patients who exhibited the typical clinical phenotype of the NF1 microdeletion and also known to be carrying deletions of the entire *NF1* gene identified by previous FISH analysis, were screened for the common deletion breakpoints by a deletion-junction PCR assay. Identification of a 3.4 kb PCR product in 10 of the 21 DNA samples from these *NF1* deletion patients, revealed the high specificity of the assay for the detection of the common breakpoint. It also confirmed the frequency (46%) of the recombination event at 17q11.2 regions as previously reported by Lopez-Correa *et al.* (2001).

The aim was to determine the frequency of the common deletion breakpoints and then to analyse the possible correlation between the occurrence of such specific deletion breakpoints and the clinical phenotype in our study group. Identification of the breakpoint hotspot in 10 of 21 NF1 patients carrying a heterozygous deletion involving the *NF1* gene and its flanking regions and exhibiting severe clinical phenotypes,

revealed a somewhat different expression of the clinical phenotype in both sporadic and familial cases. This suggests that there may be other factors that influence the variable expression of the genes involved in the deletion. The remaining 11 patients without 1.5 Mb deletion should be examined for the presence of 1.2 Mb deletion.

The wide diversity of mutation types and the wide range of variable expression of neurofibromatosis type 1 have made it difficult to establish genotype-phenotype correlations (Huson *et al.*, 1989a; Huson, 1994; Viskochil and Carey, 1992; Upadhyaya *et al.*, 1992, 1994). Nevertheless, the deletion of the entire *NF1* gene appears to be associated with a distinct phenotype that includes early onset of a large number of cutaneous neurofibromas, minor facial anomalies, and development delay (Cnossen *et al.*, 1997; Leppig *et al.*, 1997; Tonsgard *et al.*, 1997; Rasmussen *et al.*, 1998; Upadhyaya *et al.*, 1998; Riva *et al.*, 2000; Venturin *et al.*, 2004).

Up to 5% of the human genome is composed of segmental duplications (Bailey *et al.*, 2001; Shaikh *et al.*, 2001; Stankiewicz and Lupski, 2002). It is thought that segmental duplications evolved during recent hominoid evolution (during the last 35 million years) (Bailey *et al.*, 2001; Cheung *et al.*, 2003). These duplication events gave rise to the complex genomic architecture that predisposes the extant human genome both to meiotic and mitotic deletion/duplication events at specific locations. Rearrangement breakpoints are associated with LCRs far more frequently than would be expected if the rearrangements occurred randomly. Despite large stretches of high sequence identity, it appears that hotspots exist for the majority of the crossovers that occur within LCRs (Bayes *et al.*, 2003; Bi *et al.*, 2003; Lopes *et al.*, 1999; Lopez-Correa *et al.*, 2001; Reiter *et al.*, 1996; 1998; Repping *et al.*, 2002). The positional preference for strand exchange seen in NAHR may suggest the presence of additional architectural features at the hotspots that contribute to making the regions more prone to recombination. AT-rich palindromes are located near several of the hotspots, suggesting that a predisposition to double-strand breaks (DSBs) may possibly influence the location of strand exchanges (Bi *et al.*, 2003; Edelmann *et al.*, 2001; Kurahashi *et al.*, 2003; Nimmakayalu *et al.*, 2003). In support of this postulate, studies in mice have shown that large palindromes in the germline are extremely unstable and undergo stabilizing rearrangements at frequencies up to 56%, often through deletions (Akgun *et al.*, 1997; Lewis *et al.*, 1999; Zhou *et al.*, 2001).

A palindromic AT-rich repeat (17PATRR) in the *NF1* gene, which had proved almost refractory to analysis, has recently been characterized by a new method comprising a combination of PCR, cloning in a recombination-deficient *E. coli* strain, and RNA polymerase-based sequencing (Inagaki *et al.*, 2005). This palindromic AT-rich repeat exhibits size polymorphism in humans and there is some evidence for it promoting translocations perhaps through intermediate cruciform structures. It is possible that the length polymorphism could confer inter-individual variation in susceptibility to recombination at this site. This question notwithstanding, the repeat appears to be specific to humans, the great apes and the Old World monkeys (Inagaki *et al.*, 2005).

During recent years, several disorders have been found to be caused by rearrangements between LCRs (Lupski, 1998; Ji *et al.*, 2000) but the breakpoints involved have been sequenced only in Charcot-Marie-Tooth disease type 1 (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP) (Lopes *et al.*, 1999). The size of the rearranged genomic segment (1.5Mb) in CMT1A/ HNPP is very similar to that in the NF1 deletion syndrome, but there are also clear differences. The LCRs triggering 17q11.2 microdeletions are significantly larger than the CMT1A/ HNPP repeats, which are 24 kb in length (Pentao *et al.*, 1992; Kiyosawa *et al.*, 1995; Reiter *et al.*, 1997). Furthermore, in CMT1A, 92% of the duplications are generated by interchromosomal crossover during meiosis but are of paternal origin. By contrast, the rearrangements in HNPP and CMT1A that have been observed in the maternal germline are caused by unequal intrachromosomal recombination (Lopes *et al.*, 1998). Another important aspect, where NF1 differs not only from CMT1A/ HNPP rearrangements but also from other genomic disorders, is that constitutional deletions of 17q11.2 can eliminate one copy of *NF1* gene ; the somatic inactivation of the second *NF1* allele is predicted to trigger the formation of tumours associated with NF1. In patients with 17q11.2 deletions, the early onset of excess growth and a disproportionately high number of cutaneous neurofibromas have been observed, suggesting that modifier genes, which may have been co-deleted, could favour neurofibroma development in these patients. Reduced expression levels or acquired inactivation of other hemizygous genes may additionally contribute to accelerated growth leading to malignant transformation, because the incidence of malignant tumours of the peripheral nerve sheath seems to be increased among patients who have *NF1* gene deletions (Dorschner *et al.*, 2000). The identification of somatic mosaicism associated with some LCR-based deletions (Juyal *et*

al., 1996; Kasprzak et al., 1998; Kehrer-Sawatzki et al., 2004; Petek et al., 2003; Zori et al., 1993) indicates that NAHR can also occur in mitosis, suggesting that NAHR may play a significant role in the loss of heterozygosity in tumorigenesis (Lupski et al., 1996). Considering the possible role of co-deleted genes in modulating the phenotype of NF1 patients with the deletion, it is interesting to note that several genes/pseudogenes are located between the NF1-REPs of 17q11.2. Three of these are transcribed and may be functional genes (*KIAA0563*-related gene, *LOC147172*, and *LOC342662*) (De Raedt et al., 2004).

Meiotic recombination hotspots are usually 1-2 kb in length and are often associated with high GC% content, CpG islands, and the presence of poly(A) and poly(T) stretches (Nachman, 2002). The *NF1* deletion breakpoint hotspot is located in a region with high G+C content compared to the surrounding regions and it also contains a CpG island, all in concordance with the presence of a meiotic recombination hotspot (De Raedt et al., 2004; Gervasini et al., 2005). The *NF1* deletion breakpoint hotspot described by Lopez-Correa et al. (2001) is located in the chromosome 17-derived sequence.

Associations between deletions spanning the *NF1* gene and the presentation of a complex NF1 clinical phenotype have been described and commented upon in several studies (Kayes et al., 1994; Wu et al., 1995, 1997a, 1997b; Leppig et al., 1996; Riva et al., 1996; Upadhayaya et al., 1996, 1998). However, it has been shown that, although large *NF1* deletions are relatively frequent in patients with facial dysmorphism, mental retardation/learning disabilities and an early onset of multiple neurofibromas, the presence of the deletion cannot be predicted only on the basis of the clinical phenotype (Tonsgard et al., 1997). Precise characterization of the extent of the deletion is a crucial step forward in addressing genotype-phenotype correlations and investigating whether common deletion breakpoints are found in different patients. The development of junction-specific PCR assays for other putative recombination sites (such as the palindromic AT-rich repeat) will be important for diagnosis, genotype-phenotype analyses, and understanding the molecular basis for recombination-prone sites in the genome.

Additional NF1 deletion patients need to be studied and their genotype characterized in detail in order to validate any correlations between the haploinsufficiency of specific

genes and the expression of specific clinical features. Functional characterization of co-deleted genes in human cells and animal systems will be required to identify possible dosage effects of individual genes during developmental and brain maturation. Patients with rare deletion breakpoints may be useful for evaluating the presence of minor recombination hotspots and the sequences motifs underlying them. Comparing the genotype with the clinical phenotype in *NF1* microdeletion patients with different deletion sizes and breakpoints might help to identify genes or mechanisms contributing to the specific *NF1* microdeletion phenotype, such as the higher tumour load and the elevated risk of malignancy. Further investigation of the precise deletion breakpoints in multiple individuals with each type of genomic disorder could shed additional light on the mechanism of such recombination and rearrangement events.

Gene duplications have been very important during evolution (most extant genes have been derived by a process of duplication and divergence) but are also a major underlying cause of human disease. Segmental duplications represent an under-appreciated source of genetic change owing to their ability to act as substrates for aberrant genomic rearrangements. Whilst many segmental duplications in the human genome have been identified and sequenced, very little is known about their formation and amplification. To better understand segmental duplications and their effect on human disease, future efforts will need to be aimed at elucidating the mechanisms that are involved in their formation and spread in the human genome. It should also be interesting to determine whether segmental duplications have become amplified during recent human evolution, which might have served to generate polymorphic variation in the human population.

Important questions that remain to be asked when a genetic locus is being investigated are: 1) what are the neighbouring genes? Since the relative contributions of deletions/insertions and point mutations to genetic pathology will depend on factors such as the size of the gene, the incidence and severity of the effects of the lesions, and selective factors; 2) Do the processes of nearby duplications and interactions give rise to very high diversity between individuals and populations? 3) What inversions, deletions and duplications in DNA structure are promoted by these clusters? 4) What has happened on the other allele may influence phenotype and this should be thoroughly explored.

Chapter 7. Evaluation of the methylation status of the promoter region of the *NF1* gene in NF1 patient-derived tumours

7.1 Introduction

DNA methylation is essential for normal mammalian development (Ehrlich 2003; Jaenisch & Bird, 2003). It is thought to play an important role in both gene regulation and imprinting, and may serve as a cue for strand specificity in DNA replication and repair. 5-methylcytosine (5mC) is the most common form of DNA modification in the human genome. Soon after DNA synthesis is complete, target cytosines are modified by DNA methyltransferases (DNMT) using *S*-adenosylmethionine as a methyl donor. Two methyltransferases specifically, DNMT3a and DNMT3b, have been found to be highly expressed in undifferentiated embryonic stem cells whereas low levels are detectable in somatic tissues (Okano *et al.*, 1999). They are both thought to be responsible for the methylation of repetitive elements, whereas DNMT1 maintains the DNA methylation pattern after replication of a methylated DNA sequence (Chuang *et al.*, 1997). The key role fulfilled by methyltransferases in normal cells has been demonstrated in mice because embryos lacking both copies of either DNMT1 or DNMT3a die before birth whilst DNMT3b homozygous null mice die a few weeks after birth (Okano *et al.*, 1999; Li *et al.*, 1992).

Between 70% and 90% of 5mC in the human genome occurs in CpG dinucleotides, the majority of which appear to be methylated. Although it is as yet unclear how tissue-specific methylation patterns are established, they are nevertheless heritable and reproducible after transmission through the germline. The establishment of cell type-specific methylation patterns in both somatic cells and in the germline begins with global methylation of non-CpG island sequences in the embryo. The final methylation patterns are thought to be determined by a specific and highly regulated process of demethylation.

Spatially, the distribution of CpG appears to be non-random in the human genome. Indeed, about 1% of the human genome consists of stretches very rich in CpG which together account for ~15% of all CpG dinucleotides. In contrast to most of the scattered CpG dinucleotides, these CpG islands represent unmethylated domains and comprise ~50% of all non-methylated CpGs in the genome. CpG islands are often located immediately 5' to gene coding regions. In general, gene promoters containing CpG islands are unmethylated regardless of expression whereas promoters lacking CpG islands tend to lose their methylation upon transcription.

The inverse correlation between the level of DNA methylation and gene transcription has been apparent for some time. Not unexpectedly, this correlation is at its strongest in gene promoter regions where methylation of 5mC residues is presumed either to reduce the binding affinity of transcription factors or to allow the binding of transcriptional repressor proteins. Methylation of such CpG island regions serves to inactivate gene expression, a process sometimes termed "epimutation". The precise mechanism is unclear but is thought to involve the binding of methylcytosine-binding proteins and histone deacetylases to the methylated DNA (Rountree *et al.*, 2001). In heavily methylated regions, the nucleosomes (composed of histone proteins around which DNA winds) are characteristically tightly compacted and regularly spaced, a configuration that excludes transcriptional activator proteins. Whilst methylation of promoter-associated CpG islands in normal somatic tissues is a comparatively rare event, in various types of tumour, tumour suppressor genes that possess CpG islands in their promoter regions have often been found to be silenced by *de novo* DNA methylation (e.g. *CDKN2A*, *CDKN2B*, *MLH1*, *RB1*, *VHL* etc; reviewed by Baylin *et al.*, 2001; Garinis *et al.*, 2002; Herman & Baylin, 2003).

It is therefore apparent that mutation, in the strict sense of the word, is not the only mechanism responsible for inactivating tumour suppressor genes since DNA methylation also represents an important alternative (and epigenetic) mechanism for silencing these genes (Figure 7.1). It follows that molecular genetic studies of a given cancer gene ought really to take into account that gene's methylation status as well as its DNA sequence.

A wide variety of genes including those encoding tumour suppressors, DNA repair and apoptosis proteins, cell cycle regulatory proteins, and proteins with functions related to metastasis and invasion may become hypermethylated during tumorigenesis (Table 7.1). Thus, by inactivating key genes, promoter hypermethylation interferes with a variety of different cellular mechanisms and pathways including cell cycle control (*CDKN2A*), apoptosis (*DAPK1*), signal transduction (*SOCS3*), DNA repair (*MLH1*), tumour cell invasion (*APC*) and response to growth factors (*RARB*). Tumour cells possessing methylation-inactivated tumour suppressor genes probably acquire a growth advantage in much the same way as cells with activated oncogenes or mutation-inactivated tumour suppressor genes.

At the same time, overall genome hypomethylation, which is mainly due to the hypomethylation of repetitive sequences (Jurgens *et al.*, 1996; Takai *et al.*, 2000), is consistently observed in tumours (Kaneda *et al.*, 2004). Some tumours display aberrant demethylation of normally methylated CpG islands and abnormal expression of their downstream protein product, such as melanoma antigen genes (MAGEs) and cancer-testis antigen genes (Zendman *et al.*, 2003; De Smet *et al.*, 1999; Cho *et al.*, 2003).

However, there are various *caveats*: firstly, one has to be careful about interpreting studies of DNA methylation in cancer since altered levels of genome-wide methylation are not only a marker of tumorigenesis but also of cultured cells. Secondly, for a given gene, a proportion of normal healthy non-cancerous cells often exhibit some degree of promoter methylation and so it is very important to compare the methylation status of a specific gene in tumour cells with that of its counterpart in non-tumour cells. Finally, with changes in DNA methylation that accompany tumorigenesis, it is often hard to distinguish cause from consequence (Baylin and Bestor 2002). It must therefore be appreciated that even the methylation-mediated inactivation of a known tumour suppressor gene does not automatically imply that this event contributes towards neoplastic transformation – it may simply be an indirect and neutral consequence of the multiplicity of genome-wide changes in methylation resulting from the tumorigenic process.

Figure 7.1. <u>Different mechanisms by which DNA methylation can cause cancer</u>
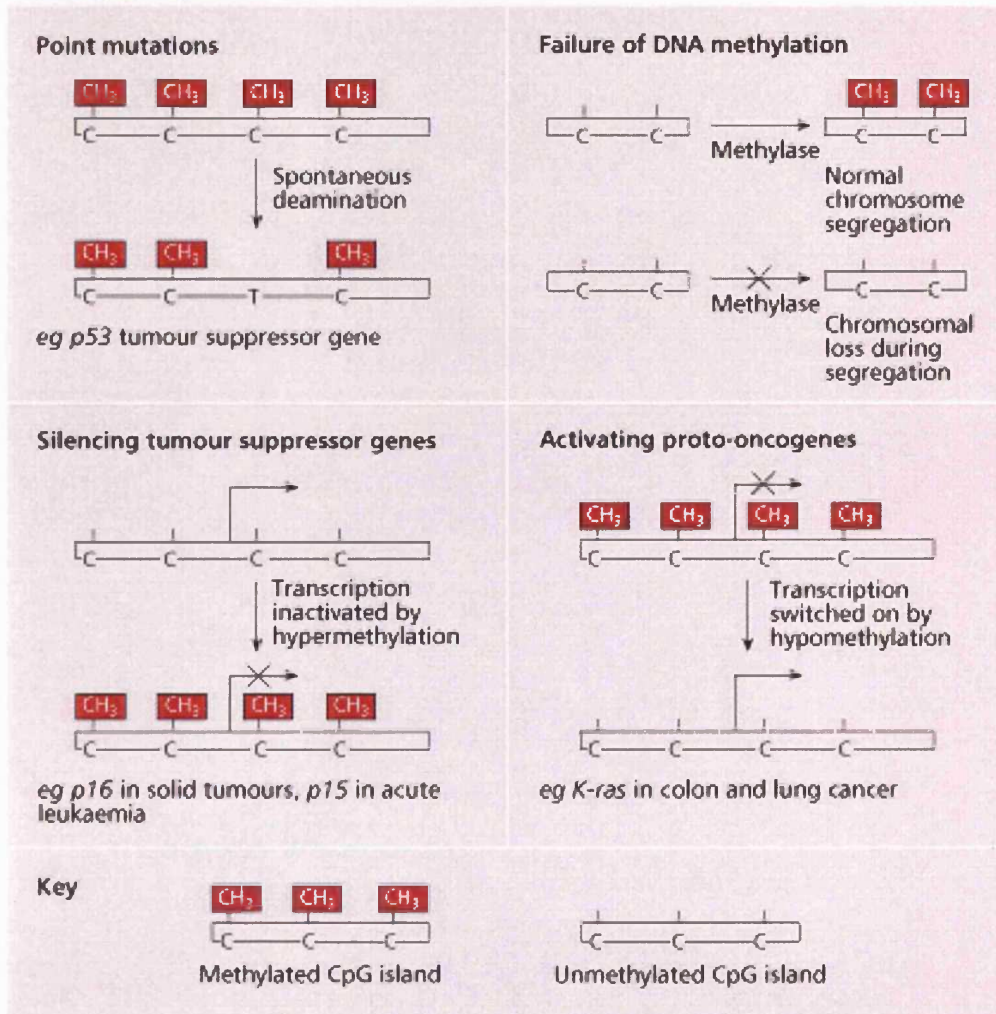
Figure 7.1: Schematic representation of possible mechanisms of DNA methylation in tumour development.

Table 7.1. Representative examples of putative suppressor genes hypermethylated in tumour samples

Gene	Tumour type	Number and % of tumours methylated	CpG island location	Gene expression	References
VHL	Renal carcinoma	5/26 (19%)	Exon 1	None	Herman et al., 1994
CDKN2A	Colorectal carcinoma	20/52 (38%)	Promoter	Not observed	Zou et al., 2002
CDKN2A	TCC	12/18 (67%)	Promoter/ Exon 1	None	Gonzalez-Zulueta et al., 1995
CDKN2A	TCC	15/18 (83%)	Promoter/ Exon 1+ Exon 2	None	Esteller et al., 1999
CDKN2A	NSCLC	9/22 (41%)	Promoter/ Exon 1	Not observed	Esteller et al., 1999
BRCA1	Mammary carcinoma	11/96 (11%)	Promoter/ Exon 1A/1B	Not observed	Catteau et al., 1999
BRCA1	Ovarian carcinoma	2/43 (5%)	Promoter/ Exon 1A/1B	Not observed	Catteau et al., 1999
MLH1	Colorectal carcinoma	4/66 (6%)	Promoter	None	Kane et al., 1997
RB1	Retinoblastoma	5/121 (4%)	Promoter/ Exon 1	Not observed	Ohtani-Fujita et al., 1997
APC	Colorectal carcinoma	11/11 (100%)	Promoter	Not observed	Hiltunen et al., 1997
DAPK	NSCLC	5/22 (23%)	Promoter	Not observed	Esteller et al., 1999
GSTP1	NSCLC	2/22 (9%)	Promoter	Not observed	Esteller et al., 1999
MGMT	NSCLC	6/22 (27%)	Promoter	Not observed	Esteller et al., 1999
CDH	Renal cell carcinoma	23/34 (67%)	Promoter	None	Nojima et al., 2001
FHIT	NSCLC	40/107 (37%)	Promoter	None	Zochbauer-Muller et al., 2001

Adapted from Horan et al. (2003).

This table represents a summary of the most representative genes in which hypermethylation of both the promoter or exon 1 regions was associated with tumour development. NSCLC, non-small cell lung carcinoma. TCC, transitional cell carcinoma.

7.1.1 NF1 gene promoter region

The *NF1* promoter was cloned and sequenced in 1994 and it was demonstrated that it possesses a CpG island-like region that contains several putative transcription factor binding sites, of which a high proportion share sequence homology with the mouse *Nf1* gene. This evolutionary conservation is supportive of the functionality of these binding sites. Although the precise length of the promoter CpG island has not yet been elucidated, the highest concentration of CpGs is contained within a 1.2 kb region of DNA sequence that encompasses the transcriptional start sites of both the human and mouse *NF1* genes. This suggests that the CpG island is at least 1200 bp in length (Hajra *et al.*, 1994).

Studies of the 5'upstream region and intron 1 of the *NF1* genes from different vertebrate species have revealed that a 24 bp sequence (NF1HCS), located 333-310 bp upstream of the translation initiation site in humans, is identical to that in both mouse and rat whilst it differs by only a single nucleotide from that in *Fugu* (Lee & Friedman, 2005). Moreover, *in vitro* studies of the *NF1* promoter region by luciferase reporter gene assay have led to the identification of possible functional elements downstream of the transcriptional start site (Viskochil *et al.*, 1998; Zhu *et al.*, 2004). These findings suggest that NF1HCS might play an important role in the control of the *NF1* gene transcription.

Mancini *et al.* (1999) showed that specific proteins contained within neuroblastoma nuclear extract, bind to three putative transcription factor-binding sequences (CRE plus two SP1 sites) specific for the *NF1* promoter. By means of oligonucleotide-specific methylation, protein binding to these sequences was shown to be significantly reduced. The same group also established the methylation status of four fragments within a 4kb region of upstream promoter sequence using neuroblastoma cell lines and a variety of normal human tissues. Methylation was shown to exist at all CpG sites within three upstream fragments, but the fragment containing the transcriptional start site was shown to be unmethylated (Mancini *et al.*, 1999). Further, Zou *et al.* (2003) identified a region within which lie the binding sites for transcription factors CREB and SP1/KLF family members that could be inactivated by site-directed mutagenesis. These authors also

162

detected a putative repressor element within the *NF1* promoter region that includes CCCTC-rich sequences which are located between the transcriptional and translational start sites.

Recently, the analysis of the methylation status of the *NF1* gene promoter in normal Schwann cells and in NF1-related plexiform neurofibromas in which no *NF1* mutation had previously been detected, revealed a low level of methylation in 18 of 21 tumour samples studied (Fishbein *et al.*, 2005). Interestingly, the majority of the methylated CpG sites identified in these studies were located to within putative transcription factor-binding sites that in accordance with *in vitro* studies (Mancini *et al.*, 1999; Zou *et al.*, 2003), could lead to the inhibition of transcription factor binding (Figure 7.2).

The development of neurofibromas would appear to involve different processes and the inactivation of the *NF1* gene probably represents the initial event. However, the malignant transformation of benign neurofibromas might be caused by other genetic and epigenetic factors. It has been proposed that the homozygous deletion of $p16^{INK4a}$ may be involved in the malignant progression of neurofibromas to neurofibrosarcomas (Koga *et al.*, 2002; Menon *et al.*, 1990; Nielsen *et al.*, 1999). In addition, Gonzalez-Gomez *et al.* (2003) demonstrated a significant degree of hypermethylation of the promoter or the exon 1 regions of at least one of the 11 different genes studied, in both sporadic and NF1-related tumours (neurofibromas and neurofibrosarcomas). Interestingly, a high frequency of DNA methylation was detected in the thrombospondin-1 (*THBS1*), the tissue inhibitor of metalloproteinase 3 (*TIMP3*) and O^6-methylguanine-DNA methyltransferase (*MGMT*) genes in both the sporadic and the NF1-related tumours, that are known to encode proteins involved in the inhibition of invasion and metastasis.

It has been shown that the loss of MGMT expression caused by *MGMT* promoter methylation resulted in an increased frequency of *TP53* gene mutations in both astrocytomas and colorectal cancer (Esteller *et al.*, 2001; Nakamura *et al.*, 2001). Therefore, it is possible that the same mechanism may play a key role in neurofibromas contributing to malignant progression.

DNA methylation analysis of the promoter regions of three tumour suppressor genes *p14*, *p15* and *p16* in both sporadic and NF1-related MPNSTs, revealed that promoter hypermethylation of these genes is not a common mechanism causing gene silencing in

MPNSTs. Methylation of one or more gene promoters occurred in only 1 of 10 (10%) NF1-related MPNSTs (Perrone *et al.*, 2003). By contrast, homozygous deletion (HD) of the 9p21 chromosomal region, which is the region in which these three gene are located, has been described as a common inactivation event in MPNSTs (Perrone *et al.*, 2003; Birindelli *et al.*, 2003; Berner *et al.*, 1999).

The small number of reported studies on the methylation status of the *NF1* gene promoter region suggest that promoter methylation is unlikely to be a major cause of *NF1* gene inactivation in tumours derived from NF1 patients (Mancini *et al.*, 1999; Horan *et al.*, 2000; 2004; Luijten *et al.*, 2000; Ebinger *et al.*, 2005).

In a recent study, methylation analysis was applied not only to the *NF1* gene promoter region but also to the 5'UTR, exon 1 and part of intron 1, in 21 NF1-associated tumours (Harder *et al.*, 2004). These analyses revealed a low level of methylation (1.35% for dermal and plexiform neurofibromas, 0.82% for MPNSTs) and an essentially random methylation pattern. In addition, these studies described *in vivo* methylation of the CRE motif at position −10 in plexiform neurofibromas and MPNSTs, suggesting that methylation of this specific motif might affect *NF1* gene expression in nerve sheath tumours by altering the cAMP levels in accordance with that described by Gutmann *et al.* (1993).

Hypomethylation of DNA is another important event that could lead to gene activation. CpG islands, normally methylated in somatic tissues, can become hypomethylated in cancer and nearby genes become activated. The frequency of hypomethylated sites might be quite high, as indicated by genomic methylation analysis of different tumours (Adorjan *et al.*, 2002; Iacobuzio-Donahue *et al.*, 2003; Oshimo *et al.*, 2003; Akiyama *et al.*, 2003; De Capoa *et al.*, 2003). It has been hypothesized that DNA hypomethylation is linked to chromosomal instability and this correlation has also been described in mouse studies of *NF1* (Eden *et al.*, 2003).

This study aimed to investigate whether the hypermethylation of the *NF1* gene promoter region was a significant causative factor for tumorigenesis in DNA derived from NF1-associated tumours.

Figure 7.2. _NF1_ promoter region
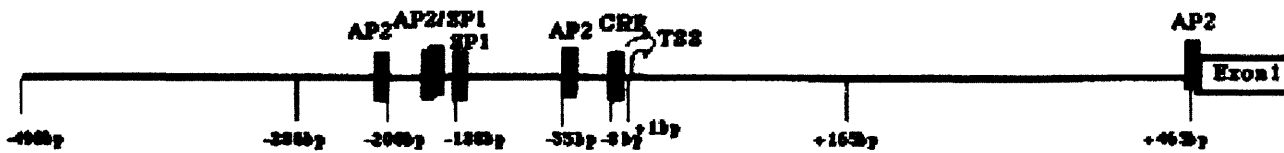
Figure 7.2. *NF1* gene promoter region. Putative transcription factor binding sites within the region are denoted by dark rectangles. TSS marks the transcriptional start site at bp +1. (Figure adapted from Fishbein *et al.*, 2005).

7.2 Results

The study involved the analysis of a total of 29 DNA samples which comprised 27 benign dermal neurofibromas, 1 plexiform neurofibroma and 1 MPNST. Bisulphite modification and methylation-specific PCR (MSP) are based on the principle that bisulphite treatment of DNA converts unmethylated cytosine residues into uracil, whereas methylated cytosine residues remain unmodified. Thus, after bisulphite conversion, methylated and unmethylated DNA sequences can be distinguished by sequence-specific primers. To assess the importance of promoter hypermethylation in *NF1* gene inactivation in NF1-related tumours, the methylation status of the *NF1* promoter region was determined by bisulphite-modified genomic sequencing in DNA tumours from NF1 patients. Bisulphite genomic DNA modification (Horan *et al.*, 2000) was used to determine the methylation status of the sense strand of a ~120bp stretch flanking the *NF1* gene transcriptional start site and containing 12 CpG dinucleotides in all tumours.

During this study, amplification of the methylated DNA sequence was revealed to be difficult. In fact, in order to use PCR primers highly specific to the methylated DNA sequence, several primer pairs had to be designed. In addition, the PCR reaction conditions required accurate optimisation. However, two sets of primers were used to amplify each region of interest.

Treatment with sodium bisulphite chemically converts unmethylated cytosine residues to uracil by hydrolytic deamination whereas methylated cytosine residues are resistant to chemical modification and remain unaffected (Clark *et al.*, 1994). PCR amplification of bisulphite-treated DNA amplifies uracil residues as thymine. For this analysis, individual DNA samples were first modified with sodium bisulphite prior to PCR amplification using specific primers for the converted sense strand of the *NF1* promoter region. The amplified DNA fragments were then sequenced so that the methylation status of each cytosine residue within CpG dinucleotides could be established within the 5' sequence. As a positive control, human lymphocyte DNA was treated with *M.SssI* CpG methylase (New England Biolabs) prior to bisulphite treatment. Non-converted DNA was used as a control for primer specificity. Primers MPR1-F and MPR1-R (Horan *et al.*, 2000) were used to control for bisulphite conversion. No methylation was found in any of the 12 CpG dinucleotides tested in any sample (Figure 7.3). However, during this study methylation analysis was applied on the sense strand of the *NF1* promoter region, therefore future investigation for the methylation status of the other strand are required for excluding hypermethylation of the *NF1* promoter region in these tumours.

Figure 7.3. Methylation analysis of the *NF1* promoter region in NF1-related tumours by MSP
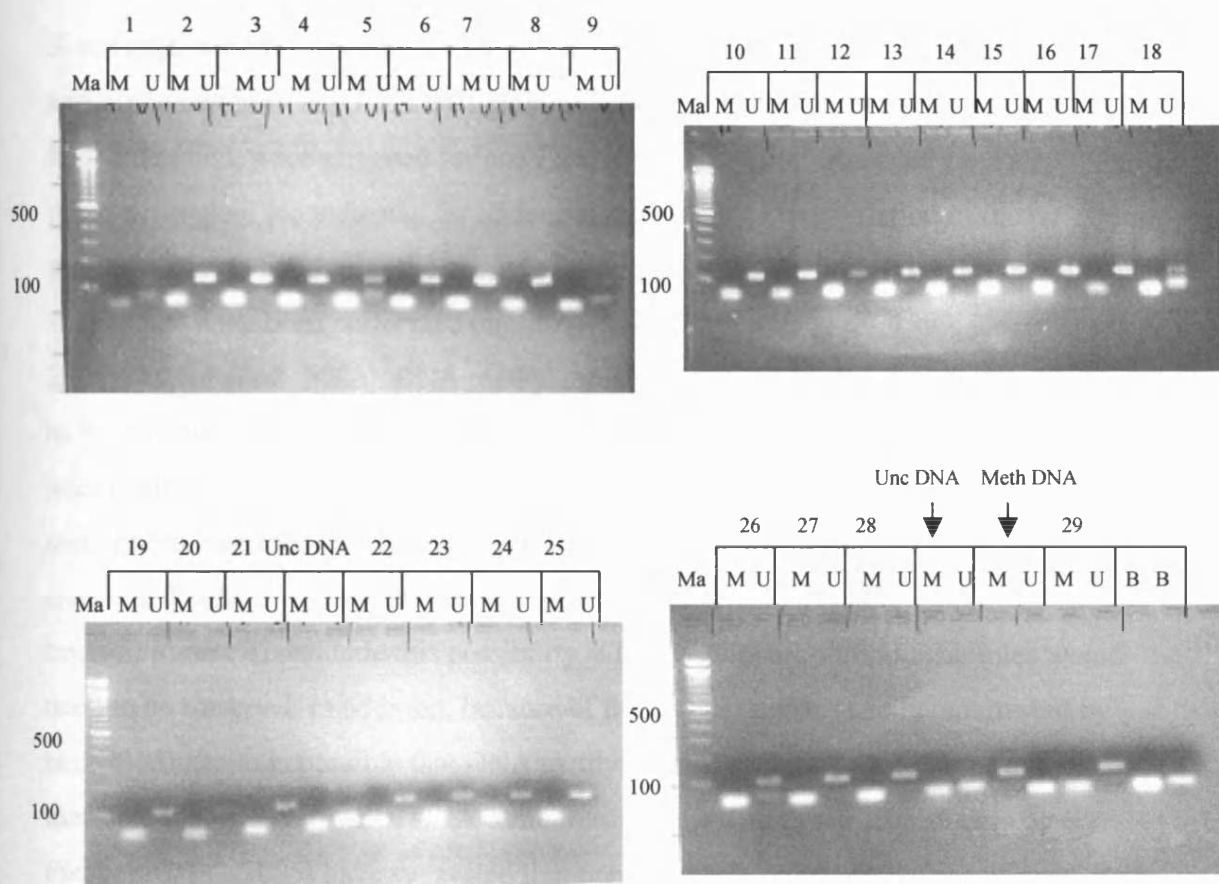
Figure 7.3 Summary of the results obtained by screening 29 NF1- related tumours for the methylation status of the *NF1* promoter region by MSP. M, amplified product with primers recognising methylated sequence. The bands of smaller size represent the primers. U, amplified product with primers recognising unmethylated sequence. The tumour samples analysed by MSP are numbered from 1-29. Unc DNA, non-converted DNA. Meth, human lymphocyte DNA treated with M.Sss1 CpG methylase (New England Biolabs). Ma, 1kb DNA ladder.

7.3 Discussion

Since epigenetic factors may lead to the silencing of the wild-type *NF1* allele, benign and malignant tumours from NF1 patients in whom no *NF1* mutation had previously been identified, were screened for possible hypermethylation status of the *NF1* promoter region. No evidence for tumour-associated hypermethylation of the *NF1* gene promoter was found during the course of this study. This finding is consistent with other studies that have been performed on the *NF1* gene (Mancini *et al.*, 1999; Horan *et al.*, 2000; Luijten *et al.*, 2000). DNA methylation cannot however be completely excluded as an epigenetic event in NF1 tumorigenesis, since the relatively small number of CpG sites analysed in this and other studies may not necessarily be representative of the methylation status of the CpG island as a whole. Hypermethylation of promoter CpG sites could still be responsible for *NF1* gene inactivation in a small proportion of neurofibromas. To exclude this possibility, a larger number of tumour samples would need to be screened. In addition, because of the cellular heterogeneity manifested by neurofibromas, it is possible that DNA methylation is specific to a cell type and therefore mis-detected when DNA tumour samples are analysed. The studies by Fishbein *et al.* (2005) have suggested that Schwann cells might be the specific cell type in which *NF1* gene promoter methylation might occur. Therefore this type of cell should be considered as the target for future investigation of the methylation status of the *NF1* gene promoter region in neurofibromas and MPNSTs.

Moreover, the method used for DNA methylation analysis might be another reason for not detecting methylated DNA. During this study, methylation-specific PCR (MSP) amplification of a bisulfite-converted DNA was adopted. This is considered very sensitive but can examine a very limited number of cytosines and a single gene per assay. The ability to integrate epigenetic DNA analyses into the available genomic microarray platform provides the means of rapidly obtaining quantitative and qualitative information of DNA methylation status across the entire genome. This application relating to the detection of alterations in methylation patterns on a genomic chromosomal scale has been used in cancer studies where DNA from tumour and corresponding normal tissue is compared (Pollack and Iyer, 2002; Zardo *et al*, 2002;

Tompa *et al*, 2002), Microarrays have been used for DNA methylation analysis in leukaemia (Adorjan *et al.*, 2002), Non-Hodgkin's lymphoma (Shi *et al.*, 2003) and gastric tumours (Hou *et al.*, 2003). More recently, an oligonucleotide-based microarray approach to the analysis of the methylation status of many individual CpG sites has been described (Mund *et al.*, 2005). The development of this technique allows the assessment of DNA methylation status in hundreds of CpG islands in a single experiment. Therefore, this method might be useful for the future methylation analysis of promoter regions in either the *NF1* gene or other tumour suppressor genes in NF1-related neurofibromas.

Genome-wide mechanisms involved in tumour-associated hypomethylation development may also exist within NF1-related tumours and may be responsible for benign neurofibromas developing into the more invasive malignant peripheral nerve sheath tumours. Therefore, further studies are required on tumour tissues to identify whether malignant transformation is associated with a state of global DNA hypomethylation within various subsets of NF1-specific tumours.

To date, only very limited experimental analysis of *NF1* transcriptional regulation has been attempted and additional studies *in vitro* and in knockout mice are necessary. In addition, further work will be needed to understand how both genetic and epigenetic factors can regulate *NF1* gene expression, and how these pathways can be targeted perhaps to modulate NF1 expression at the level of transcription.

There is substantial evidence that DNA methylation plays a critical role in silencing specific genes during development and cell differentiation. The intrinsic mutagenicity of 5-mC, the activation of proto-oncogenes through hypomethylation, the transcriptional inactivation of tumour suppressor genes through hypermethylation, and defects in chromosomal segregation due to failure of *de novo* methylation may all contribute to neoplasia. In addition, it is possible that cellular defects of malignant cells induce methylation. Changes in DNA methylation may therefore also be a consequence of genetic alterations rather than simply a cause (French *et al.*, 2002). Finally, the silencing of a tumour suppressor gene by promoter methylation does not need to be complete in order for it to exert an effect. For example, it has been reported that a 50% reduction in

the expression of one allele in the *APC* tumour suppressor can still result in the development of familial adenomatous polyposis coli (Yan *et al.*, 2001).

The role of *NF1* as a tumour suppressor gene, and its possession of a CpG island in its promoter region, is compatible with this gene being a candidate for epigenetic inactivation through promoter hypermethylation. Current models suggest that DNA methylation inhibits transcription by interfering with the initiation of transcription (Clark and Melki, 2002; Jones, 2002) or through changes in chromatin structure, and histone acetylation levels mediated by histone deacetylases (Robertson, 2002). Aberrant DNA methylation has been shown to occur frequently in tumours within the promoter regions of a wide variety of other tumour suppressor genes, where methylation provides one of the hits postulated by Knudson's two-hit hypothesis to inactivate tumour suppressor genes (Plass and Soloway, 2002). The CpG island in the promoter region of the cell cycle kinase inhibitor p16 (*CDKN2A*) gene is methylated in various tumours (e.g. Merlo *et al.*, 1995), which correlates with the downregulation of *p16* expression. The CpG island of *MLH1* is methylated in the majority of colon cancers with microsatellite instability (MSI) and correlates with downregulation of *MLH1* expression (Herman *et al.*, 1998). Kino *et al.* (2001) have reported that suppressed expression by methylation could be an alternative mechanism for inactivation of the *NF2* gene.

Chapter 8. General Discussion

The mutational analysis of the *NF1* gene is very complex owing to the large size of the gene, the presence of pseudogenes and the great variety displayed in terms of the nature of the underlying molecular lesions. Although a few possible mutation hotspots have recently been described (Fahsold *et al.*, 2000; Ars *et al.*, 2003), the *NF1* gene mutations that have so far been detected seem to be distributed fairly evenly along the entire length of the gene. Thus, the detection of somatic and germline mutations requires the analysis of the all 60 *NF1* exons and flanking regions in individuals affected by NF1. The variety of the *NF1* gene mutations detected ranges from gross chromosomal aberrations to single base-pair substitutions (Upadhyaya & Cooper, 1998; Fahsold *et al.*, 2000) (Appendix). However, the majority of mutations identified in the *NF1* gene lead to a truncated protein as similarly described for other tumour-suppressor genes. Truncating mutations represent approximately 100% for *TSC1* and approximately 95% for *APC* (Jones *et al.* 1999; Suzuki *et al.* 1998), 80% for *RB1*, *BRCA1* and *ATM* (Lohmann *et al.* 1996; 1997; Miki *et al.* 1994; Sandoval *et al.*, 1999), and 60% for *TSC2* (Jones *et al.* 1999).

The analysis of the gene mutational spectrum is critically important for identifying possible genotype-phenotype correlations in any disease. However, such correlations appear thus far to be very limited in NF1. Different sequence variants within a gene may exert differential effects on the structure and function of the encoded protein and may therefore result in inter-individual variation in the clinical phenotype. In some genes, different mutations give rise to different phenotypes. One example is the *DMD* gene in which nonsense mutations, and deletions or insertions that cause frameshifts, can eliminate the *DMD* product causing Duchenne muscular dystrophy. By contrast, missense mutations and in-frame deletions alter but do not eliminate the gene product and these cause the less severe Becker form of muscular dystrophy. Another example of the importance of the relationship between mutant genotype and clinical phenotype, is provided by a form of retinal degeneration, retinitis pigmentosa, in which different rhodopsin gene mutations can cause autosomal dominant disease in some patients and an autosomal recessive form in others.

During the course of this study, in order to detect the large variety of *NF1* gene lesions in both atypical NF1 and SNF1 patients, a combination of different mutation detection techniques was used (Figure 8.1). DHPLC was used for the analysis of the entire *NF1* coding region and flanking intronic sequences to detect the presence of single base-pair substitutions and small deletions and insertions of up to 20bp. Subsequently, a deletion-junction PCR assay (Lopez-Correa *et al.*, 2001) was used to detect the common 1.5Mb deletion of the entire *NF1* gene and adjacent regions. This method was adopted for both the detection of the large deletion in patients in whom no mutation was identified by DHPLC and also for evaluating the deletion breakpoints in NF1 patients known to be carrying the large gene deletion, as previously identified by FISH. When pairs of lymphocyte/fibroblast DNA samples were available from the same SNF1 patient, then LOH and MSI studies for the detection of gross gene rearrangements were applied. RNA samples extracted from fibroblasts that were derived from both affected and unaffected skin of SNF1 patients were screened by long-range PCR and cDNA-DHPLC analysis. RNA samples from affected and unaffected skin were analysed using an Affymetrix array to detect whether there is any variation in the expression of genes in these subgroups of samples.

Figure 8.1. Flow diagram for the mutational analysis of the *NF1* gene as performed during this study
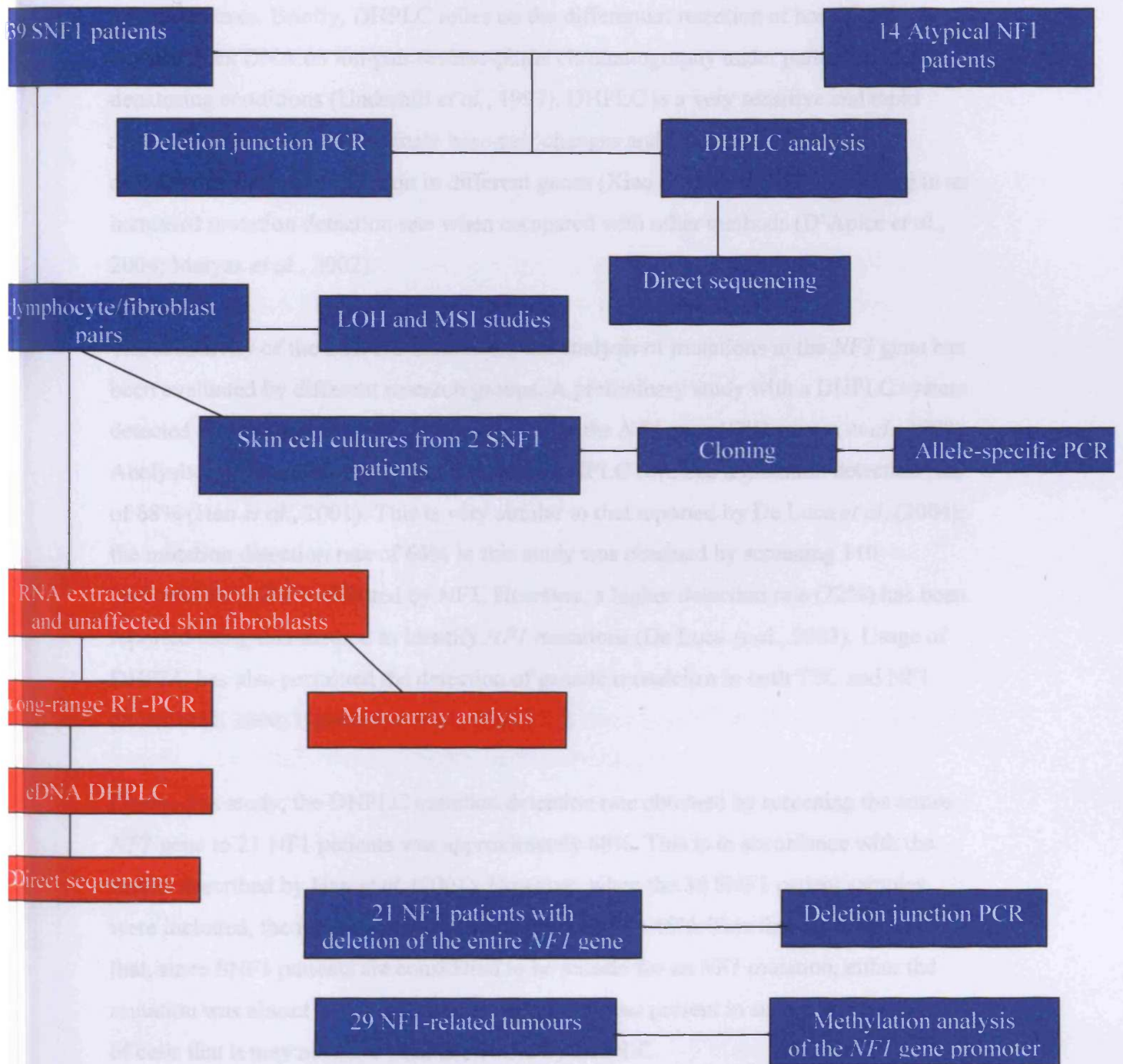
Figure 8.1. A summary of the mutation detection methods adopted for the analysis of the *NF1* gene in this study. The genomic DNA analysis is coloured blue, whereas the RNA studies are in red.

The main technique used for the mutational analysis of the *NF1* gene on DNA samples from all study groups was DHPLC. This method detects mutations on the basis of mismatches between amplified PCR fragments that result in the formation of heteroduplexes. Briefly, DHPLC relies on the differential retention of homo- and heteroduplex DNA on ion-pair reverse-phase chromatography under partially denaturing conditions (Underhill *et al.*, 1997). DHPLC is a very sensitive and rapid method for the detection of single base-pair changes and it has been applied to the detection of sequence variation in different genes (Xiao & Oefner, 2001), resulting in an increased mutation detection rate when compared with other methods (D'Apice *et al.*, 2004; Matyas *et al.*, 2002).

The sensitivity of the DHPLC method for the analysis of mutations in the *NF1* gene has been evaluated by different research groups. A preliminary study with a DHPLC system detected all known mutations within exon 16 of the *NF1* gene (O'Donovan *et al.*, 1998). Analysis of 50 unrelated NF1 individuals by DHPLC revealed a mutation detection rate of 68% (Han *et al.*, 2001). This is very similar to that reported by De Luca *et al.* (2004); the mutation detection rate of 66% in this study was obtained by screening 110 unrelated individuals affected by NF1. However, a higher detection rate (72%) has been reported using this method to identify *NF1* mutations (De Luca *et al.*, 2003). Usage of DHPLC has also permitted the detection of genetic mosaicism in both TSC and NF1 (Jones et al, 2000; Upadhyaya *et al.*, 2004).

During this study, the DHPLC mutation detection rate obtained by screening the entire *NF1* gene in 21 NF1 patients was approximately 68%. This is in accordance with the results described by Han *et al.* (2001). However, when the 36 SNF1 patient samples were included, the mutation detection rate decreased to 45%. This finding suggested that, since SNF1 patients are considered to be mosaic for an *NF1* mutation, either the mutation was absent in the cell lines analysed or it was present in such a low percentage of cells that it may not have been detectable by DHPLC.

Identification of false positives by DHPLC analysis was a recurring problem during the course of this study as previously reported (Matyas *et al.*, 2002; Groos *et al.*, 2000; Spiegelman *et al.*, 2000). One reason for DHPLC false positive results is the presence of non-specific PCR fragments that are not always detectable by agarose gel analysis or

due to unsatisfactory quality of DNA. Nevertheless, it could be possible that false positives represent a mutation that is not detectable by sequencing due, for example, to somatic mosaicism. In support of the latter idea, it has been shown that direct sequencing often fails to detect mutant alleles present at low frequency such as in somatic mosaicism (Kwiatkowska *et al.*, 1999; Jones *et al.*, 2001).

The major aim of this project was to identify possible genetic and molecular mechanisms responsible for the clinical phenotype of individuals affected by SNF1. Since patients affected by SNF1 display the typical NF1 clinical features but limited to one or more body segments (Huson & Ruggieri, 2000; Gutman *et al.*, 1997; Moss *et al.*, 1994; Viskochil & Carey, 1994), the molecular basis of this clinical phenotype is thought to be due to somatic mosaicism for mutations in the *NF1* gene. Furthermore, it was hypothesized that the different distribution of NF1 clinical features, from a generalized to a more limited clinical phenotype, may be influenced by the time at which the *NF1* mutation occurred during embryogenesis dictating the differential involvement of specific cell types exhibiting the mutation. Although somatic mosaicism for an heterozygous deletion of the entire *NF1* gene has been demonstrated at the molecular level in one SNF1 patient (Tinschert *et al.*, 2000), the pathogenesis of this disorder remains unclear.

Both germline and somatic mosaicism have been described in NF1, suggesting that somatic mosaicism is a relatively common event in this disease. Lazaro *et al.* (1994) identified germ-line mosaicism for a 12kb deletion in an unaffected individual with two NF1-affected children. Somatic mosaicism for deletions of exons 4-39, exons 27-41 and the entire *NF1* gene have also been described in individuals affected by NF1 (Colman *et al.*, 1996; Ainsworth *et al.*, 1997; Wu *et al.*, 1997a). A better understanding of the frequency of such mosaicism may help to predict recurrence risk in these families.

To date, the majority of SNF1-affected individuals published in the literature may be considered sporadic. However, rather fewer familial cases have so far been described. During this study, tissue samples from both sporadic and familial SNF1 cases were obtained. This has allowed us to study the possible different mechanisms underlying the two types of inheritance of the clinical features of SNF1. Seven of the SNF1 familial cases included in this study had a child affected by full-blown NF1. The approach used

for the mutational analysis of the *NF1* gene in those 7 families was first to screen the entire *NF1* gene in lymphocyte DNA samples from the children affected by full-blown NF1 using DHPLC. After identification of germline *NF1* lesions in those patients, DNAs from both lymphocytes and skin fibroblasts from the SNF1 parents were screened for both germline and somatic mutations in the specific fragment in which the *NF1* change was identified in the child. In cases where an *NF1* gene lesion was not detected in the children, the entire gene was analysed by DHPLC in the SNF1 parents when DNA samples were available.

Using this approach, four different *NF1* mutations (R1947X, 2396del L, Y489C, IVS45ds+2 T→C) and three previously described polymorphisms (L234L, IVS3+41 A→G, IVS10b-35del T) were detected in lymphocyte DNAs from full-blown NF1 individuals but *NF1* gene lesions were not detected in DNAs from both lymphocytes and cultured fibroblasts from SNF1 parents. If there was a mosaic pattern of the detected *NF1* mutations in the SNF1-affected individuals, DHPLC might not be able to detect it. Therefore, the need for cell cloning was essential in those cases. In fact, by cloning the specific *NF1* gene fragment from DNA derived from lymphocytes and both fibroblasts and keratinocytes from different areas of the SNF1 patient's body, the nonsense mutation in exon 31 was identified in 9% and 20% respectively in the pathological tissues but not in lymphocytes and normal areas of the skin by allele-specific PCR and direct sequencing. These results are consistent with gonosomal mosaicism for a nonsense *NF1* mutation in one family in whom a full-blown NF1 child has inherited a *NF1* nonsense mutation from the SNF1 parent mosaic for the mutation. (Consoli *et al.*, 2005).

This study has demonstrated that cell lineage, as well as method of analysis are critical to obtaining a meaningful diagnostic result. Owing to the unavailability of facilities, melanocytes from skin biopsies were not studied. It is speculated that the original embryonic mutation was transmitted to all skin cell types within limited region. Subsequent embryonic migration of the daughter cells led to some scattering. Biopsy from a site defined by the melanocyte abnormality would be expected to exhibit the mutation in most melanocytes and many keratinocytes, but only in a smaller proportion of fibroblasts. Biopsy from a clinically normal skin site remote from the lesion, however, is unlikely to pick up any mutant cells.

In future, considering the apparent higher percentage of keratinocytes harbouring the mutation compared with fibroblasts, further investigation of this type of family should involve the analysis of keratinocyte and melanocyte cell lines, as well as fibroblasts. In addition, when neurofibromas from SNF1 patients are available, then Schwann cells isolated from this benign tumour should be specifically screened for *NF1* mutations.

These findings demonstrate for the first time gonosomal mosaicism in a SNF1 patient and this will have important implications for calculating the risk that for SNF1 patient with an NF1 child and in molecular diagnosis. With the availability of more sensitive quantitative assays that are capable of detecting low-levels of mosaic mutations against a normal background, it should become possible to elucidate the molecular basis of the pathology in the majority of NF1/SNF1 families, and to offer successful antenatal testing, if required.

The difficulty in obtaining tissue samples from the SNF1 patients was the major problem encountered during this study. However, on the basis that an *NF1* mutation might occur very early on in embryogenesis and would thus affect both germline and somatic cell lineages, lymphocyte DNAs from these patients were analysed for mutations of the *NF1* gene.
SNF1 represents an interesting model for expression studies. Patients with SNF1 (which is considered to be due to somatic mosaicism) will provide material to enable us to study three genetically distinct tissues with respect to NF1 genotype: wild type (+/+, unaffected skin), heterozygous (+/- affected skin) and mutant homozygous (-/-, tumour from affected area). The availability of all three genotypes will be useful for expression studies since it will preclude the need to obtain tissue from different individuals which may reduce variability due to interindividual differences in gene expression.
A pilot expression profiling study based on fibroblast RNA derived from affected and unaffected skin using Affymetrix chips did not reveal any significant difference in the expression pattern.

The second part of this study aimed to understand the role of *NF1* gene mutations in patients with clinical phenotypes that overlap between NF1 and other apparently unrelated disorders. The observation of similar clinical features among affected members of a few families with disorders known as 'NF1 variants', Watson syndrome

(Allanson *et al.*, 1991), familial café-au-lait spots (Abeliovich *et al.*, 1995), or familial spinal neurofibromas (Ars *et al*, 1998; Polyhonem *et al.*, 1997; Pulst *et al.*, 1991), is consistent with an allele-phenotype correlation. However, no consistent type of *NF1* mutation was found in the families with these or other phenotypic variant.

The mutational analysis of the *NF1* gene in 14 atypical NF1 patients by DHPLC succeeded in identifying three small deletions, two of them in-frame (2970-2972 del AAT, 4312del GAA, 3525-3526del AA), a small frameshift insertion (4095ins TG), a nonsense mutation (R1513X), a missense mutation (H553R) and a silent change (F1289F). A previously reported 3 base-pair deletion in exon 17 (2970-2972del AAT), which is predicted to remove a methionine from the neurofibromin protein, was identified in NF1 affected members of two families who did not exhibit neurofibromas. Moreover, the same mutation has been identified in two other NF1 patients in our laboratory during diagnostic screening. One of those two individuals had a family history of NF1 but exhibited only CALs, whereas the other had no family history of NF1. It has also been found that this same mutation segregates with the disease in another apparently unrelated family with history of NF1 referred to our laboratory (Sian Griffiths, personal communication). The fact that the NF1 patients with the mutation in exon 17 did not have neurofibromas suggests that the mutation might play a key role in the formation of these benign tumours. Furthermore, this mutation lies within a region upstream of the NF1-GRD domain that has been proposed to be a possible second functional domain in the protein (Fahsold *et al.*, 2000). This region represents a cysteine/serine-rich domain as defined by Izaka *et al.* (1996) and presents three potential cAMP-dependent protein kinase A (PKA) binding sites (Marchuk *et al.*, 1991) that suggests that this region might be involved in cAMP signalling. Further functional analysis on the role of this small in-frame deletion to the formation of neurofibromas may provide new insights into this unusual genotype-phenotype correlation.

Aberrant exon skipping resulting from missense, nonsense or translationally silent single base-pair substitutions is frequently caused by disruption of a critical ESE. There is evidence that the disruption of functional ESE sequences is frequently the mechanism underlying mutation-associated exon skipping in other diseases, for example the exon skipping caused by nonsense, missense and even silent mutations in the *BRCA1* gene can result from ESE disruption (Liu *et al.*, 2001). The silent change in exon 22

identified in an NF1 patient who also exhibits the MoyaMoya syndrome phenotype, resulted in the disruption of the ESE- SC35 motif and score reduction for SRp40 motif. However, this silent change did not affect splice site recognition. The disruption or variation of one or more ESE motifs may be compensated for by the simultaneous creation of another one. However, the results obtained from the mutated sequences did not reveal the presence of any new motifs when compared with the wild-type. If this mutation disrupts an ESE, however, the transcripts may be spliced incorrectly, effectively deleting an entire exon-encoded segment of the protein. In order to evaluate whether this DNA sequence change affects the splicing process, we would need to further investigate the patient at the RNA level. However, no RNA sample from this patient was available and therefore a possible effect of this DNA variant on the mRNA transcriptional event was not evaluated.

Intragenic polymorphisms may also affect gene function (Cargill *et al.*, 1999). Because individual exons comprise multiple positive and negative *cis*-acting elements that affect splicing, some exonic polymorphisms may influence splicing efficiency or accuracy, and therefore gene expression levels. Mutations of the *NF1* gene which affect pre-mRNA splicing and therefore result in exon skipping and the consequent loss of one allele, have been described (Ars *et al.*, 2000a; Fahsold *et al.*, 2000 Zatkova *et al.*, 2004). Understanding the clinical significance of missense and silent mutations and also the relationship between structure and function in neurofibromin should be useful in helping to identify the diverse mechanisms and pathways that ultimately connect the mutant NF1 genotype to the highly variable NF1 phenotype.

Since epigenetic factors may lead to the silencing of the wild-type *NF1* allele, benign and malignant tumours from NF1 patients in whom no *NF1* mutation had previously been identified, were screened for possible hypermethylation status of the *NF1* promoter region (Upadhyaya *et al.*, 2004). This analysis did not however reveal any hypermethylation in a ~120-bp region flanking the *NF1* gene transcriptional start site and containing 12 CpG dinucleotides using the bisulphite conversion method (Horan *et al.*, 2000). These findings suggest that epigenetic inactivation of the *NF1* gene promoter is not a common event in NF1-related tumours (Horan *et al.*, 2001; Harder *et al.* 2005). However, recently a low level of methylation was found in 12 of 18 NF1-related plexiform tumours analysing cultured Schwann cells which suggests that the *NF1* gene

promoter may be a target for hypermethylation in Schwann cells (Fishbein *et al.*, 2005). In addition, site-specific methylation, involving transcription factor binding sites SP1, CRE and AP-2, has been observed in a plexiform neurofibroma and an MPNST (Harder *et al.*, 2005).

8.1 Conclusions

This study has demonstrated for the first time gonosomal mosaicism for a nonsense mutation (R1947X) in the *NF1* gene in an SNF1 affected parent with a full-blown NF1 affected child, suggesting that this might be one of the mechanisms underlying the SNF1 phenotype in families in whom an NF1 patient has a parent affected by SNF1 (Consoli *et al.*, 2005). These findings will facilitate genetic counselling and molecular diagnosis in such families.

The analysis of *NF1* mutations in SNF1 sporadic patients identified a very small number of NF1-causing changes. Given the hypothesis that the SNF1 phenotype is caused by somatic mosaicism for an *NF1* gene mutation, the failure to identify *NF1* gene mutations in most of the SNF1 patients analysed by DHPLC suggests that this screening method is not suitable for the analysis of low levels of mosaicism and that other techniques, e.g. Schwann cell purification or the molecular cloning and sequencing of the mutant alleles may be required and, indeed, may be essential for the detection of such low levels of mosaicism. Another possible explanation is that the incorrect tissue types may have been studied. The analysis therefore of several different tissues that are readily available from SNF1 patients may be useful in order to try to understand more about the mutational spectrum and tissue/cellular distribution of the somatic *NF1* gene lesions associated with SNF1. A pilot expression profiling analysis of RNAs isolated from CAL fibroblasts derived from five SNF1 patients however failed to reveal any significant gene expression differences between the affected and unaffected cell lines. This does suggest that fibroblasts may not be the optimal cells to be targeted for such gene expression changes in SNF1. Gene expression analysis of different patient cell lines, such as melanocytes and keratinocytes, may be better in helping to identify the mechanisms underlying the SNF1 clinical phenotype.

It is hoped that the 2970-2972 del AAT in-frame micro-deletion identified in NF1 patients who did not exhibit neurofibromas in two different families, might provide new insights into the genotype-phenotype relationship in NF1.

The identification of *NF1* gene mutations in approximately 65% of the patients exhibiting an atypical phenotype allows us to conclude that the nature of the mutations detected are on their own inadequate to the task of explaining the unusual clinical phenotypes observed in these patients. Other factors, both of genetic and perhaps environmental origin, must influence the highly unusual clinical characteristics seen in these patients. It cannot be excluded however that some of the unidentified mutations in this study may have been large deletions, multi-exon deletions, or large duplications and inversions whose nature and location could have provided further information about the relationship between genotype and phenotype. Similarly, incomplete assessment of intronic and regulatory sequences may account for other undetected mutations. However, *NF1* promoter mutations seem to be rare and the sequence variants identified so far appear unlikely to cause the disease phenotype (Osborn *et al.*, 2000; Horan *et al.*, 2004). It nevertheless remains possible that hitherto undetected mutations could be responsible for the unusual clinical phenotypes associated with these atypical NF1 patients.

8.2 Future directions

8.2.1 Mutation detection methods

DHPLC has proven to be a very sensitive method for screening the entire *NF1* gene and boasts a mutation detection rate of between 66% (De Luca *et al.*, 2004) and 72% (De Luca *et al.*, 2003). However, some improvements may further increase its sensitivity. As described by Narayanaswami & Taylor (2001), a hybridisation procedure involving GC-clamped primers in PCR and 3M betaine may well help to improve the efficiency of mutation detection by DHPLC. In addition, the amount of PCR product injected into the column influences DHPLC sensitivity. Thus, using 50-200ng of amplified DNA could help to increase the mutation detection rate. Finally, increased throughput of DHPLC analysis might be obtained by using capillary array HPLC systems interfaced with

multi-colour fluorescence detection and labelling different fragments which share a common melting temperature (Huber *et al.*, 2001; Xiao *et al.*, 2001; Guipponi *et al.*, 2005).

There is urgent need for the development of efficient, cost effective mutation detection techniques that are rapidly able to screen the entire *NF1* gene and the additional flanking genes.

8.2.2 Mouse Models

Significant advances in this field have been made in developing animal models. Mouse models exhibiting a phenotype which reflects a number of human clinical features have been developed. Mice in which the *NF1* gene is disrupted, either in specific cell types and/or during specific time window, have been developed. Mice in which multiple genes have been disrupted, have also been modelled.

It has been shown to be possible to rescue the learning deficits observed in $Nf^{+/-}$ mice by genetic and pharmacologic manipulations that decrease Ras activity. Possible therapy has been suggested and phase 1 and 2 clinical trials on those new drugs are ongoing. Animal models continue to provide important information on the biology, and pathology of this disease.

8.2.3 Gene expression analysis

Analysis of gene expression profiles in different pathological tissues from NF1 affected individuals should provide information on which gene or genes are up- or down-regulated and hence whether they are likely to play a key role in the development and differentiation of specific NF1 tumours. However, during the course of this study, fibroblast cells cultured from both affected and unaffected body areas from five SNF1 affected patients did not reveal any significant differentially expressed genes. This might depend on the cell type analysed and also on the small number of samples studied. In the future, using microarray technologies for gene expression analysis on Schwann cells from neurofibromas and melanocytes from CALs might be helpful for identifying up- or down-regulated genes in both NF1 and SNF1 patients. Recently, an MPLA method has been used for gene expression measurements to define both

182

inflammation and the apoptosis response (Eldering *et al.*, 2003). This approach might be more practical than microarray technologies because a predefined set of genes is targeted instead of thousands of genes, leading to easier interpretation of the results. Data generated from such studies will further offer targets for potential therapeutic intervention, and also provide novel information on the identity and function of genes that are altered due to the loss of neurofibromin expression in Schwann cells. This could lead to the identification of new prognostic, diagnostic and potential therapeutic targets for NF1.

8.2.4 Array CGH

Array CGH can identify any gene copy number change in tumour genome by comparing tumour human DNA with normal non-tumour DNA. CGH has been developed to permit genome-wide analysis of DNA sequence copy number in a single experiment. Recently reported, the full coverage 32K human genomic array is a very efficient tool for copy number analysis in genetics. This array covers practically the entire genome in a overlapping contiguous set of 32450 BAC clones This array platform allows one to perform multiple levels of analysis, including gene dosage profiling and epigenetic analysis (Ishkanian *et al*, 2004; Chen *et al*, 2005). Use of this technology might unravel certain loci which interact with neurofibromin and may be involved in modulating the phenotype.

8.2.5 Modifying genes

As hypothesized by Easton *et al.* (1993), several modifying genes may interact with the *NF1* gene, thereby resulting in variation of the clinical phenotype, as is seen even in individuals with the same *NF1* mutation. The possible interaction of the *NF1* gene with other genes was described. In a family affected by NF1 and intestinal neuronal dysplasia type B (IND B), mutations in both the *NF1* and the glial cell line-derived neurotrophic factor (*GDNF*) genes were identified (Bahuau *et al.*, 2001). Both *NF1* and *GDNF* are involved in the signalling pathway of Ras; *NF1* gene disruption generates activated GTP-RAS, while low *GDNF* maintains GDP-RAS. In addition, the three genes

embedded within intron 27b of the *NF1* gene may also influence the clinical phenotype in NF1 patients.

Identification of modifying loci and their location will help in explaining the variable expressivity of the gene. However, no systematic, comprehensive studies have been performed to determine the clinical spectrum, phenotype/genotype correlations, or prognostic utility of NF1 microdeletions. It is proposed that co-deletion of NF1 and an unknown modifying gene (NPL) may function in concert to potentiate neurofibromagenesis, either by increasing the frequency of NF1 second hit mutations, or by selecting for the increased growth of neurofibromin-negative Schwann cells. In addition, in order to understand more about the specific and more severe phenotype seen in NF1 patients carrying the deletion of the entire *NF1* gene and flanking regions, the analysis of the three embedded genes (*EVI2A, EVI2B* and *OMGP*) within intron 27b of the *NF1* gene, as well as many of the seventeen genes known to flank the NF1 locus and to be variably co-deleted in such microdeletion patients may provide useful information on both the function of those genes and on their role in specifying some of the clinical features characteristic of these patients, for example, the early onset of neurofibromas.

Evidence that the *NF1* gene is a mutational target in mismatch repair-deficient cells has come from the description of patients homozygous for mutations in either the *MLH1* or the *MSH2* genes, as well as other genes within the mismatch repair (MMR) pathway, who developed certain clinical features of NF1, along with a number of other symptoms. Thus, the study of these MMR genes may be good candidates for future modifying gene analysis in NF1 patients. Furthermore, the many genes directly and indirectly involved in the various Ras pathways are also obvious candidates for functioning as modifying loci, and should therefore also be screened

8.2.6 Functional analysis

To date, only 3 functional domains of neurofibromin have been identified, these include the CSRD (cysteine serine rich domain), the GRD (GAP related domain) and the Sec 14p-like domain. Identification of additional protein domains will better define the

functional basis of the protein and also help to elucidate with which other proteins neurofibromin is likely to interact.

8.2.7 Clinical trials

Before starting any clinical trials, we must understand the basic science well enough to target the underlying mechanism.
 The clinical phenotype of NF1 is associated with varied clinical features and complications, ie scoliosis, bone abnormalities and learning disability. The success of clinical trials is dependent on defining treatment end points for specific clinical features. In order to ascertain the effectiveness of the treatment, it will therefore be important that the developmental progress of that particular disease feature, in the absence of therapeutic interventions, is well characterised.
What clinical features will be more amenable to potential treatments? Clearly, plexiform neurofibroma, which represents major cause of patient morbidity, should be considered first. Over the past 5 years several chemotherapy trials have been conducted to develop treatments for plexiform neurofibroma. Both cis-retinoic acid and interferon-alpha have been used in phase 2 clinical trials. Other phase 1 studies have employed farnesyl protein transferase inhibitors (R115777), which block the post-translational isoprenylation of Ras and inhibit Ras activity The drug pirfenidone, an antifibrotic which modulates cytokine action has also been used in phase 2 clinical trials.
In neurofibroma, Schwann cells over secrete a stem cell factor and this factor attracts mast cells due to the presence of c-Kit tyrosine kinase receptor on their surface. A compound which directly affects the c-Kit receptor has been identified and is being tested in NF1 mouse models.

Learning disability affects some 40% of all NF1 patients and is clearly a target for therapeutic intervention. Mice model studies have produced some promising results. Statins, used to lower cholesterol levels in humans, have exhibited a dramatic effect on the learning disability observed in $Nf^{+/-}$ mice as studied in a maze experiment. Hence statins are being used in the clinical trials.

8.2.8 Several questions remain unanswered

Should one prevent tumour growth or merely attempt to reduce the overall tumour load?

What is the effective methodology for the measurement of tumour size and number?

Which are the key genes involved in different types of NF1-associated tumours?

What are the molecular mechanisms involved in the transformation of benign tumour to malignancy?

Which biomarkers for plexiform neurofibromas likely to progress to malignancy or for optic gliomas requiring medical intervention?

Finally, NF1 like tuberous sclerosis is a disease model where successful cloning of the gene has resulted in enhanced basic research, translational research and clinical trials, and further exciting developments are awaited in this field.

References

Abeliovich D, Gelman-Kohan Z, Silverstein S, Lerer I, Chemke J, Merin S, Zlotogora J (1995). Familial cafè-au-lait spots : A variant of neurofibromatosis type 1. *J Med Genet* 32: 985-986.

Abernathy CR, Colman SD, Kousseff BG, Wallace MR (1994). Two NF1 mutations: frameshift in the GAP-related domain, and loss of two codons toward the 3' end of the gene. *Hum Mutat* 3: 347-352.

Abernathy C R, Rasmussen S A, Stalker H J, Zori R, Driscoll D J, Williams C A, Kousseff B G, Wallace M R (1997). *NF1* mutation analysis using a combined heteroduplex/SSCP approach. *Hum Mutat* 9: 548-554.

Abuelo DN, Meryash DL (1988). Neurofibromatosis with fully expressed Noonan syndrome. *Am J Med Genet.* 29: 937-941.

Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, Merril CR, Wu A, Olde B, Moreno RF (1991). Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* 252: 1651-1656.

Adjei AA (2001a). Farnesyltransferase inhibitors. *Cancer Chemother Biol Response Modif* 19: 149-164.

Adjei AA (2001b). Ras signaling pathway proteins as therapeutic targets. *Curr Pharm Des* 7: 1581-1594.

Agesen TH, Florenes VA, Molenaar WM, Lind GE, Berner JM, Plaat BE, Komdeur R, Myklebost O, van den Berg E, Lothe RA (2005). Expression patterns of cell cycle components in sporadic and neurofibromatosis type 1-related malignant peripheral nerve sheath tumours. *J Neuropathol Exp Neurol* 64: 74-81.

Ahlbom BE, Dahl N, Zetterqvist P, Anneren G (1995). Noonan syndrome with cafe-au-lait spots and multiple lentigines syndrome are not linked to the neurofibromatosis type 1 locus. *Clin Genet* 48: 85-89.

Ahmadian MR, Hoffmann U, Goody RS, Wittinghofer A (1997). Individual rate constants for the interaction of Ras proteins with GTPase-activating proteins determined by fluorescence spectroscopy. *Biochemistry* 36: 4535-4541.

Ainsworth PJ, Chakraborty PK, Weksberg R (1997). Example of somatic mosaicism in a series of de novo neurofibromatosis type 1 cases due to a maternally derived deletion. *Hum Mutat.* 9: 452-457.

Ainsworth PJ, Rodenhiser DI, Costa MT (1993). Identification and characterization of sporadic and inherited mutations in exon 31 of the neurofibromatosis (*NF1*) gene. *Hum Genet* 91: 151-156.

Akenside M (1768). Observations on cancers. *Med Trans Coll Phys Lond* 1: 64-92.

Akgun E, Zahn J, Baumes S, Brown G, Liang F, Romanienko PJ, Lewis S, Jasin M (1997). Palindrome resolution and recombination in the mammalian germ line. *Mol Cell Biol* 17: 5559-5570.

Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM (2000). Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403: 503-511.

Allanson JE, Hall JG, Van Allen MI (1985). Noonan phenotype associated with neurofibromatosis. *Am J Med Genet* 21: 457-462.

Allanson JE, Upadhyaya M, Watson GH, Partington M, MacKenzie A, Lahey D, MacLeod H, Sarfarazi M, Broadhead W, Harper PS (1991). Watson syndrome: is it a subtype of type 1 neurofibromatosis? *J Med Genet.* 28: 752-756.

Alwine JC, Kemp DJ, Stark GR (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci U S A* 74: 5350-5354.

Amos-Landgraf JM, Ji Y, Gottlieb W, Depinet T, Wandstrat AE, Cassidy SB, Driscoll DJ, Rogan PK, Schwartz S, Nicholls RD (1999). Chromosome breakage in the Prader–Willi and Angelman syndromes involves recombination between large, transcribed repeats at proximal and distal breakpoints. *Am J Hum Genet* 65: 370–386.

Andersen LB, Ballester R, Marchuk DA, Chang E, Gutmann DH, Saulino AM, Camonis J, Wigler M, Collins FS (1993a). A conserved alternative splice in the von Recklinghausen neurofibromatosis (NF1) gene produces two neurofibromin isoforms, both of which have GTPase-activating protein activity. *Mol Cell Biol* 13: 487-495.

Andersen LB, Fountain JW, Gutmann DH, Tarle SA, Glover TW, Dracopoli NC, Housman DE, Collins FS (1993b). Mutations in the neurofibromatosis 1 gene in sporadic malignant melanoma cell lines. *Nat Genet* 3: 118-121.

Anglani F, Murgia A, Bedin S, Bresin E, Bernardi F, Clementi M, Tenconi R (1993). A new disease-causing mutation in the GAP-related domain of the *NF1* gene. *Hum Mol Genet* 2: 1057-1059.

Apostolski S, Sadiq SA, Hays A, Corbo M, Suturkova-Milosevic L, Chaliff P, Stefansson K, LeBaron RG, Ruoslahti E, Hays AP (1994). Identification of Gal(beta 1-3)GalNAc bearing glycoproteins at the nodes of Ranvier in peripheral nerve. *J Neurosci Res* 38: 134-141.

Aravind L, Neuwald AF, Ponting CP (1999).Sec14p-like domains in NF1 and Dbl-like proteins indicate lipid regulation of Ras and Rho signaling. *Curr Biol* 9: R195-197.

Arbiser JL, Flynn E, Barnhill RL (1998). Analysis of vascularity of human neurofibromas. *J Am Acad Dermatol* 38: 950–954.

Arfan-ul-Bari, Simeen-ber-Rahman (2003). Segmental neurofibromatosis [NF type - v]. *J Coll Physicians Surg Pak* 13: 717-718.

Armour JA, Sismani C, Patsalis PC, Cross G (2000). Measurement of locus copy number by hybridisation with amplifiable probes. *Nucleic Acids Res* 28: 605-609.

Arnsmeier SL, Riccardi VM, Paller AS (1994). Familial multiple cafe au lait spots. *Arch Dermatol.* 130: 1425-1426.

Ars E, Kruyer H, Gaona A, Casquero P, Rosell J, Volpini V, Serra E, Lazaro C, Estivill X (1998). A clinical variant of neurofibromatosis type 1: Familial spinal neurofibromatosis with a frameshift mutation in the *NF1* gene. *Am J Hum Genet* 62: 834-841.

Ars E, Kruyer H, Morell M, Pros E, Serra E, Ravella A, Estivill X, Lazaro C (2003). Recurrent mutations in the *NF1* gene are common among neurofibromatosis type 1 patients. *J Med Genet* 40: e82.

Ars E, Serra E, Garcia J, Kruyer H, Gaona A, Lazaro C, Estivill X (2000). Mutations affecting mRNA splicing are the most common molecular defects in patients with neurofibromatosis type 1. *Hum Mol Genet* 9: 237-247.

Atit RP, Crowe MJ, Greenhalgh DG, Wenstrup RJ, Ratner N (1999). The Nf1 tumor suppressor regulates mouse skin wound healing, fibroblast proliferation, and collagen deposited by fibroblasts. *J Invest Dermatol* 112: 835-842.

Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, Shark KB, Grande WJ, Hughes KM, Kapur V, Gregersen PK, Behrens TW (2003).Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci U S A* 100: 2610-2615.

Bagnall RD, Waseem N, Green PM, Giannelli F (2002). Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood.* 99: 168-174.

Bahuau M, Flintoff W, Assouline B, Lyonnet S, Le Merrer M, Prieur M, Guilloud-Bataille M, Feingold N, Munnich A, Vidaud M, Vidaud D (1996). Exclusion of allelism of Noonan syndrome and neurofibromatosis-type 1 in a large family with Noonan syndrome-neurofibromatosis association. *Am J Med Genet.* 66: 347-355.

Bahuau M, Houdayer C, Assouline B, Blanchet-Bardon C, Le Merrer M, Lyonnet S, Giraud S, Recan D, Lakhdar H, Vidaud M, Vidaud D (1998). Novel recurrent nonsense mutation causing neurofibromatosis type 1 (NF1) in a family segregating both NF1 and Noonan syndrome. *Am J Med Genet.* 75: 265-272.

Bailey JA, Yavor AM, Massa HF, Trask BJ, Eichler EE (2001). Segmental duplications: organization and impact within the current human genome project assembly. *Genome Res* 11: 1005-1017.

Bajenaru ML, Donahoe J, Corral T, Reilly KM, Brophy S, Pellicer A, Gutmann DH (2001). Neurofibromatosis 1 (*NF1*) heterozygosity results in a cell-autonomous growth advantage for astrocytes. *Glia* 33: 314-323.

Bajenaru ML, Garbow JR, Perry A, Hernandez MR, Gutmann DH (2005). Natural history of neurofibromatosis 1-associated optic nerve glioma in mice. *Ann Neurol* 57: 119-127.

Bajenaru ML, Hernandez MR, Perry A, Zhu Y, Parada LF, Garbow JR, Gutmann DH (2003). Optic nerve glioma in mice requires astrocyte Nf1 gene inactivation and Nf1 brain heterozygosity. *Cancer Res* 63: 8573-8577.

Baldwin DN, Vanchinathan V, Brown PO, Theriot JA (2003). A gene-expression program reflecting the innate immune response of cultured intestinal epithelial cells to infection by Listeria monocytogenes. *Genome Biol* 4: R2.

Ballester R, Marchuk D, Boguski M, Saulino A, Letcher R, Wigler M, Collins F (1990). The *NF1* locus encodes a protein functionally related to mammalian GAP and Yeast IRA proteins. *Cell* 63: 852–859.

Barrall JL, Summers CG (1996). Ocular ischemic syndrome in a child with moyamoya disease and neurofibromatosis. *Surv Ophthalmol* 40: 500–504.

Baralle D, Mattocks C, Kalidas K, Elmslie F, Whittaker J, Lees M, Ragge N, Patton MA, Winter RM, Ffrench-Constant C (2003). Different mutations in the *NF1* gene are associated with Neurofibromatosis-Noonan syndrome (NFNS). *Am J Med Genet* 119A: 1-8.

Barker D, Wright K, Nguyen L, Fain CP, Goldgar D, Bishop DT, Carey J, Baty B, Kiviin J, Willard H, Waye JS, Greig G, Leinwand L, Nakamura Y, O'Connell P, Leppert M, Lalouel JM, White R, Skolnichk M (1987). Gene for von Recklinghausen neurofibromatosis is in the pericentromeric region of chromosome 17. *Science* 236:1100-1102.

Barrall JL, Summers CG (1996). Ocular ischemic syndrome in a child with Moyamoya disease and neurofibromatosis. *Surv Ophthalmol.*;40:500–504.

Basu TN, Gutmann DH, Fletcher JA, Glover TW, Collins FS, Downward J (1992). Aberrant regulation of ras proteins in malignant tumor cells from type 1 neurofibromatosis patients. *Nature* 356: 713–715.

Baurmann H, Cherif D, Berger R (1993). Interphase cytogenetics by fluorescent in situ hybridization (FISH) for characterization of monosomy-7-associated myeloid disorders. *Leukemia* 7: 384-391.

Bayes, M., Magano, L.F., Rivera, N., Flores, R. and Perez Jurado, L.A (2003). Mutational mechanisms of Williams -Beuren syndrome deletions. *Am J Hum Genet* 73: 131-151.

Bell AC, Felsenfeld G (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. *Nature* 405: 482–485.

Benedict PH, Szabo G, Fitzpatrick TB, Sinesi SJ (1969). Melanotic macules in Albright's syndrome and in neurofibromatosis. *JAMA* 205: 72-80.

Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, Pascual V (2003). Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. *J Exp Med* 197: 711-723.

Berk AJ, Sharp PA (1977).Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids.*Cell* 12: 721-732.

Bernards A (1995). Neurofibromatosis type 1 and Ras-mediated signaling: filling in the GAPs. *Biochim Biophys Acta* 1242: 43–59.

Bernards A, Snijders AJ, Hannigan GE, Murthy AE, Gusella JF (1993). Mouse neurofibromatosis type 1 cDNA sequence reveals high degree of conservation of both coding and non-coding mRNA segments. *Hum Mol Genet* 2: 645-650.

Bertola DR, Pereira AC, Passetti F, de Oliveira PS, Messiaen L, Gelb BD, Kim CA, Krieger JE (2005). Neurofibromatosis-Noonan syndrome: molecular evidence of the concurrence of both disorders in a patient. *Am J Med Genet* A136: 242-245.

Bi W, Park S-S, Shaw CJ, Withers MA, Patel PI, Lupski JR (2003). Reciprocal crossovers and a positional preference for strand exchange in recombination events resulting in deletion or duplication of chromosome 17p11.2. *Am J Hum Genet* 73: 1302–1315.

Biagi F, Campanella J, Alvisi C, Versino M, Corazza GR (2005). Unusual association of neurofibromatosis type 1 and coeliac disease in a single patient. *Funct Neurol* 20: 33-34.

Bird A (2002). DNA methylation patterns and epigenetic memory. *Genes Dev* 16: 6–21.

Birindelli S, Perrone F, Oggionni M, Lavarino C, Pasini B, Vergani B, Ranzani GN, Pierotti MA, Pilotti S (2001). Rb and TP53 pathway alterations in sporadic and NF1-related malignant peripheral nerve sheath tumours. *Lab Invest* 81: 833–844.

Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, Simon R, Yakhini Z, Ben-Dor A, Sampas N, Dougherty E, Wang E, Marincola F, Gooden C, Lueders J, Glatfelter A, Pollock P, Carpten J, Gillanders E, Leja D, Dietrich K, Beaudry C, Berens M, Alberts D, Sondak V (2000). Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 406: 536-540.

Boddrich A, Griesser J, Horn D, Kaufmann D, Krone W, Nurnberg P (1995). Reduced neurofibromin content but normal GAP activity in a patient with neurofibromatosis type 1 caused by a five base pair duplication in exon 12b of the *NF1* gene. *Biochem Biophys Res Commun* 214: 895-904.

Boddrich A, Robinson PN, Schulke M, Buske A, Tinschert S, Nurnberg P (1997). New evidence for a mutation hotspot in exon 37 of the *NF1* gene. *Hum Mutat* 9: 374-377.

Boldrick JC, Alizadeh AA, Diehn M, Dudoit S, Liu CL, Belcher CE, Botstein D, Staudt LM, Brown PO, Relman DA (2002). Stereotyped and specific gene expression programs in human innate immune responses to bacteria. *Proc Natl Acad Sci U S A* 99: 972-977.

Bollag G, Clapp DW, Shih S, Adler F, Zhang YY, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T, Shannon K (1996). Loss of NF1 results in activation of Ras signaling pathway and leads to aberrant growth in haematopoietic cells. *Nat Genet* 12: 144–148.

Bollag G, and McCormick F (1991). Regulators and effectors of *ras* proteins. *Ann Rev Cell Biol* 7: 601–632.

Bollag G, McCormick F, Clark R (1993). Characterization of full-length neurofibromin: tubulin inhibits Ras GAP activity. *EMBO J* 12: 1923-1927.

Bonneau F, D'Angelo I, Welti S, Stier G, Ylanne J, Scheffzek K (2004). Expression, purification and preliminary crystallographic characterization of a novel segment from the neurofibromatosis type 1 protein. *Acta Crystallogr D Biol Crystallogr* 60: 2364-2367.

Boltshauser E, Stocker H, Machler M (1989). Neurofibromatosis type 1 in a child of a parent with segmental neurofibromatosis (NF5) *Neurofibromatosis* 2: 244-245.

Borochowitz Z, Berant N, Dar H, Berant M (1989). The neurofibromatosis-Noonan syndrome: genetic heterogeneity versus clinical variability. Case report and review of the literature. *Neurofibromatosis.* 2: 309-314.

Boucneau J, De Schepper S, Vuylsteke M, Van Hummelen P, Naeyaert JM, Lambert J (2005). Gene expression profiling of cultured human NF1 heterozygous (NF1+/-) melanocytes reveals downregulation of a transcriptional cis-regulatory network mediating activation of the melanocyte-specific dopachrome tautomerase (DCT) gene. *Pigment Cell Res* 18: 285-299.

Bourneville DM (1880). Sclérose tubéreuse des circonvolution cérébrales: Idiotie et épilepsie hemiplégique. Archives de neurologie, Paris 1: 81-91.

Bosch EP, Murphy MJ, Cancilla PA (1981). Peripheral neurofibromatosis and peroneal muscular atrophy. *Neurology* 31: 1408-1414.

Boveri T (1929). *The origin of malignant tumours.* Williams & Willkins, Baltimore, Maryland.

Brannan CI, Perkins AS, Vogel KS, Ratner N, Nordlund ML, Reid SW, Buchberg AM, Jenkins NA, Parada LF, Copeland NG (1994). Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. *Genes Dev* 8: 1019-1029.

Brem RB, Yvert G, Clinton R, Kruglyak L (2002).Genetic dissection of transcriptional regulation in budding yeast. *Science* 296: 752-755.

Brunner HG, Hulsebos T, Steijlen PM, der Kinderen DJ, von der Steen A, Hamel BC (1993). Exclusion of the neurofibromatosis 1 locus in a family with inherited café-au-lait spots. *Am J Med Genet* 46: 472-474.

Buehning L, Curry CJ (1995). Neurofibromatosis-Noonan syndrome. *Pediatr Dermatol* 12: 267-271.

Burwell RG, James NJ, Johnston DI (1982). Cafe-au-lait spots in school children. *Arch Dis Child* 57:631-632.

Buske A, Gewies A, Lehmann R, Ruther K, Algermissen B, Nurnberg P, Tinschert S (1999). Recurrent NF1 gene mutation in a patient with oligosymptomatic neurofibromatosis type 1 (NF1). *Am J Med Genet* 86: 328-330.

Carey JC (1998). Neurofibromatosis-Noonan syndrome. *Am J Med Genet* 75: 263-264.

Carey JC, Baty BJ, Johnson JP, Morrison T, Skolnick M, Kivlin J (1986). The genetic aspects of neurofibromatosis. *Ann N Y Acad Sci* 486: 45-56.

Carey, JC.; Stevenson, DA.; Ota, M.; Neil, S.; Viskochil, DH (1997): Is there an Noonan syndrome: Part 2. Documentation of the clinical and molecular aspects of an important family. *Proc. Greenwood Genet. Center* 17: 52-53.

Carey JC, Viskochil DH (1999). Neurofibromatosis type 1. A model condition for the study of the molecular basis of variable expressivity in human disorders. *Am J Med Genet* 89: 7–13.

Carlomagno F, Salvatore G, Cirafici AM, De Vita G, Melillo RM, de Franciscis V, Billaud M, Fusco A, Santoro M (1997). The different RET-activating capability of mutations of cysteine 620 or cysteine 634 correlates with the multiple endocrine neoplasia type 2 disease phenotype. *Cancer Res.* 57: 391-395.

Castle B, Baser ME, Huson SM, Cooper DN, Upadhyaya M (2003). Evaluation of genotype-phenotype correlations in neurofibromatosis type 1. *J Med Genet* 40: e109.

Cawthon RM, Andersen LB, Buchberg AM, Xu GF, O'Connell P, Viskochil D, Weiss RB, Wallace MR, Marchuk DA, Culver M (1991). cDNA sequence and genomic

structure of EV12B, a gene lying within an intron of the neurofibromatosis type 1 gene. *Genomics* 9: 446-460.

Cawthon RM, O'Connell P, Buchberg AM, Viskochil D, Weiss RB, Culver M, Stevens J, Jenkins NA, Copeland NG, White R (1990a).Identification and characterization of transcripts from the neurofibromatosis 1 region: the sequence and genomic structure of *EVI2* and mapping of other transcripts. *Genomics* 7: 555-565.

Cawthon RM, Weiss R, Xu GF, Viskochil D, Culver M, Stevens J, Robertson M, Dunn D, Gesteland R, O'Connell P,White R (1990b). A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* 62: 193–201.

Celis JE, Ostergaard M, Basse B, Celis A, Lauridsen JB, Ratz GP, Andersen I, Hein B, Wolf H, Orntoft TF, Rasmussen HH (1996). Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas. *Cancer Res* 56: 4782-4790.

Chance PF, Abbas N, Lensch MW, Pentao L, Roa BB, Patel PI, Lupski JR (1994). Two autosomal dominant neuropathies result from reciprocal DNA duplication/deletion of a region on chromosome 17. *Hum Mol Genet* 3: 223–228.

Charrow J, Listernick R, Ward K (1993). Autosomal dominant multiple café-au-lait spots and neurofibromatosis 1: Evidence of non-linkage. *Am J Med Genet* 45: 606-608.

Cheadle JP, Reeve MP, Sampson JR, Kwiatkowski DJ (2000). Molecular genetic advances in tuberous sclerosis. *Hum Genet* 107: 97-114.

Chen KS, Manian P, Koeuth T, Potocki L, Zhao Q, Chinault AC, Lee CC, Lupski JR (1997). Homologous recombination of a flanking repeat gene cluster is a mechanism for a common contiguous gene deletion syndrome. *Nature Genet* 17: 154–163.

Chen SH, Schoof JM, Weinmann AF, Thompson AR (1995).Heteroduplex screening for molecular defects in factor IX genes from haemophilia B families. *Br J Haematol* 89: 409-412.

Chen YW, Zhao P, Borup R, Hoffman EP (2000). Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. *J Cell Biol* 151: 1321-1336.

Cheung J, Estivill X, Khaja R, MacDonald JR, Lau K, Tsui LC, Scherer SW (2003). Genome-wide detection of segmental duplications and potential assembly errors in the human genome sequence. *Genome Biol* 4: R25.

Cho B, Lee H, Jeong S, Bang YJ, Lee HJ, Hwang KS, Kim HY, Lee YS, Kang GH, Jeoung DI (2003). Promoter hypomethylation of a novel cancer/testis antigen gene CAGE is correlated with its aberrant expression and is seen in premalignant stage of gastric carcinoma. *Biochem Biophys Res Commun* 307: 52–63.

Christian, S. L., Fantes, J. A., Mewborn, S. K., Huang, B. & Ledbetter, D. H (1999). Large genomic duplicons map to sites of instability in Prader–Willi/Angelman syndrome chromosome region (15q11–q13). *Hum Mol Genet.* 8: 1025–1037.

Cichowski K, Shih TS, Jacks T (1996). Nf1 gene targeting: toward models and mechanisms. *Semin Cancer Biol* 7: 291-298.

Cichowski K, Jacks T (2001). NF1 tumour suppressor gene function: narrowing the GAP. *Cell* 104: 593-604.

Cichowski K, Shih TS, Schmitt E, Santiago S, Reilly K, McLaughlin ME, Bronson RT, Jacks T (1999). Mouse models of tumour development in neurofibromatosis type 1. *Science* 286: 2172–2176.

Cnossen MH, de Goede-Bolder A, van den Broek KM, Waasdorp CM, Oranje AP, Stroink H, Simonsz HJ, van den Ouweland AM, Halley DJ, Niermeijer MF (1998). A prospective 10 year follow up study of patients with neurofibromatosis type 1. *Arch Dis Child* 78: 408-412.

Cnossen MH, van der Est MN, Breuning MH, van Asperen CJ, Breslau-Siderius EJ, van der Ploeg AT, de Goede-Bolder A, van den Ouweland AM, Halley DJ, Niermeijer MF (1997). Deletions spanning the neurofibromatosis type 1 gene: implications for genotype-phenotype correlations in neurofibromatosis type 1? *Hum Mutat.* 9: 458-464.

Colapietro P, Gervasini C, Natacci F, Rossi L, Riva P, Larizza L (2003). *NF1* exon 7 skipping and sequence alterations in exonic splice enhancers (ESEs) in a neurofibromatosis 1 patient. *Hum Genet* 113: 551-554.

Colby RS, Saul RA (2003). Is Jaffe-Campanacci syndrome just a manifestation of neurofibromatosis type 1? *Am J Med Genet* A 123: 60-63.

Colley A, Donnai D, Evans DRG (1996). Neurofibromatosis/Noonan phenotype : a variable feature of type 1 neurofibromatosis. *Clin Genet* 49: 59-64.
Colman SD, Abernathy CR, Ho VT, Wallace MR (1997). Four frameshift mutations in neurofibromatosis type 1 caused by small insertions. *J Med Genet* 34: 579-581.

Colman SD, Collins FS, Wallace MR (1993). Characterization of a single base-pair deletion in neurofibromatosis type 1. *Hum Mol Genet* 2: 1709-1711.

Colman SD, Rasmussen SA, Ho VT, Abernathy CR, Wallace MR (1996). Somatic mosaicism in a patient with neurofibromatosis type 1. *Am J Hum Genet* 58: 484–490.

Colman SD, Williams CA, Wallace MR (1995). Benign neurofibromas in type 1 neurofibromatosis (NF1) show somatic deletions of the NF1 gene. *Nat Genet* 11: 90-92.

Consoli C, Moss C, Green S, Balderson D, Cooper DN, Upadhyaya M (2005). Gonosomal Mosaicism for a Nonsense Mutation (R1947X) in the NF1 Gene in Segmental Neurofibromatosis Type 1. *J Invest Dermatol* 125: 463-466.

Cooper DN (2002). Human gene mutation in pathology and evolution. *J Inherit Metab Dis.* 25: 157-182.

Cooper DN and Krawczak M (1993). *Human Gene Mutation*. Bios Scientific, Oxford.

Corral T, Jimenez M, Hernandez-Munoz I, Perez de Castro I, Pellicer A (2003). NF1 modulates the effects of Ras oncogenes: evidence of other NF1 function besides its GAP activity. *J Cell Physiol* 197: 214-224.

Costa RM, Federov NB, Kogan JH, Murphy GG, Stern J, Ohno M, Kucherlapati R, Jacks T, Silva AJ (2002). Mechanism for the learning deficits in a mouse model of neurofibromatosis type 1. *Nature* 415: 526–530.

Costa RM, Silva AJ (2003). Mouse models of neurofibromatosis type I: bridging the GAP. *Trends Mol Med.* 9: 19-23.

Costa RM, Yang T, Huynh DP, Pulst SM, Viskochil DH, Silva AJ, Brannan CI (2001). Learning deficits, but normal development and tumour predisposition, in mice lacking exon 23a of *Nf1*. *Nat Genet* 27: 399–405.

Cotton RGH, Rodrigues NR, Campbell RD (1988). Activity of cytosine and thymidine in single bas-pair mismatches with hydroxylamine and osmium tetraoxide and its application to the study of mutations. *Proc Natl Acad Sci USA* 85: 3997-4401.

Crowe FW, Schull WJ (1953). Diagnostic importance of café-au-lait spot in neurofibromatosis. *AMA Arch Intern Med.* 91: 758-766.

Crowe FW, Schull WJ, Neel JV (1956). *A Clinical, Pathological and Genetic Study of Multiple Neurofibromatosis*. Springfield, III: Charles C Thomas Publishers.

Cunha KS, Barboza EP, Da Fonseca EC (2003). Identification of growth hormone receptor in localised neurofibromas of patients with neurofibromatosis type 1. *J Clin Pathol* 56: 758-763.

Das R, Hammamieh R, Neill R, Melhem M, Jett M (2001). Expression pattern of fatty acid-binding proteins in human normal and cancer prostate cells and tissues. *Clin Cancer Res* 7: 1706-1715.

Daschner K, Assum G, Eisenbarth I, Krone W, Hoffmeyer S, Wortmann S, Heymer B, Kehrer-Sawatzki H (1997). Clonal origin of tumor cells in a plexiform neurofibroma with LOH in NF1 intron 38 and in dermal neurofibromas without LOH of the *NF1* gene. *Biochem Biophys Res Commun* 234: 346-350.

Dasgupta B, Dugan LL, Gutmann DH (2003). The Neurofibromatosis 1 Gene Product Neurofibromin Regulates Pituitary Adenylate Cyclase-Activating Polypeptide-Mediated Signaling in Astrocytes. *J Neurosci* 23: 8949-8954.

Dasgupta B, Gutmann DH (2003). Neurofibromatosis 1: closing the GAP between mice and men. *Curr Opin Genet Dev* 13: 20-27.

Dasgupta B, Li W, Perry A, Gutmann DH (2005). Glioma formation in neurofibromatosis 1 reflects preferential activation of K-RAS in astrocytes. *Cancer Res* 65: 236-145.

Dasgupta B, Yi Y, Chen DY, Weber JD, Gutmann DH (2005a). Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in neurofibromatosis 1-associated human and mouse brain tumours. *Cancer Res* 65: 2755-2760.

Daston MM, Scrable H, Nordlund M, Sturbaum AK, Nissen LM, Ratner N (1992). The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, Schwann cells, and oligodendrocytes. *Neuron* 8: 415-428.

Daston MM, Ratner N (1992). Neurofibromin, a predominantly neuronal GTPase activating protein in the adult, is ubiquitously expressed during development. *Dev Dyn* 195: 216-226.

DeBella K, Szudek J, Friedman JM (2000). Use of the National Institutes of Health Criteria for diagnosis of neurofibromatosis1 in children. *Pediatrics* 105: 608–614.

DeClue JE, Cohen BD, Lowy DR (1991). Identification and characterization of the neurofibromatosis type 1 protein product. *Proc Natl Acad Sci U S A* 88: 9914-9918.

DeClue JE, Heffelfinger S, Benvenuto G, Ling B, Li S, Rui W, Vass WC, Viskochil D, Ratner N (2000). Epidermal growth factor receptor expression in neurofibromatosis type 1-related tumors and NF1 animal models. *J Clin Invest* 105: 1233–1241.

DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowy DR (1992). Abnormal regulation of mammalian p21ras contributes to malignant tumour growth in von Recklinghausen (type 1) neurofibromatosis. *Cell* 69: 265–273.

de Koning Gans PA, Ginjaar I, Bakker E, Yates JR, den Dunnen JT (1999). A protein truncation test for Emery-Dreifuss muscular dystrophy (EMD): detection of N-terminal truncating mutations. *Neuromuscul Disord* 9: 247-250.

De Luca A, Bottillo I, Sarkozy A, Carta C, Neri C, Bellacchio E, Schirinzi A, Conti E, Zampino G, Battaglia A, Majore S, Rinaldi MM, Carella M, Marino B, Pizzuti A, Digilio MC, Tartaglia M, Dallapiccola B (2005). NF1 Gene Mutations Represent the Major Molecular Event Underlying Neurofibromatosis-Noonan Syndrome. *Am J Hum Genet* 77: 1092-1101.

De Luca A, Buccino A, Gianni D, Mangino M, Giustini S, Richetta A, Divona L, Calvieri S, Mingarelli R, Dallapiccola B (2003). *NF1* gene analysis based on DHPLC. *Hum Mutat* 21: 171-172.

De Luca A, Schirinzi A, Buccino A, Bottillo I, Sinibaldi L, Torrente I, Ciavarella A, Dottorini T, Porciello R, Giustini S, Calvieri S, Dallapiccola B (2004). Novel and recurrent mutations in the NF1 gene in Italian patients with neurofibromatosis type 1. *Hum Mutat* 23: 629.

DeRisi JL, Iyer VR, Brown PO (1997).Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278: 680-686.

De Raedt T, Brems H, Lopez-Correa C, Vermeesch JR, Marynen P, Legius E (2004). Genomic organization and evolution of the NF1 microdeletion region. *Genomics* 84: 346-360.

De Raedt T, Brems H, Wolkenstein P, Vidaud D, Pilotti S, Perrone F, Mautner V, Frahm S, Sciot R, Legius E (2003). Elevated risk for MPNST in NF1 microdeletion patients. *Am J Hum Genet* 72: 1288-1292.

De Schepper S, Boucneau J, Lambert J, Messiaen L, Naeyaert JM (2005). Pigment cell-related manifestations in neurofibromatosis type 1: an overview. *Pigment Cell Res* 18: 13-24.

De Smet C, Lurquin C, Lethe B, Martelange V, Boon T (1999). DNA methylation is the primary silencing mechanism for a set of germ line- and tumour-specific genes with a CpG-rich promoter. *Mol Cell Biol* 19: 7327–35.

Dib C, Faure S, Fizames C, Samson D, Drouot N, Vignal A, Millasseau P, Marc S, Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J, Weissenbach J (1996). A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* 380: 152-154.

Dietz HC, Pyeritz RE, Puffenberger EG, Kendzior RJ Jr, Corson GM, Maslen CL, Sakai LY, Francomano CA, Cutting GR (1992). Marfan phenotype variability in a family segregating a missense mutation in the epidermal growth factor-like motif of the fibrillin gene. *J Clin Invest.* 89: 1674-80.

Dobson-Stone C, Cox RD, Lonie L, Southam L, Fraser M, Wise C, Bernier F, Hodgson S, Porter DE, Hamish A, Simpson RW, Monaco AP (2000). Comparison of fluorescent single-strand conformation polymorphism analysis and denaturing high-performance liquid chromatography for detection of *EXT1* and *EXT2* mutations in hereditary multiple exostoses. *Eur J Hum Genet* 8: 24-32.

Dorschner MO, Sybert VP, Weaver M, Pletcher BA, Stephens K (2000). *NF1* microdeletion breakpoints are clustered at flanking repetitive sequences. *Hum Mol Genet* 9: 35-46.

Dublin S, Riccardi VM, Stephens K (1995). Methods for rapid detection of a recurrent nonsense mutation and documentation of phenotypic features in neurofibromatosis type 1 patients. *Hum Mutat* 5: 81-85

Easton DF, Ponder MA, Huson SM, Ponder BAJ (1993). An analysis of variation in expression of neurofibromatosis type 1 (NF1): evidence for modifying genes. *Am J Hum Genet* 53: 305-313.

Edelmann, L., Pandita, R. K. & Morrow, B. E (1999). Low-copy repeats mediate the common 3-Mb deletion in patients with velo-cardio-facial syndrome. *Am J Hum Genet* 64: 1076–1086.

Edelmann L, Spiteri E, Koren K, Pulijaal V, Bialer MG, Shanske A, Goldberg R, Morrow BE (2001). AT-rich palindromes mediate the constitutional t(11;22) translocation. *Am J Hum Genet* 68: 1–13.

Edman AB, Dahl N, Zetterquist P, Anneren G (1995). Noonan syndrome with café au lait spots and multiple lentigines are not linked to the neurofibromatosis type 1 locus. *Clin Genet* 48: 85-89.

Edwards-Brown MK, Quets JP (1997). Midwest experience with Moyamoya disease. *Clin Neurol Neurosurg* 99: S36 –S38.

Eichler EE, Budarf ML, Rocchi M, Deaven LL, Doggett NA, Baldini A, Nelson DL, Mohrenweiser HW (1997). Interchromosomal duplications of the adrenoleukodystrophy locus: a phenomenon of pericentromeric plasticity. *Hum Mol Genet* 6: 991–1002.

Eichler EE, Lu F, Shen Y, Antonacci R, Jurecic V, Doggett NA, Moyzis RK, Baldini A, Gibbs RA, Nelson DL (1996). Duplication of a gene rich cluster between 16p11.1 and Xq28: a novel pericentromeric directed mechanism for paralogous genome evolution. *Hum. Mol. Genet* 5: 899–912.

phylloides. *Neurofibromatosis* 2: 121-124.

Erickson RP, Woolliscroft J, Allen RJ (1980). Familial occurrence of intracranial arterial occlusive disease (Moyamoya) in neurofibromatosis. *Clin Genet* 18: 191-196.

Evans DG, Baser ME, McGaughran J, Sharif S, Howard E, Moran A (2002). Malignant peripheral nerve sheath tumors in neurofibromatosis 1. *J Med Genet* 39: 311-314.

Evans DG, Huson SM, Donnai D, Neary W, Blair V, Teare D, Newton V, Strachan T, Ramsden R, Harris R (1992). A genetic study of type 2 neurofibromatosis in the United Kingdom. I. Prevalence, mutation rate, fitness, and confirmation of maternal transmission effect on severity. *J Med Genet* 29: 841-846.

Fahsold R, Hoffmeyer S, Mischung C, Gille C, Ehlers C, Kucukceylan N, Abdel-Nour M, Gewies A, Peters H, Kaufmann D, Buske A, Tinschert S, Nurnberg P (2000). Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. *Am J Hum Genet* 66: 780-818.

Fang L, Chalhoub N, Li W, Feingold J, Ortenberg J, Lemieux B, Thirion JP (2001). Genotype analysis of the *NF1* gene in the French Canadians from the Quebec population. *Am J Med Genet* 104: 189-198.

Fay JC, McCullough HL, Sniegowski PD, Eisen MB (2004).Population genetic variation in gene expression is associated with phenotypic variation in Saccharomyces cerevisiae. *Genome Biol* 5: R26.

Ferner RE, Hughes RAC, Johnson MR (1995).. Neurofibromatosis 1 and multiple sclerosis. *J Neurol Neurosurg Psychiatry* 58: 582–585.

Feuillet L, Boudinet H, Casseron W, Uzenot D, Pelletier J, Ali Cherif A (2004). [Multiple sclerosis associated with neurofibromatosis type I]. *Rev Neurol* (Paris) 160: 447-451.

Fieber LA (1998). Ionic currents in normal and neurofibromatosis type 1-affected human Schwann cells: induction of tumor cell K current in normal Schwann cells by cyclic AMP. *J Neurosci Res* 54: 495-506.

Flintoff WF, Bahuau M, Lyonnet S, Gilgenkrantz S, Lacombe D, Marcon F, Levilliers J, Kachaner J, Munnich A, Le Merrer M (1993). No evidence for linkage to the type 1 or type 2 neurofibromatosis loci in Noonan syndrome families. *Am J Med Genet* 46: 700-705.

Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D (1991).Light-directed, spatially addressable parallel chemical synthesis. *Science* 251: 767-773.

Forbes SH, Dorschner MO, Le R, Stephens K (2004). Genomic context of paralogous recombination hotspots mediating recurrent *NF1* region microdeletion. *Genes Chromosomes Cancer* 41: 12-25.

Frahm S, Mautner VF, Brems H, Legius E, Debiec-Rychter M, Friedrich RE, Knofel WT, Peiper M, Kluwe L (2004). Genetic and phenotypic characterization of tumor cells derived from malignant peripheral nerve sheath tumors of neurofibromatosis type 1 patients. *Neurobiol Dis* 16: 85-91.

Frank-Raue K, Hoppner W, Frilling A, Kotzerke J, Dralle H, Haase R, Mann K, Seif F, Kirchner R, Rendl J, Deckart HF, Ritter MM, Hampel R, Klempa J, Scholz GH, Raue F (1996). Mutations of the ret protooncogene in German multiple endocrine neoplasia families: relation between genotype and phenotype. German Medullary Thyroid Carcinoma Study Group. *J Clin Endocrinol Metab.* 81:1780-1783.

Friedman JM (2002). Neurofibromatosis 1: clinical manifestations and diagnostic criteria. *J Child Neurol* 17: 548-554.

Friedman JM, Birch PH (1997). Type 1 neurofibromatosis: a descriptive analysis of the disorder in 1,728 Patients. *Am J Med Genet* 70: 138–143.

Friedman JM, Riccardi VM (1999). Clinical and epidemiological features. In: Neurofibromatosis: phenotype, natural history and pathogenesis (Friedman J, Gutmann D, MacCollin M, Riccardi VM, eds), 3rd edn. Baltimore: John Hopkins University Press,: 29–86.

Fujimura T, Terui T, Kusaka Y, Tagami H (2004). Neurofibromatosis 1 associated with an intracranial artery abnormality, Moyamoya disease and bilateral congenital large hairy pigmented macules. *Br J Dermatol* 150: 611–613.

Gaasterland T, Bekiranov S (2000). Making the most of microarray data. *Nat Genet* 24: 204-206.

Gallinger S, Aronson M, Shayan K, Ratcliffe EM, Gerstle JT, Parkin PC, Rothenmund H, Croitoru M, Baumann E, Durie PR, Weksberg R, Pollett A, Riddell RH, Ngan BY, Cutz E, Lagarde AE, Chan HS (2004). Gastrointestinal cancers and neurofibromatosis type 1 features in children with a germline homozygous MLH1 mutation. *Gastroenterology* 126: 576-585.

Gammel JA., (1931). Localized neurofibromatosis. *Arch Dermatol* 24: 712-713.

Ganguly A, Rock MJ, Prockop DJ (1993). Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and

DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci U S A* 90: 10325-10329.

Gasparini P, D'Agruma L, Pio de Cillis G, Balestrazzi P, Mingarelli R, Zelante L (1996). Scanning the first part of the neurofibromatosis type 1 gene by RNA-SSCP: identification of three novel mutations and of two new polymorphisms. *Hum Genet* 97: 492-495.

Gasparini P, Grifa A, Origone P, Coviello D, Antonacci R, Rocchi M (1993).Detection of a neurofibromatosis type I (*NF1*) homologous sequence by PCR: implications for the diagnosis and screening of genetic diseases. *Mol Cell Probes* 7: 415-418.

Gaul U, Mardon G, Rubin GM (1992). A putative Ras GTPase activating protein acts as a negative regulator of signaling by the Sevenless receptor tyrosine kinase. *Cell* 68: 1007-1019.

Geiss GK, Bumgarner RE, An MC, Agy MB, van 't Wout AB, Hammersmark E, Carter VS, Upchurch D, Mullins JI, Katze MG (2000). Large-scale monitoring of host cell gene expression during HIV-1 infection using cDNA microarrays. *Virology* 266: 8-16.

Gervasini C, Venturin M, Orzan F, Friso A, Clementi M, Tenconi R, Larizza L, Riva P (2005). Uncommon Alu-mediated NF1 microdeletion with a breakpoint inside the NF1 gene. *Genomics* 85: 273-279.

Gille JJ, Hogervorst FB, Pals G, Wijnen JT, van Schooten RJ, Dommering CJ, Meijer GA, Craanen ME, Nederlof PM, de Jong D, McElgunn CJ, Schouten JP, Menko FH (2002. Genomic deletions of MSH2 and MLH1 in colorectal cancer families detected by a novel mutation detection approach. *Br J Cancer* 87:892-897.

Ginsberg SD, Hemby SE, Lee VM, Eberwine JH, Trojanowski JQ (2000). Expression profile of transcripts in Alzheimer's disease tangle-bearing CA1 neurons. *Ann Neurol* 48: 77-87.

Girodon-Boulandet E, Pantel J, Cazeneuve C, Gijn MV, Vidaud D, Lemay S, Martin J, Zeller J, Revuz J, Goossens M, Amselem S, Wolkenstein P (2000). *NF1* gene analysis focused on CpG-rich exons in a cohort of 93 patients with neurofibromatosis type 1. *Hum Mutat* 16: 274-275.

Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD, Lander ES (1999).Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 286: 531-537.

Golubic M, Harwalkar JA, Bryant SS, Sundaram V, Jove R, Lee JH (1998). Differential regulation of neurofibromin and p120 GTPase-activating protein by nutritionally relevant fatty acids. *Nutr Cancer* 30: 97-107.

Gonzalez-Martin J, Glover S, Dixon S, Fryer A, Carty H, Smith C, Kaye SB, Verbov J (2000). Neurofibromatosis type 1 and McCune-Albright syndrome occurring in the same patient. *Br J Dermatol* 143: 1288-1291.

Gott JM, Emeson RB (2000). Functions and mechanisms of RNA editing. *Annu Rev Genet* 34: 499-531.

Gottlieb B, Beitel LK, Trifiro MA (2001). Comment in: *Trends Genet* 17: 627-628. Somatic mosaicism and variable expressivity. *Trends Genet* 17: 79-82.

Gregory PE, Gutmann DH, Mitchell A, Park S, Boguski M, Jacks T, Wood DL, Jove R, Collins FS (1993). Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. *Somat Cell Mol Genet* 19: 265-274.

Groves C, Lamlum H, Crabtree M, Williamson J, Taylor C, Bass S, Cuthbert-Heavens D, Hodgson S, Phillips R, Tomlinson I (2002). Mutation cluster region, association between germline and somatic mutations and genotype-phenotype correlation in upper gastrointestinal familial adenomatous polyposis. *Am J Pathol.* 160: 2055-2061.

Gross E, Arnold N, Goette J, Schwarz-Boeger U, Kiechle M (1999). A comparison of *BRCA1* mutational analysis by direct sequencing, SSCP and DHPLC. *Hum Genet* 66:780-818.

Guo HF, The I, Hannan F, Bernards A & Zhong Y (1997). Requirement of *Drosophila* NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. *Science* 276: 795-798.

Guo HF, Tong J, Hannan F, Luo L, Zhong Y (2000). A neurofibromatosis-1-regulated pathway is required for learning in Drosophila. *Nature* 403: 895-898.

Gould SR (1991). Hamartomatous rectal polyps are common in tuberous sclerosis. *Ann N Y Acad Sci* 615: 71-80.

Guipponi M, Herbert S, Toh MY, Poetter K, Forrest S, Scott HS (2005).Universal fluorescent labeling of PCR products for DHPLC analysis: reducing cost and increasing sample throughput. *Biotechniques* 39: 34, 36, 38, 40.

Guran S, Safali M (2005).A case of neurofibromatosis and breast cancer: loss of heterozygosity of NF1 in breast cancer. *Cancer Genet Cytogenet* 156: 86-88.

Gutmann DH (2001). The neurofibromatoses: When less is more. *Hum Mol Genet* 10: 747-755.

Gutmann DH, Aylsworth A, Carey JC, Korf B, Marks J, Pyeritz RE, Rubenstein A, Viskochil D (1997). The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. *JAMA* 278: 51–57.

Gutmann DH, Collins FS (2001). Neurofibromatosis 1. In: The Metabolic and Molecular Bases of Inherited Disease. 8th edn. CR Scriver , AL Beaudet, WS Sly, DV Alle (eds.) Mc Graw Hill. New York, pp. 877-896.

Gutmann DH, Collins FS (1993). Recent progress toward understanding the molecular biology of von Recklinghausen neurofibromatosis. *Ann Neurol* 31: 555-561.

Gutmann DH, Geist RT, Rose K, Wright DE (1995). Expression of two new protein isoforms of the neurofibromatosis type 1 gene product, neurofibromin, in muscle tissues. *Dev Dyn* 202: 302-311.

Gutmann DH, Hedrick NM, Li J, Nagarajan R, Perry A, Watson MA (2002). Comparative gene expression profile analysis of neurofibromatosis 1-associated and sporadic pilocytic astrocytomas. *Cancer Res* 62: 2085-2091.

Gutmann DH, Winkeler E, Kabbarah O, Hedrick N, Dudley S, Goodfellow PJ, Liskay RM (2003). Mlh1 deficiency accelerates myeloid leukemogenesis in neurofibromatosis 1 (*Nf1*) heterozygous mice. *Oncogene.* 22: 4581-4585.

Gutmann DH, Zhang Y, Hirbe A (1999). Developmental regulation of a neuron-specific neurofibromatosis 1 isoform. *Ann Neurol* 46: 777-782.

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999).Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 19: 1720-1730.

Haab BB (2001). Advances in protein microarray technology for protein expression and interaction profiling. *Curr Opin Drug Discov Devel* 4: 116-123

Habib AA, Gulcher JR, Hognason T, Zheng L, Stefansson K (1998). The OMgp gene, a second growth suppressor within the NF1 gene. *Oncogene* 16:1525-1531.

Haeussler J, Haeusler J, Striebel AM, Assum G, Vogel W, Furneaux H, Krone W (2000). Tumour antigen HuR binds specifically to one of five protein-binding segments in the 3'-untranslated region of the neurofibromin messenger RNA. *Biochem Biophys Res Commun* 267: 726-732.

Hager CM, Cohen PR, Tschen JA (1997). Segmental neurofibromatosis: Case reports and review. *J Am Acad Dermatol* 37: 864–869.

Hajra A, Martin-Gallardo A, Tarle SA, Freedman M, Wilson-Gunn S, Bernards A, Collins FS (1994). DNA sequences in the promoter region of the *NF1* gene are highly conserved between human and mouse. *Genomics* 21: 649-52.

Hakimi MA, Speicher DW, Shiekhattar R (2002). The motor protein kinesin-1 links neurofibromin and merlin in a common cellular pathway of neurofibromatosis. *J Biol Chem* 277: 36909-36912.

Hall JG (1988). Review and hypotheses: somatic mosaicism: observations related to clinical genetics. *Am J Hum Genet* 43: 355-363.

Hall JG & Byers PH (1987). Genetics of tuberous sclerosis. *Lancet* 1: 751.

Halling KC, Scheithauer BW, Halling AC, Nascimento AG, Ziesmer SC, Roche PC, Wollan PC (1996). p53 expression in neurofibroma and malignant peripheral nerve sheath tumuor. An immunohistochemical study of sporadic and NF1-associated tumours. *Am J Clin Pathol* 106: 282-288.

Han S, Cooper DN, Upadhyaya M. (2001) Evaluation of denaturing high performance liquid chromatography (DHPLC) for the mutational analysis of the neurofibromatosis type 1 (*NF1*) gene. *Hum Genet* 109: 487-497.

Han JW, McCormick F, Macara IG (1991). Regulation of Ras-GAP and the neurofibromatosis-1 gene product by eicosanoids. *Science* 252: 576-579.

Happle R (2000). Principles of genetics, mosaicism and molecular biology. In Harper J, Oranje JM, Rose M, eds. *Textbook of Pediatric Dermatology*. Vol.2. Oxford: Blackwell Science: pp1037-1056.

Harder A, Rosche M, Reuss DE, Holtkamp N, Uhlmann K, Friedrich R, Mautner VF, von Deimling A (2004). Methylation analysis of the neurofibromatosis type 1 (*NF1*) promoter in peripheral nerve sheath tumours. *Eur J Cancer* 40: 2820-2828.

Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM (2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the *H19/Igf2* locus. *Nature* 405: 486–489.

Hatta N, Horiuchi T, Fujita S (1994). Analysis of *NF1* gene mutations in neurofibromatosis type 1 patients in Japan. *Biochem Biophys Res Commun* 199: 207-212.

Hatta N, Horiuchi T, Watanabe I, Kobayashi Y, Shirakata Y, Ohtsuka H, Minami T, Ueda K, Kokoroishi T, Fujita S (1995). *NF1* gene mutations in Japanese with neurofibromatosis 1 (NF1). *Biochem Biophys Res Commun* 212: 697-704.

Hattori S, Ohmi N, Maekawa M, Hoshino M, Kawakita M, Nakamura S (1991). Antibody against neurofibromatosis type 1 gene product reacts with a triton-insoluble GTPase activating protein toward ras p21. *Biochem Biophys Res Commun* 177: 83-89.

Hayashi K, Yandell DW (1993). How sensitive is PCR-SSCP? *Hum Mutat* 2: 338-346.

Hazan J, Seboun E, Lathrop M, Gyapay G, Morissette J, Weissenbach J (1996). A comprehensive genetic map of the human genome based on 5,264 microsatellites. Comment in: *Nature*. 1996 380: 111-112. *Nature* 380: 152-154.

Heim RA, Kam-Morgan LNW, Binnie CG, Corns DD, Cayouette MC, Farber RA, Aylsworth AS, Silverman LM, Luce M C (1995). Distribution of 13 truncating mutations in the neurofibromatosis gene. *Hum Mol Genet* 4: 975-981.

Henkemeyer M, Rossi DJ, Holmyard DP, Puri MC, Mbamalu G, Harpal K, Shih TS, Jacks T, Pawson T (1995). Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein. *Nature* 377: 695-701.

Herman JG, Baylin SB (2003). Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 349: 2042–2054.

Hiatt KK, Ingram DA, Zhang Y, Bollag G, ClappDW (2001). Neurofibromin GTPase-activating protein-related domains restore normal growth in NF1$^{-/-}$ cells. *J Biol Chem* 276: 7240–7245.

Hix K, Rothner D, Cohen B (1998). Segmental neurofibromatosis: analysis of 14 cases. *Ann Neurol* 44: 575–576.

Hoffmeyer S, Assum G, Griesser J, Kaufmann D, Nurnberg P, Krone W (1995). On unequal allelic expression of the neurofibromin gene in neurofibromatosis type 1. *Hum Mol Genet* 4: 1267-1272.

Hoffmeyer S, Assum G, Kaufmann D, Krone W (1994a). Unequal expression of *NF1* alleles. *Nat Genet* 6: 331.

Hoffmeyer S, Assum G, Kaufmann D, Schwenk K, Krone W (1994b). A deletion in the 5'-region of the neurofibromatosis type 1 (*NF1*) gene. *Hum Genet* 94: 97-100.

Hoffmeyer S, Nurnberg P, Ritter H, Fahsold R, Leistner W, Kaufmann D, Krone W (1998). Nearby stop codons in exons of the neurofibromatosis type 1 gene are disparate splice effectors. *Am J Hum Genet* 62: 269-277.

Holtkamp N, Reuss DE, Atallah I, Kuban RJ, Hartmann C, Mautner VF, Frahm S, Friedrich RE, Algermissen B, Pham VA, Prietz S, Rosenbaum T, Estevez-Schwarz L, von Deimling A (2004). Subclassification of nerve sheath tumors by gene expression profiling. *Brain Pathol* 14: 258-264.

Hollox EJ, Atia T, Cross G, Parkin T, Armour JA (2002). High throughput screening of human subtelomeric DNA for copy number changes using multiplex amplifiable probe hybridisation (MAPH). *J Med Genet* 39: 790-795.

Holmgren C, Kanduri C, Dell G, Ward A, Mukhopadhya R, Kanduri M, Lobanenkov V, Ohlsson R (2001). CpG methylation regulates the *Igf2/H19* insulator. *Curr Biol* 11: 1128–1130.

Horan MP, Cooper DN, Upadhyaya M (2000). Hypermethylation of the neurofibromatosis type 1 (*NF1*) gene promoter is not a common event in the inactivation of the NF1 gene in NF1-specific tumours. *Hum Genet* 107: 33-39.

Horan MP, Osborn M, Cooper DN, Upadhyaya M. (2004) Functional analysis of polymorphic variation within the promoter and 5' untranslated region of the neurofibromatosis type 1 (*NF1*) gene. *Am J Med Genet* 131: 227-231.

Horiuchi T, Hatta N, Watanabe I, Kobayashi Y, Wallace MR, Shirakata Y, Ohtsuka H, Fujita S (1998). Identification of two novel mutations (1448delA and Q682X) in the *NF1* gene and analysis for nonsense mutations in patients with neurofibromatosis type 1. *Hum Mutat* Suppl 1: S47-49.

Horn D, Robinson PN, Boddrich A, Buske A, Tinschert S, Nurnberg P (1996). Three novel mutations of the *NF1* gene detected by temperature gradient gel electrophoresis of exons 5 and 8. *Electrophoresis* 17: 1559-1563.

Hsueh YP, Roberts AM, Volta M, Sheng M, Roberts RG (2001).Bipartite interaction between neurofibromatosis type I protein (neurofibromin)and syndecan transmembrane heparan sulfate proteoglycans. *J Neurosci* 21: 3764-3770.

Hudson J, Wu CL, Tassabehji M, Summers EM, Simon S, Super M, Donnai D, Thakker N (1997). Novel and recurrent mutations in the neurofibromatosis type 1 (*NF1*) gene. *Hum Mutat* 9: 366-367.

Hulsebos TJ, Bijleveld EH, Riegman PH, Smink LJ, Dunham I (1996). Identification and characterization of NF1-related loci on human chromosomes 22,14 and 2. *Hum Genet* 98: 7-11.

Huson SM, Compston DA, Clark P, Harper PS (1989a). A genetic study of von Recklinghausen neurofibromatosis in south east Wales. I. Prevalence, fitness, mutation rate, and effect of parental transmission on severity. *J Med Genet* 26: 704-711.

Huson SM, Compston DA, Harper PS (1989b). A genetic study of von Recklinghausen neurofibromatosis in south east Wales. II. Guidelines for genetic counselling. *J Med Genet* 26: 712-721.

Huson SM, Harper PS, Compston DAS (1988). Von Recklinghausen neurofibromatosis: a clinical and population study in South East Wales. *Brain* 111: 1355-1381.

Huson SM, Hughes RAC (1994). *The Neurofibromatoses: Pathogenic and Clinical Overview*. London: Chapman and Hall.

Huson SM, Ruggieri M (2000). The neurofibromatoses. In: Harper J, Oranje JM, Rose M, eds. Textbook of pediatric dermatology.Vol II. Oxford: Blackwell Science:1204–1224.

Hutter P, Antonarakis SE, Delozier-Blanchet CD, Morris MA (1994). Exon skipping associated with A-->G transition at +4 of the IVS33 splice donor site of the neurofibromatosis type 1 (*NF1*) gene. *Hum Mol Genet* 3: 663-665.

Ikeda H, Sasaki T, Yoshimoto T, Fukui M, Arinami T (1999). Mapping of a familial moyamoya disease gene to chromosome 3p24.2-p26. *Am J Hum Genet* 64: 533-537.

Ikezaki K, Han DH, Kawano T, Kinukawa N, Fukui M (1997). A clinical comparison of definite moyamoya disease between South Korea and Japan. *Stroke.* 28: 2513-2517.

Imokawa G (2004). Autocrine and paracrine regulation of melanocytes in human skin and in pigmentary disorders. *Pigment Cell Res* 17: 96-110.

Inagaki H, Ohye T, Kogo H, Yamada K, Kowa H, Shaikh TH, Emanuel BS, Kurahashi H (2005). Palindromic AT-rich repeat in the NF1 gene is hypervariable in humans and evolutionarily conserved in primates. *Hum Mutat* 26: 332-342.

Ingordo V, D'Andria G, Mendicini S, Grecucci M, Baglivo A (1995). Segmental neurofibromatosis: is it uncommon or underdiagnosed ? *Arch Dermatol* 131: 959–960.

Ingram DA, Yang FC, Travers JB, Wenning MJ, Hiatt K, New S, Hood A, Shannon K, Williams DA, Clapp DW (2000). Genetic and biochemical evidence that haploinsufficiency of the Nf1 tumour suppressor gene modulates melanocyte and mast cell fates in vivo. *J Exp Med* 191: 181-188.

Inoki K, Corradetti MN, Guan KL (2005). Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 37: 19-24.

Inoki K, Li Y, Zhu T, Wu J, Guan KL (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 4: 648-657.

Inoue K, Lupski JR (2002). Molecular mechanisms for genomic disorders. *Annu Rev Genomics Hum Genet* 3: 199-242.

Inoue K, Osaka H, Thurston VC, Clarke JTR, Yoneyama A, Rosenbarker L, Bird TD, Hodes ME, Shaffer LG, Lupski JR (2002). Genomic rearrangements resulting in *PLP1* deletion occur by nonhomologous end joining and cause different dysmyelinating phenotypes in males and females. *Am J Hum Genet* 71: 838–853.

Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA (1994). Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. *Nat Genet* 7: 353-361.

Jenne DE, Tinschert S, Dorschner MO, Hameister H, Stephens K, Kehrer-Sawatzki H (2003). Complete physical map and gene content of the human NF1 tumor suppressor region in human and mouse. *Genes Chromosomes Cancer* 37: 111-120.

Jenne DE, Tinschert S, Reimann H, Lasinger W, Thiel G, Hameister H, Kehrer-Sawatzki H (2001). Molecular characterization and gene content of breakpoint boundaries in patients with neurofibromatosis type 1 with 17q11.2 microdeletions. *Am J Hum Genet* 69: 516-527.

Jenne DE, Tinschert S, Stegmann E, Reimann H, Nurnberg P, Horn D, Naumann I, Buske A, Thiel G (2000). A common set of at least 11 functional genes is lost in the majority of NF1 patients with gross deletions. *Genomics* 66: 93-97.

Jessel TM (1988). Adhesion molecules and the hierarchy of neural development. *Neuron* 1: 3–13.

Ji, Y., Eichler, E. E., Schwartz, S. & Nicholls, R. D (2000). Structure of chromosomal duplicons and their role in mediating human genomic disorders. *Genome Res* 10: 597–610.

Johannessen CM, Reczek EE, James MF, Brems H, Legius E, Cichowski K (2005). The *NF1* tumour suppressor critically regulates *TSC2* and mTOR. *Proc Natl Acad Sci U S A* 102: 8573-8578.

John AM, Ruggieri M, Ferner R, Upadhyaya M (2000). A search for evidence of somatic mutations in the NF1 gene. *J Med Genet* 37: 44-49.

Johnson MR, DeClue JE, Felzmann S, Vass WC, Xu G, White R & Lowy DR (1994). Neurofibromin can inhibit Ras-dependent growth by a mechanism independent of its GTPase accelerating function. *Mol Cell Biol* 14: 641-645.

Johnson MR, Ferner RE, Bobrow M, Hughes RA (2000). Detailed analysis of the oligodendrocyte myelin glycoprotein gene in four patients with neurofibromatosis 1 and primary progressive multiple sclerosis. *J Neurol Neurosurg Psychiatry* 68: 643-646.

Jones AC, Sampson JR, Cheadle JP (2001). Low level mosaicism detectable by DHPLC but not by direct sequencing. *Hum Mutat* 17: 233-234.

Jones AC, Shyamsundar MM, Thomas MW, Maynard J, Idziaszczyk S, Tomkins S, Sampson JR, Cheadle JP (1999).Comprehensive mutation analysis of TSC1 and TSC2- and phenotypic correlations in 150 families with tuberous sclerosis.*Am J Hum Genet* 64: 1305-1315.

Jones PA, Baylin SB (2002). The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 3: 415–428.

Joseph D. Andrews, Debora N. Mancini, Shiva M. Singh and David I. Rodenhiser (1996). Site and sequence specific DNA methylation in the neurofibromatosis (*NF1*) gene includes C5839T: the site of the recurrent substitution mutation in exon 31. *Hum Mol Genet* 5: 503–507.

Joslyn G, Carlson M, Thliveris A, Albertsen H, Gelbert L, Samowitz W, Groden J, Stevens J, Spirio L, Robertson M, *et al* (1991). Identification of deletion mutations and three new genes at the familial polyposis locus. *Cell* 66: 601-613.

Jurgens B, Schmitz-Drager BJ, Schulz WA (1996). Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. *Cancer Res* 56: 5698–703.

Juyal RC, Kuwano A, Kondo I, Zara F, Baldini A, Patel PI (1996). Mosaicism for del(17)(p11.2p11.2) underlying the Smith-Magenis syndrome. *Am J Med Genet* 66: 193-196.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D (1992).Comparative genomic hybridization for molecular cytogenetic analysis of solid tumours. *Science* 258: 818-821.

Kammula US, Marincola FM, Rosenberg SA (2000). Real-time quantitative polymerase chain reaction assessment of immune reactivity in melanoma patients after tumor peptide vaccination. *J Natl Cancer Inst* 92: 1336-1344.

Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, Jessup JM, Kolodner R (1997). Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res* 57: 808–11.

Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, Tatematsu M, Ushijima T (2004). Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. *Cancer Sci* 95: 58–64.

Kasprzak L, Der Kaloustian VM, Elliott AM, Shevell M, Lejtenyi C, Eydoux P (1998).Deletion of 22q11 in two brothers with different phenotype. *Am J Med Genet* 75: 288-291.

Kaufmann D, Bartelt B, Hoffmeyer S, Muller R (1999). Posttranslational regulation of neurofibromin content in melanocytes of neurofibromatosis type 1 patients. *Arch Dermatol Res* 291: 312-317.

Kaufmann D, Muller R, Bartelt B, Wolf M, Kunzi-Rapp K, Hanemann CO, Fahsold R, Hein C, Vogel W, Assum G (2001). Spinal neurofibromatosis without café-au-lait macules in two families with null mutations of the *NF1* gene. *Am J Hum Genet.* 69: 1395-1400.

Kayes LM, Burke W, Riccardi VM, Bennett R, Ehrlich P, Rubenstein A, Stephens K (1994). Deletions spanning the neurofibromatosis 1 gene: identification and phenotype of five patients. *Am J Hum Genet* 54: 424-436.

Kayes LM, Riccardi VM, Burke W, Bennett RL, Stephens K (1992). Large *de novo* DNA deletion in a patient with sporadic neurofibromatosis, mental retardation and dysmorphism. *J Med Genet* 29: 686-690.

Keen J, Lester D, Inglehearn C, Curtis A, Bhattacharya S (1991). Rapid detection of single base mismatches as heteroduplexes on Hydrolink gels. *Trends Genet* 7: 5.

Kehrer-Sawatzki H, Haussler J, Krone W, Bode H, Jenne DE, Mehnert KU, Tummers U, Assum G (1997a).The second case of a t(17;22) in a family with neurofibromatosis type 1: sequence analysis of the breakpoint regions. *Hum Genet* 99: 237-247.

Kehrer-Sawatzki H, Kluwe L, Funsterer C, Mautner VF (2005). Extensively high load of internal tumours determined by whole body MRI scanning in a patient with neurofibromatosis type 1 and a non-LCR-mediated 2-Mb deletion in 17q11.2. *Hum Genet* 116: 466-475.

Kehrer-Sawatzki H, Kluwe L, Sandig C, Kohn M, Wimmer K, Krammer U, Peyrl A, Jenne DE, Hansmann I, Mautner VF (2004). High frequency of mosaicism among patients with neurofibromatosis type 1 (NF1) with microdeletions caused by somatic recombination of the JJAZ1 gene. *Am J Hum Genet* 75: 410-423.

Kehrer-Sawatzki H, Maier C, Moschgath E, Elgar G, Krone W (1998).Genomic characterization of the Neurofibromatosis Type 1 gene of *Fugu rubripes*. *Gene* 222: 145-153.

Kehrer-Sawatzki H, Schwickardt T, Assum G, Rocchi M, Krone W (1997b). A third neurofibromatosis type 1 (*NF1*) pseudogene at chromosome 15q11.2. *Hum Genet* 100: 595-600.

Kehrer-Sawatzki H, Tinschert S, Jenne DE (2003). Heterogeneity of breakpoints in non-LCR-mediated large constitutional deletions of the 17q11.2 NF1 tumour suppressor region. *J Med Genet* 40: e116.

Kell DB, King RD (2000). On the optimization of classes for the assignment of unidentified reading frames in functional genomics programmes: the need for machine learning. *Trends Biotechnol* 18: 93-98.

Kim HA, Ratner N, Roberts TM, Stiles CD (2001). Schwann cell proliferative responses to cAMP and Nf1 are mediated by cyclin D1. *J Neurosci* 21: 1110-1116.

Kim HA, Rosenbaum T, Marchionni MA, Ratner N, DeClue JE (1995). Schwann cells from neurofibromin deficient mice exhibit activation of p21ras, inhibition of cell proliferation and morphological changes. *Oncogene* 11: 325-335.

Kindblom LG, Ahlden M, Meis-Kindblom JM, Stenman G (1995). Immunohistochemical and molecular analysis of p53, MDM2, proliferating cell nuclear antigen and Ki67 in benign and malignant peripheral nerve sheath tumours. *Virchows Arch* 427: 19-26.

Kiyosawa H, Lensch MW, Chance PF (1995). Analysis of the CMT1A-REP repeat: mapping crossover breakpoints in CMT1A and HNPP. *Hum Mol Genet* 4: 2327-2334.

Klose A, Ahmadian MR, Schuelke M, Scheffzek K, Hoffmeyer S, Gewies A, Schmitz F, Kaufmann D, Peters H, Wittinghofer A, Nurnberg P (1998a). Selective disactivation

of neurofibromin GAP activity in neurofibromatosis type 1. *Hum Mol Genet* 7: 1261-1268.

Klose A, Robinson N, Gewies A, Kluwe L, Kaufmann D, Buske A, Tinschert S, Peters H (1998b). Two novel mutations in exons 19a and 20 and a BsaBI [correction of BsaI] polymorphism in a newly characterized intron of the neurofibromatosis type 1 gene. *Hum Genet* 102: 367-371.

Kluwe L, Friedrich RE, Korf B, Fahsold R, Mautner VF (2002). NF1 mutations in neurofibromatosis 1 patients with plexiform neurofibromas. *Hum Mutat* 19: 309.

Kluwe L, Friedrich R, Mautner VF (1999). Loss of *NF1* allele in Schwann cells but not in fibroblasts derived from an NF1-associated neurofibroma. *Genes Chromosomes Cancer* 24: 283–285.

Kluwe L, Friedrich RE, Peiper M, Friedman J, Mautner VF (2003a). Constitutional NF1 mutations in neurofibromatosis 1 patients with malignant peripheral nerve sheath tumours. *Hum Mutat* 22: 420.

Kluwe L, Nygren AO, Errami A, Heinrich B, Matthies C, Tatagiba M, Mautner V (2005). Screening for large mutations of the NF2 gene. *Genes Chromosomes Cancer* 42: 384-391.

Kluwe L, Tatagiba M, Fünsterer C, Mautner VF (2003b). NF1 mutations and clinical spectrum in patients with spinal neurofibromas. *J Med Genet* 40: 368–371.

Knudson AG Jr (1971). Mutation and cancer: Statistical study of retinoblastoma. Proc *Natl Acad Sci USA* 68: 820–823.

Kocer U, Uysal A, Sungur N, Karaaslan O, Kankaya Y, Tiftikcioglu Y (2003). Familial neurofibromatosis-1 and Gilbert syndrome. *Dermatol Surg* 29: 759-765.

Koestenberger M, Kroisel PM, Lackner H, Urban C (2003). Simultaneous occurrence of retinoblastoma and neurofibromatosis I in a young child. *Med Pediatr Oncol* 40: 124-125.

Korf BR (1992). Diagnostic outcome in children with multiple café au lait spots. *Pediatrics*. 90: 924-927.

Korf BR (1998). The *NF1* Genetic Analysis Consortium. Chap. 4 in: Upadhayaya M, Cooper DN (eds) *Neurofibromatosis Type 1: from Genotype to Phenotype*. BIOS Scientific, Oxford, pp 57-62.

Korf BR (1999). Plexiform neurofibromas. *Am J Med Genet* 89: 31-37.

Kourea HP, Cordon-Cardo C, Dudas M, Leung D, Woodruff JM (1999). Expression of p27-Kip and other cell cycle regulators in malignant peripheral nerve sheath tumours and neurofibromas: the emerging role of p27-Kip in malignant transformation of neurofibromas. *Am J Pathol* 155: 1885–1891.

Krkljus S, Abernathy CR, Johnson JS, Williams CA, Driscoll DJ, Zori R, Stalker HJ, Rasmussen SA, Collins FS, Kousseff BG, Baumbach L, Wallace MR (1998). Analysis of CpG C-to-T mutations in neurofibromatosis type 1. Mutations in brief no. 129. *Hum Mutat* 11: 411.

Kukita Y, Tahira T, Sommer SS, Hayashi K (1997). SSCP analysis of long DNA fragments in low pH gel. *Hum Mutat* 10: 400-407.

Kurahashi H, Emanuel BS (2001). Long AT-rich palindromes and the constitutional t(11;22) breakpoint. *Hum Mol Genet* 10: 2605-2617.

Kurahashi H, Shaikh T, Takata M, Toda T, Emanuel BS (2003). The constitutional t(17;22): another translocation mediated by palindromic AT-rich repeats. *Am J Hum Genet* 72: 733-738.

Lakich D, Kazazian HH Jr, Antonarakis SE, Gitschier J (1993). Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. *Nat Genet*. 5: 236-241.

Lakkis MM, Epstein JA (1998). Neurofibromin modulation of ras activity is required for normal endocardial-mesenchymal transformation in the developing heart. *Development* 125: 4359-4367.

Lakkis MM, Golden JA, O'Shea KS, Epstein JA (1999). Neurofibromin deficiency in mice causes exencephaly and is a modifier for Splotch neural tube defects. *Dev Biol* 212: 80-92.

Lalic T, Vossen RH, Coffa J, Schouten JP, Guc-Scekic M, Radivojevic D, Djurisic M, Breuning MH, White SJ, den Dunnen JT (2005).Deletion and duplication screening in the DMD gene using MLPA. *Eur J Hum Genet* Jul 20; (Epub ahead of print).

Latil A, Bieche I, Chene L, Laurendeau I, Berthon P, Cussenot O, Vidaud M (2003). Gene expression profiling in clinically localized prostate cancer: a four-gene expression model predicts clinical behavior. *Clin Cancer Res* 9: 5477-5485.

Lau N, Feldkamp MM, Roncari L, Loehr AH, Shannon P, Gutmann DH, Guha A (2000). Loss of neurofibromin is associated with activation of RAS/MAPK and PI3-K/AKT signaling in a neurofibromatosis 1 astrocytoma. *J Neuropathol Exp Neurol* 59: 759-767.

Lazaro C, Gaona A, Lynch M, Kruyer H, Ravella A, Estivill X (1995a).Molecular characterization of the breakpoints of a 12-kb deletion in the NF1 gene in a family showing germ-line mosaicism. *Am J Hum Genet* 57: 1044-1049.

Lazaro C, Gaona A, Ainsworth P, Tenconi R, Vidaud D, Kruyer H, Ars E, Volpini V, Estivill X (1996). Sex differences in mutational rate and mutational mechanism in the *NF1* gene in neurofibromatosis type 1 patients. *Hum Genet.* 98: 696-699.

Lazaro C, Gaona A, Ravella A, Volpini V, Casals T, Fuentes JJ, Estivill X (1993). Novel alleles, hemizygosity and deletions at an Alu-repeat within the neurofibromatosis type 1 (*NF1*) gene. *Hum Mol Genet* 2: 725-730.

Lazaro C, Kruyer H, Gaona A, Estivill X (1995b). Two further cases of mutation R1947X in the *NF1* gene: screening for a relatively common recurrent mutation. *Hum Genet* 96: 361-363.

Lazaro C, Ravella A, Gaona A, Volpini V, Estivill X (1994). Neurofibromatosis type 1 due to germ-line mosaicism in a clinically normal father. *New Eng J Med* 331: 1403-1407.

Ledbetter DH, Rich DC, O'Connell P, Leppert M, Carey JC (1989). Precise localization of NF1 to 17q11.2 by balanced translocation. *Am J Hum Genet* 44: 20-25.

Lee TC, Sung ML, Chen JS (1994). Tuberous sclerosis associated with neurofibromatosis: report of a case. *J Formos Med Assoc* 93: 797-801.

Legius E, Hall BK, Wallace MR, Collins FS, Glover TW (1994). Ten base pair duplication in exon 38 of the *NF1* gene. *Hum Mol Genet* 3: 829-830.

Legius E, Marchuk DA, Collins FS, Glover TW (1993). Somatic deletion of the neurofibromatosis type 1 gene in a neurofibrosarcoma supports a tumor suppressor gene hypothesis. *Nat Genet* 3: 122–126.

Legius E, Marchuk DA, Hall BK, Andersen LB, Wallace MR, Collins FS, Glover TW (1992). NF1-related locus on chromosome 15. *Genomics* 13: 1316-1318.

Legius E, Wu R, Eyssen M, Marynen P, Fryns JP, Cassiman JJ (1995). Encephalocraniocutaneous lipomatosis with a mutation in the NF1 gene. *J Med Genet* 32: 316-319.

Leppig KA, Kaplan P, Viskochil D, Weaver M, Ortenberg J, Stephens K (1997). Familial neurofibromatosis 1 microdeletions: cosegregation with distinct facial phenotype and early onset of cutaneous neurofibromata. *Am J Med Genet* 73: 197-204.

Leppig KA, Viskochil D, Neil S, Rubenstein A, Johnson VP, Zhu XL, Brothman AR, Stephens K (1996). The detection of contiguous gene deletions at the neurofibromatosis 1 locus with fluorescence in situ hybridization. *Cytogenet Cell Genet* 72: 95-98.

Leuer M, Oldenburg J, Lavergne JM, Ludwig M, Fregin A, Eigel A, Ljung R, Goodeve A, Peake I, Olek K (2001). Somatic mosaicism in hemophilia A: a fairly common event. *Am J Hum Genet* 69: 75-87.

Levine AJ (1993). The tumour suppressor genes. *Annu Rev Biochem* 62: 623-651.

Lewis RA, Riccardi VM (1981). Von Recklinghausen neurofibromatosis: prevalence of iris hamartoma. *Ophthalmology* 88: 348-354.

Lewis S, Akgun E, Jasin M (1999). Palindromic DNA and genome stability. Further studies. *Ann N Y Acad Sci* 870: 45-57.

Levy P, Vidaud D, Leroy K, Laurendeau I, Wechsler J, Bolasco G, Parfait B, Wolkenstein P, Vidaud M, Bieche I (2004a). Molecular profiling of malignant peripheral nerve sheath tumors associated with neurofibromatosis type 1, based on large-scale real-time RT-PCR. *Mol Cancer* 3:20.

Levy P, Bieche I, Leroy K, Parfait B, Wechsler J, Laurendeau I, Wolkenstein P, Vidaud M, Vidaud D (2004b). Molecular profiles of neurofibromatosis type 1-associated plexiform neurofibromas: identification of a gene expression signature of poor prognosis. *Clin Cancer Res* 10: 3763-3771.

Li E (2002). Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* 3: 662–73.

Li Y, Bollag G, Clark R, Stevens J, Conroy L, Fults D, Ward K, Friedman E, Samowitz W, Robertson M (1992). Somatic mutations in the neurofibromatosis 1 gene in human tumours. *Cell* 69: 275-281.

Li Y, O'Connell P, Breidenbach HH, Cawthon R, Stevens J, Xu G, Neil S, Robertson

M, White R, Viskochil D (1995). Genomic organisation of the neurofibromatosis 1 gene (*NF1*). *Genomics* 25: 9–18.

Li Y, White R (1996). Suppression of a human colon cancer cell line by introduction of an exogenous *NF1* gene. *Cancer Res* 56: 2872-2876.

Liang P, Pardee AB (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257: 967-971.

Liapis H, Marley EF, Lin Y, Dehner LP (1999). p53 and Ki-67 proliferating cell nuclear antigen in benign and malignant nerve sheath tumours in children. *Pediatr Dev Pathol* 2: 377–384.

Lippman, Z., Gendrel, A. V., Colot, V., and Martienssen, R. (2005). Profiling DNA methylation patterns using genomic tiling microarrays. *Nat Methods* 2: 219-224.

Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ (1999). High density synthetic oligonucleotide arrays. *Nat Genet* 21: 20-24.

Lisch K (1937). Ueber Beteiligung der Augen, insbesondere das Vorkommen von Irisknotchen bei der Neurofibromatose (Recklinghausen). *Z. Augenheilkunde* 93: 137-143.

Listernick R, Charrow J, Gutmann DH (1999). Intracranial gliomas in neurofibromatosis type 1. *Am J Med Genet* 89: 38-44.

Listernick R, Mancini AJ, Charrow J (2003). Segmental neurofibromatosis in childhood. *Am J Med Genet* 121A: 132-135.

Liu B, Farrington SM, Petersen GM, Hamilton SR, Parsons R, Papadopoulos N, Fujiwara T, Jen J, Kinzler KW, Wyllie AH (1995). Genetic instability occurs in the majority of young patients with colorectal cancer. *Nat Med* 1: 348-352.

Liu MT, Su JS, Huang CY, Tsai SF (2003). Novel mutations involving the *NF1* gene coding sequence in neurofibromatosis type 1 patients from Taiwan. *J Hum Genet* 48: 545-549.

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 14: 1675-1680.

Lopes J, Ravise N, Vandenberghe A, Palau F, Ionasescu V, Mayer M, Levy N, Wood N, Tachi N, Bouche P, Latour P, Ruberg M, Brice A, LeGuern E (1998). Fine mapping of de novo CMT1A and HNPP rearrangements within CMT1A-REPs evidences two distinct sex-dependent mechanisms and candidate sequences involved in recombination. *Hum Mol Genet* 7: 141-148.

Lopes J, Tardieu S, Silander K, Blair I, Vandenberghe A, Palau F, Ruberg M, Brice A, LeGuern E (1999). Homologous DNA exchanges in humans can be explained by the yeast double-strand break repair model: a study of 17p11.2 rearrangements associated with CMT1A and HNPP. *Hum Mol Genet* 8: 2285-2292.

Lopez Correa C, Brems H, Lazaro C, Estivill X, Clementi M, Mason S, Rutkowski JL, Marynen P, Legius E (1999). Molecular studies in 20 submicroscopic neurofibromatosis type 1 gene deletions. *Hum Mutat* 14: 387-393.

Lopez Correa C, Brems H, Lazaro C, Marynen P, Legius E (2000). Unequal meiotic crossover: a frequent cause of NF1 microdeletions. *Am J Hum Genet* 66: 1969-1974.

Lopez-Correa C, Dorschner M, Brems H, Lazaro C, Clementi M, Upadhyaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns JP, Marynen P, Stephens K, Legius E (2001). Recombination hotspot in NF1 microdeletion patients. *Hum Mol Genet* 10: 1387-1392.

Lothe RA, Slettan A, Saeter G, Brogger A, Borresen AL, Nesland JM (1995). Alterations at chromosome 17 loci in peripheral nerve sheath tumours. *J Neuropathol Exp Neurol* 54: 65-73.

Lott IT, Richardson EP Jr (1981). Neuropathological findings and the biology of neurofibromatosis. *Adv Neurol* 29: 23-32.

Lowe J, Amos LA (1998). Crystal structure of the bacterial cell-division protein FtsZ. *Nature* 391: 203-206.

Lu AL, Hsu IC (1992). Detection of single DNA base mutations with mismatch repair enzymes.*Genomics* 14: 249-255.

Lubs ML, Bauer MS, Formas ME, Djokic B (1991). Lisch nodules in neurofibromatosis type 1. *N Engl J Med* 324: 1264-1266.

Luijten M, Redeker S, van Noesel MM, Troost D, Westerveld A, Hulsebos TJM (2000). Microsatellite instability and promoter methylation as possible causes of *NF1* gene inactivation in neurofibromas. *Eur J Hum Genet* 8: 939–945.

Lupski JR (1996). DNA diagnostics for Charcot-Marie-Tooth disease and related inherited neuropathies.*Clin Chem* 42: 995-998.

Lupski JR (1998). Genomic disorders: structural features of the genome can lead to DNA rearrangements and human disease traits. *Trends Genet* 14: 417-422.

Lupski JR, Pentao L, Williams LL, Patel PI (1993). Stable inheritance of the CMT1A DNA duplication in two patients with CMT1 and NF1. *Am J Med Genet* 45: 92-96.

Lynch TM, Gutmann DH (2002). Neurofibromatosis 1. *Neurol Clin* 20: 841-865.

MacCollin M, Kwiatkowski D (2001). Molecular genetic aspects of the phakomatoses: tuberous sclerosis complex and neurofibromatosis 1. *Curr Opin Neurol* 14: 163-169.

Mahgoub N, Taylor BR, Gratiot M, Kohl NE, Gibbs JB, Jacks T, Shannon KM (1999). In vitro and in vivo effects of a farnesyltransferase inhibitor on Nf1-deficient hematopoietic cells. *Blood* 94: 2469-2476.

Mancini DN, Singh SM, Archer TK, Rodenhiser DI (1999). Site-specific DNA methylation in the neurofibromatosis (NF1) promoter interferes with binding of CREB and SP1 transcription factors. *Oncogene* 18: 4108-4119.

Mandigers CM, Meijerink JP, Raemaekers JM, Schattenberg AV, Mensink EJ (1998). Graft-versus-lymphoma effect of donor leucocyte infusion shown by real-time quantitative PCR analysis of t(14;18). *Lancet* 352: 1522-1523.

Marchuk DA, Saulino AM, Tavakkol R, Swaroop M, Wallace MR, Andersen LB, Mitchell AL, Gutmann DH, Boguski M, Collins FS (1991). cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the NF1 gene product. *Genomics* 11: 931-940.

Marchuk DA, Tavakkol R, Wallace MR, Brownstein BH, Taillon-Miller P, Fong CT, Legius E, Andersen LB, Glover TW, Collins FS (1992). A yeast artificial chromosome contig encompassing the type 1 neurofibromatosis gene. *Genomics* 13: 672-680.

Marshall CJ (1991). Tumour suppressor genes. *Cell* 64:313-326.

Marshall M (1995). Interactions between Ras and Raf: key regulatory proteins in cellular transformation. *Mol Reprod Dev* 42: 493-499.

Martin GA, Viskochil D, Bollag g, McCabe PC, Crosier WJ, Haubruck H, Conroy L, Clark R, O'Connell P, Cawthon RM (1990). The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell* 16: 843–849.

Mashour GA, Wang HL, Cabal-Manzano R, Wellstein A, Martuza RL, Kurtz A (1999). Aberrant cutaneous expression of the angiogenic factor midkine is associated with neurofibromatosis type-1. *J Invest Dermatol* 113: 398-402.

Masson C, Colombani JM (1997). [Neurofibromatosis 1 and multiple sclerosis. Apropos of a case]. *Rev Neurol* (Paris) 153: 684-686.

Mantripragada KK, Thuresson AC, Piotrowski A, Diaz de Stahl T, Menzel U, Grigelionis G, Ferner RE, Griffiths S, Bolund L, Mautner V, Nordling M, Legius E, Vetrie D, Dahl N, Messiaen L, Upadhyaya M, Bruder CE, Dumanski JP (2006). Identification of novel deletion breakpoints bordered by segmental duplications in the *NF1* locus using high resolution array-CGH. *J Med Genet* 43: 28-38.

Mantripragada KK, Buckley PG, de Stahl TD, Dumanski JP (2004).Genomic microarrays in the spotlight. *Trends Genet* 20: 87-94.

Mattocks C, Baralle D, Tarpey P, ffrench-Constant C, Bobrow M, Whittaker J (2004). Automated comparative sequence analysis identifies mutations in 89% of NF1 patients and confirms a mutation cluster in exons 11-17 distinct from the GAP related domain. *J Med Genet* 41: e48.

Mattocks C, Tarpey P, Bobrow M, Whittaker J (2000). Comparative sequence analysis (CSA): a new sequence-based method for the identification and characterization of mutations in DNA. *Hum Mutat* 16: 437-443.

Mazzarella R, Schlessinger D (1998). Pathological consequences of sequence duplications in the human genome. *Genome Res* 8: 1007-1021.

Maxam AM, Gilbert W (1977). A new method for sequencing DNA. *Proc Natl Acad Sci U S A* 74: 560-564.

Maynard J, Krawczak M, Upadhyaya M (1997). Characterization and significance of nine novel mutations in exon 16 of the neurofibromatosis type 1 (NF1) gene. *Hum Genet* 99: 674-676.

McGaughran J M, Harris D I, Donnai D, Teare D, MacLeod R, Westerbeek R, Kingston H, Super M, Harris R, Evans D G R (1999). A clinical study of type I neurofibromatosis in north west England. *J Med Genet* 36: 197-203.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D (1995). 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* 1: 686–92.

Messiaen L, Callens T, De Paepe A, Craen M, Mortier G (1997). Characterisation of two different nonsense mutations, C6792A and C6792G, causing skipping of exon 37 in the *NF1* gene. *Hum Genet* 101: 75-80.

Messiaen LM, Callens T, Mortier G, Beysen D, Vandenbroucke I, Van Roy N, Speleman F, Paepe AD (2000). Exhaustive mutation analysis of the NF1 gene allows identification of 95% of mutations and reveals a high frequency of unusual splicing defects. *Hum Mutat* 15: 541-555.

Messiaen L, Riccardi V, Peltonen J, Maertens O, Callens T, Karvonen SL, Leisti EL, Koivunen J, Vandenbroucke I, Stephens K, Poyhonen M (2003). Independent NF1 mutations in two large families with spinal neurofibromatosis. *J Med Genet.* 40: 122-126.

Metheny LJ, Skuse GR (1996). *NF1* mRNA isoform expression in PC12 cells: modulation by extrinsic factors. *Exp Cell Res* 228: 44–49.

Mikol DD, Gulcher JR, Stefansson K (1990). The oligodendrocyte myelin glycoprotein belongs to a distinct family of proteins and contains the HNK-1 carbohydrate. *J Cell Biol* 110: 471–479.

Milburn MV, Tong L, deVos AM, Brunger A, Yamaizumi Z, Nishimura S, Kim SH (1990). Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic ras proteins. *Science* 247: 939-945.

Miller SJ, Li H, Rizvi TA, Huang Y, Johansson G, Bowersock J, Sidani A, Vitullo J, Vogel K, Parysek LM, DeClue JE, Ratner N (2003). Brain lipid binding protein in axon-Schwann cell interactions and peripheral nerve tumorigenesis. *Mol Cell Biol* 23: 2213-2224.

Miller SM, Sparkes RS (1977). Segmental neurofibromatosis. *Arch Dermatol* 113: 837-838.

Mirnics K, Middleton FA, Marquez A, Lewis DA, Levitt P (2000). Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex. *Neuron* 28: 53-67.

Morse RP (1999). Neurofibromatosis type 1. *Arch Neurol* 56: 364-365.

Moss C, Green SH (1994). What is segmental neurofibromatosis? *Br J Dermatol* 130: 106-110.

Muhonen MG, Godarsky JC van, Gilder JC (1991). Cerebral aneurysms associated with neurofibromatosis. *Surg Neurol* 36: 470–475.

Myers RM, Larin Z, Maniatis T (1985). Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. *Science* 230: 1242-1246.

Nachman MW (2002). Variation in recombination rate across the genome: evidence and implications. *Curr Opin Genet Dev* 12: 657-663.

Nakamura M, Tangoku A, Kusanagi H, Oka M, Suzuki T (1998). Breast cancer associated with Recklinghausen's disease: report of a case. *Nippon Geka Hokan* 67: 3-9.

Naylor JA, Nicholson P, Goodeve Anne, Hassock S, Peake I, Giannelli F (1996). A novel DNA inversion causing severe hemophilia A. *Blood* 87: 3255-3261.

Nielsen GP, Stemmer-Rachamimov AO, Ino Y, Moller MB, Rosenberg AE, Louis DN (1999). Malignant transformation of neurofibromas in neurofibromatosis 1 is associated with CDKN2A/p16 inactivation. *Am J Pathol* 155: 1879–1884.

Nickerson ML, Weirich G, Zbar B, Schmidt LS (2000). Signature-based analysis of *MET* proto-oncogene mutations using DHPLC. *Hum Mutat* 16: 68-76.

Nicholls EM (1969). Somatic variation and multiple neurofibromatosis. *Hum Hered* 19: 473-479.

Nimmakayalu MA, Gotter AL, Shaikh TH, Emanuel BS (2003). A novel sequence-based approach to localize translocation breakpoints identifies the molecular basis of a t(4;22). *Hum Mol Genet* 12: 2817-2825.

Nishi T, Lee PS, Oka K, Levin VA, Tanase S, Morino Y, Saya H (1991). Differential expression of two types of the neurofibromatosis type 1 (NF1) gene transcripts related to neuronal differentiation. *Oncogene* 6: 1555–1559.

Nogales E, Downing KH, Amos LA, Lowe J (1998). Tubulin and FtsZ form a distinct family of GTPases. *Nat Struct Biol* 5: 451-458.

Nordlund M, Gu X, Shipley MT, Ratner N (1993). Neurofibromin is enriched in the endoplasmic reticulum of CNS neurons. *J Neurosci* 13: 1588-600.

North KN, Riccardi V, Samango-Sprouse C, Ferner R, Moore B, Legius E, Ratner N, Denckla MB (1997). Cognitive function and academic performance in neurofibromatosis. 1: consensus statement from the NF1 Cognitive Disorders Task Force. *Neurology* 48: 1121-1127.

Ober WB (1978). Selected items from the history of pathology. Mark Akenside, MD (1721-1770): first recorded description of multiple neurofibromatosis. *Am J Pathol* 92: 315.

Obringer AC, Meadows AT, Zackai EH (1989). The diagnosis of neurofibromatosis-1 in the child under the age of 6 years. *Am J Dis Child* 143: 717-719.

O'Connell P, Leach R, Cawthon RM, Culver M, Stevens J, Viskochil D, Fournier RE, Rich DC, Ledbetter DH, White R (1989). Two von Recklinghausen neurofibromatosis translocations map within a 600 kb region of 17q11.2. *Science* 244: 1087-1088.

O'Donovan M.C., Oefner P.J., Roberts S.C., Austin J, Hoogendoorn B, Guy C, Speight G, Upadhyaya M, Sommer S.S., McGuffin P (1998). Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. *Genomics* 52: 44-49.

Oguzkan S, Cinbis M, Ayter S, Anlar B, Aysun S (2004). Familial segmental neurofibromatosis. *Child Neurol* 19: 392-394.

Okazaki M, Yoshimura K, Suzuki Y, Uchida G, Kitano Y, Harii K, Imokawa G (2003). The mechanism of epidermal hyperpigmentation in cafe-au-lait macules of neurofibromatosis type 1 (von Recklinghausen's disease) may be associated with dermal fibroblast-derived stem cell factor and hepatocyte growth factor. *Br J Dermatol* 148: 689-697.

Oktenli C, Gul D, Deveci MS, Saglam M, Upadhyaya M, Thompson P, Consoli C, Kocar IH, Pilarski R, Zhou XP, Eng C (2004). Unusual features in a patient with neurofibromatosis type 1: Multiple subcutaneous lipomas, a juvenile polyp in ascending colon, congenital intrahepatic portosystemic venous shunt, and horseshoe kidney. *Am J Med Genet*127A: 298-301.

Oktenli C, Saglam M, Demirbas S, Thompson P, Upadhyaya M, Consoli C, Ulucan H, Koz C, Durukan AH, Bozkurt A, Koc B, Kocar IH, Gul D (2003). A large deletion (1.5 Mb) encompassing the neurofibromatosis type 1 (NF1) gene in a patient with sporadic NF1 associated with dysmorphism, mental retardation, and unusual ocular and skeletal features. *Clin Dysmorphol* 12: 199-201.

Okubo K, Hori N, Matoba R, Niiyama T, Fukushima A, Kojima Y, Matsubara K (1992). Large scale cDNA sequencing for analysis of quantitative and qualitative aspects of gene expression. *Nat Genet* 2: 173-179.

Oleksiak MF, Churchill GA, Crawford DL (2002). Variation in gene expression within and among natural populations. *Nat Genet* 32: 261-266.

Origone P, De Luca A, Bellini C, Buccino A, Mingarelli R, Costabel S, La Rosa C, Garre C, Coviello DA, Ajmar F, Dallapiccola B, Bonioli E (2002). Ten novel mutations in the human neurofibromatosis type 1 (*NF1*) gene in Italian patients. *Hum Mutat* 20: 74-75.

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci U S A* 86: 2766-2770.

Orntoft TF, Thykjaer T, Waldman FM, Wolf H, Celis JE (2002). Genome-wide study of gene copy numbers, transcripts, and protein levels in pairs of non-invasive and invasive human transitional cell carcinomas. *Mol Cell Proteomics* 1: 37-45.

Osawa M, Kanai N, Kawai M, Fukuyama Y (1992). Clinical genetic study on the idiopathic occlusion of the circle of Willis. In: The Research Committee on Spontaneous Occlusion of the Circle of Willis (Moyamoya Disease) of the Ministry of Health and Welfare Japan: Annual Report Tokyo, Japan: Ministry of Health and Welfare Japan: 147–152.

Osborn MJ, Upadhyaya M (1999). Evaluation of the protein truncation test and mutation detection in the NF1 gene: mutational analysis of 15 known and 40 unknown mutations. *Hum Genet* 105: 327-332.

Pai EF, Krengel U, Petsko GA, Goody RS, Kabsch W, Wittinghofer A (1990).Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. *EMBO J* 9: 2351-2359.

Pandey PK, Narayanan R, Chaudhuri Z, Chadha V, Jain S (2002). An unusual case of neurofibromatosis fulfilling the diagnostic criteria for types I and II. *J Pediatr Ophthalmol Strabismus* 39: 313-316.

Papp AC, Pinsonneault JK, Cooke G, Sadee W (2003). Single nucleotide polymorphism genotyping using allele-specific PCR and fluorescence melting curves. *Biotechniques* 34: 1068-1072.

Parada LF (2000). Neurofibromatosis type 1. *Biochm Biophys Acta* 1471: M13-19.

Paradis V, Bieche I, Dargere D, Laurendeau I, Laurent C, Bioulac Sage P, Degott C, Belghiti J, Vidaud M, Bedossa P (2003). Molecular profiling of hepatocellular

carcinomas (HCC) using a large-scale real-time RT-PCR approach: determination of a molecular diagnostic index. *Am J Pathol* 163: 733-741.

Park KC, Choi HO, Han WS, Hwang JH, Park KH, Kim KH, Chung JH, Eun HC (2000). Identification of two novel frame shift mutations of the *NF1* gene in Korean patients with neurofibromatosis type 1. *J Korean Med Sci* 15: 542-544.

Park VM, Kenwright KA, Sturtevant DB, Pivnick EK (1998a). Alternative splicing of exons 29 and 30 in the neurofibromatosis type 1 gene. *Hum Genet* 103: 382-385.

Park VM, Pivnick EK (1998b). Neurofibromatosis type 1 (NF1): a protein truncation assay yielding identification of mutations in 73% of patients. *J Med Genet* 35: 813-820.

Parry DM, Eldridge R, Kaiser-Kupfer MI, Bouzas EA, Pikus A, Patronas N (1994). Neurofibromatosis 2 (NF2): clinical characteristics of 63 affected individuals and clinical evidence for heterogeneity. *Am J Med Genet* 52: 450-461.

Parsons R, Myeroff LL, Liu B, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B (1995). Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. *Cancer Res* 55: 5548-5550.

Peltonen S, Ylä-Outinen H, Peltonen J (2005). Gene expression profile of skin fibroblasts with a mutation in *NF1* gene. *11th European Neurofibromatosis meeting* July.Göteborg, Sweden.

Pentao L, Wise CA, Chinault AC, Patel PI, Lupski JR (1992). Charcot-Marie-Tooth type 1A duplication appears to arise from recombination at repeat sequences flanking the 1.5 Mb monomer unit. *Nat Genet* 2: 292-300.

Peoples R, Franke Y, Wang YK, Perez-Jurado L, Paperna T, Cisco M, Francke U (2000). A physical map, including a BAC/PAC clone contig, of the Williams–Beuren syndrome — deletion region at 7q11.23. *Am J Hum Genet* 66: 47–68.

Perez Jurado LA, Wang YK, Peoples R, Coloma A, Cruces J, Francke U (1998). A duplicated gene in the breakpoint regions of the 7q11.23 Williams–Beuren syndrome deletion encodes the initiator binding protein TFII-I and BAP-135, a phosphorylation target of BTK. *Hum Mol Genet* 7: 325–334.

Perini P, Gallo P (2001). The range of multiple sclerosis associated with neurofibromatosis type 1. *J Neurol Neurosurg Psychiatry* 71: 679-681.

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000). Molecular portraits of human breast tumours. *Nature* 406: 747-752.

Perrin G, Morris MA, Antonarakis SE, Boltshauser E, Hutter P (1996). Two novel mutations affecting mRNA splicing of the neurofibromatosis type 1 (*NF1*) gene. *Hum Mutat* 7: 172-175.

Perry A, Roth KA, Banerjee R, Fuller CE, Gutmann DH (2001). *NF1* deletions in S-100 protein-positive and negative cells of sporadic and neurofibromatosis 1 (NF1)-associated plexiform neurofibromas and MPNSTs. *Am J Pathol* 159: 57–61.

Petek E, Jenne DE, Smolle J, Binder B, Lasinger W, Windpassinger C, Wagner K, Kroisel PM, Kehrer-Sawatzki H (2003). Mitotic recombination mediated by the JJAZF1 (KIAA0160) gene causing somatic mosaicism and a new type of constitutional NF1 microdeletion in two children of a mosaic female with only few manifestations. *J Med Genet* 40: 520-525.

Peters H, Luder A, Harder A, Schuelke M, Tinschert S (1999). Mutation screening of neurofibromatosis type 1 (*NF1*) exons 28 and 29 with single strand conformation polymorphism (SSCP): five novel mutations, one recurrent transition and two polymorphisms in a panel of 118 unrelated NF1 patients. *Hum Mutat* 13: 258.

Phillips CM, Rye B (1994). Neurofibromatosis type 1 and tuberous sclerosis: a case of double phakomatosis. *J Am Acad Dermatol* 31: 799-800.

Piccoli F, Demma M, Pastore L, Lupo I (1977). Su di un caso di sclerosi tuberosa associata a malattia di Recklinghausen: tentativo di revisione del problema delle facomatosi. *Acta Neurologica* 32: 389-398.

Pinson S (2002). Neurofibromatosis type 1 or Recklinghausen's disease. Neurofibromatosis type 1. Orphanet encyclopedia.

Platten M, Giordano MJ, Dirven CM, Gutmann DH, Louis DN (1996). Up-regulation of specific *NF1* gene transcripts in sporadic pilocytic astrocytomas. *Am J Pathol* 149: 621-627.

Pollack JR, Iyer VR (2002). Characterizing the physical genome. *Nat Genet* 32 Suppl: 515-521.

Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brown PO (1999). Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat Genet* 23: 41-46.

Poyhonen M, Leisti EL, Kytola S, Leisti J (1997). Hereditary spinal neurofibromatosis: a rare form of NF1? *J Med Genet* 34: 184-187.

Potocki L, Chen KS, Park SS, Osterholm DE, Withers MA, Kimonis V, Summers AM, Meschino WS, Anyane-Yeboa K, Kashork CD, Shaffer LG, Lupski JR (2000). Molecular mechanism for duplication 17p11.2- the homologous recombination reciprocal of the Smith-Magenis microdeletion. *Nat Genet* 24: 84-87.

Preiser SA, Davenport CB (1918). Multiple neurofibromatosis (von Recklinghausen disease) and ist inheritance. *Am J Med Sc* 156: 507-541.

Prendergast GC, Oliff A (2000). Farnesyltransferase inhibitors: antineoplastic properties, mechanisms of action, and clinical prospects. *Semin Cancer Biol* 10: 443-452.

Puisieux A (1999). HNPCC syndrome, microsatellite instability and NF1 gene alteration. *Bull Cancer* 86: 812-814.

Pulst SM, Riccardi VM, Fain P, Korenberg JR (1991). Familial spinal neurofibromatosis: clinical and DNA linkage analysis. *Neurology* 41: 1923-1927.

Purandare SM, Huntsman Breidenbach H, Li Y, Zhu XL, Sawada S, Neil SM, Brothman A, White R, Cawthon R, Viskochil D (1995). Identification of neurofibromatosis 1 (*NF1*) homologous loci by direct sequencing, fluorescence in situ hybridization, and PCR amplification of somatic cell hybrids. *Genomics* 30: 476-485.

Purandare SM, Lanyon WG, Connor JM (1994). Characterisation of inherited and sporadic mutations in neurofibromatosis type-1. *Hum Mol Genet* 3: 1109-1115.
Quattrin T, McPherson E, Putnam T (1987). Vertical transmission of the neurofibromatosis/Noonan syndrome. *Am J Med Genet*. 26: 645-649.

Raevaara TE, Gerdes AM, Lonnqvist KE, Tybjaerg-Hansen A, Abdel-Rahman WM, Kariola R, Peltomaki P, Nystrom-Lahti M (2004). HNPCC mutation MLH1 P648S makes the functional protein unstable, and homozygosity predisposes to mild neurofibromatosis type 1. *Genes Chromosomes Cancer* 40: 261-265.

Rasmussen SA, Colman SD, Ho VT, Abernethy CR, Arn PH, Weiss L, Schwartz C, Saul RA, Wallace MR (1998). Constitutional and mosaic large *NF1* gene deletions in neurofibromatosis type 1. *J Med Genet* 35: 468-471.

Rasmussen SA, Friedman JM (2000). NF1 gene and neurofibromatosis 1. *Am J Epidemiol* 151: 33-40.

Rasmussen HH, Orntoft TF, Wolf H, Celis JE (1996). Towards a comprehensive database of proteins from the urine of patients with bladder cancer. *J Urol* 155: 2113-2119.

Rasmussen SA, Overman J, Thomson SA, Colman SD, Abernathy CR, Trimpert RE, Moose R, Virdi G, Roux K, Bauer M, Rojiani AM, Maria BL, Muir D, Wallace MR (2000). Chromosome 17 loss-of-heterozygosity studies in benign and malignant tumours in neurofibromatosis type 1. *Genes Chromosomes Cancer* 28: 425-431.

Rasmussen SA, Yang Q, Friedman JM (2001). Mortality in neurofibromatosis 1: an analysis using U.S. death certificates. *Am J Hum Genet* 68: 1110–1118.

Regnier V, Meddeb M, Lecointre G, Richard F, Duverger A, Nguyen VC, Dutrillaux B, Bernheim A, Danglot G (1997). Emergence and scattering of multiple neurofibromatosis (NF-1)-related sequences during hominoid evolution suggest a process of pericentromeric interchromosomal transposition. *Hum. Mol. Genet* 6: 9–16.

Reid AG, Tarpey PS, Nacheva EP (2003).High-resolution analysis of acquired genomic imbalances in bone marrow samples from chronic myeloid leukemia patients by use of multiple short DNA probes. *Genes Chromosomes Cancer* 37: 282-290.

Reilly KM, Loisel DA, Bronson RT, McLaughlin ME, Jacks T (2000). Nf1;Trp53 mutant mice develop glioblastoma with evidence of strain-specific effects. *Nat Genet* 26: 109-113.

Reiter LT, Hastings PJ, Nelis E, De Jonghe P, Van Broeckhoven C, Lupski JR (1998). Human meiotic recombination products revealed by sequencing a hotspot for homologous strand exchange in multiple HNPP deletion patients. *Am J Hum Genet* 62: 1023-1033.

Reiter LT, Murakami T, Koeuth T, Pentao L, Muzny DM, Gibbs RA, Lupski JR (1996). A recombination hotspot responsible for two inherited peripheral neuropathies is located near a mariner transposon-like element. *Nat Genet* 12: 288-297.

Reiter LT, Murakami T, Koeuth T, Gibbs RA, Lupski JR (1997). The human COX10 gene is disrupted during homologous recombination between the 24 kb proximal and distal CMT1A-REPs. *Hum Mol Genet* 6: 1595-603.

Rendtorff ND, Bjerregaard B, Frodin M, Kjaergaard S, Hove H, Skovby F, Brondum-Nielsen K, Schwartz M; Danish Tuberous Sclerosis Group.Analysis of 65 tuberous sclerosis complex (TSC) patients by TSC2 DGGE, TSC1/TSC2 MLPA, and TSC1 long-range PCR sequencing, and report of 28 novel mutations. *Hum Mutat* 26: 374-383.

Repping, S, Skaletsky, H, Lange J, Silber S, Van Der Veen F, Oates RD, Page DC, and Rozen S (2002). Recombination between palindromes P5 and P1 on the human Y chromosome causes massive deletions and spermatogenic failure. *Am J Hum Genet* 71: 906-922.

Resat H, Straatsma TP, Dixon DA, Miller JH (2001).The arginine finger of RasGAP helps Gln-61 align the nucleophilic water in GAP-stimulated hydrolysis of GTP. *Proc Natl Acad Sci U S A* 98: 6033-6038.

Riccardi VM (1993). Genotype, malleotype, phenotype, and randomness: lessons from neurofibromatosis-1 (NF-1). *Am J Hum Genet* 53: 301–304.

Riccardi VM (2000). Histogenesis control genes: embryology, wound-healing, and NF1. *Teratology* 62: 4.

Riccardi VM, (1982). Neurofibromatosis: clinical heterogeneity. *Curr Probl Cancer* 7: 1-34.

Riccardi VM (1992). *Neurofibromatosis: Phenotype, Natural History and Pathogenesis. Johns Hopkins University*, Baltimore.

Riccardi VM (1999). Skeletal system. In: Neurofibromatosis: Phenotype, Natural History and Pathogenesis (Friedman J, Gutmann D, MacCollin M, Riccardi VM, eds), 3rd edn. Baltimore: John Hopkins University Press, 250–273.

Riccardi VM (1981). Von Recklinghausen neurofibromatosis. *N Engl J Med* 305: 1617-1627.

Riccardi VM, Lewis RA (1988). Penetrance of von Recklinghausen neurofibromatosis: a distinction between predecessors and descendants. *Am J Hum Genet* 42: 284-289.

Ricciardone MD, Ozcelik T, Cevher B, Ozdag H, Tuncer M, Gurgey A, Uzunalimoglu O, Cetinkaya H, Tanyeli A, Erken E, Ozturk M (1999). Human MLH1 deficiency predisposes to hematological malignancy and neurofibromatosis type 1. *Cancer Res* 59: 290-293.

Riva P, Corrado L, Natacci F, Castorina P, Wu BL, Schneider GH, Clementi M, Tenconi R, Korf BR, Larizza L (2000). NF1 microdeletion syndrome: refined FISH characterization of sporadic and familial deletions with locus-specific probes. *Am J Hum Genet* 66: 100-109.

Riva P, Castorina P, Manoukian S, Dalpra L, Doneda L, Marini G, den Dunnen J, Larizza L (1996). Characterization of a cytogenetic 17q11.2 deletion in an NF1 patient with a contiguous gene syndrome. *Hum Genet* 98: 646-650.

Robinson PN, Boddrich A, Peters H, Tinschert S, Buske A, Kaufmann D, Nurnberg P (1995). Two recurrent nonsense mutations and a 4 bp deletion in a quasi-symmetric element in exon 37 of the *NF1* gene. *Hum Genet* 96: 95-98.

Robinson PN, Buske A, Neumann R, Tinschert S, Nurnberg P (1996). Recurrent 2-bp deletion in exon 10c of the *NF1* gene in two cases of von Recklinghausen neurofibromatosis. *Hum Mutat* 7: 85-88.

Rodenhiser DI, Jung JH, Gillett JM, Hovland K, Andrews J, Ainsworth PJ, Coulter-Mackie M, Singh SM (1997). A five-basepair deletion (7118 delTTTTA) identified within neurofibromatosis (*NF1*) exon 39. *Hum Mutat* 9: 473

Roest PA, Roberts RG, Sugino S, van Ommen GJ, den Dunnen JT (1993).Protein truncation test (PTT) for rapid detection of translation-terminating mutations. *Hum Mol Genet* 2: 1719-1721.

Roth DB, Wilson JH (1986). Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. *Mol Cell Biol* 6: 4295-4304.

Rouleau GA, Wertelecki W, Haines JL, Hobbs M (1987). Genetic linkage of bilateral acoustic neurofibromatosis to a DNA marker on chromosome. *Nature* 329: 246-248.

Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Xuan K, Demczuk S, Desmaze C, Plougastel B (1993). Alteration in a new gene encoding a

putative membrane-organizing protein causes neurofibromatosis type 2. *Nature* 363: 515-521.

Rouleau G, Werteleki W, Seizinger BR, Martuza RL, Suprnau DW, Conneally PM, Gusella JF (1987). Genetic linkage analysis of bilateral acoustic neurofibromatosis to a DNA marker on chromosome 22. *Nature* 329: 246-248.

Rowan AJ, Bodmer WF (1997). Introduction of a myc reporter tag to improve the quality of mutation detection using the protein truncation test. *Hum Mutat* 9: 172-176.

Rubenstein AE, Bader Jl, Aron AA, Wallace S (1983). Familial transmission of segmental neurofibromatosis. *Neurology* 33 : 76.

Ruggieri M (2001). Mosaic (segmental) neurofibromatosis type 1 (NF1) and type 2 (NF2): no longer neurofibromatosis type 5 (NF5). *Am J Med Genet* 101: 178–180.

Ruggieri M, Huson SM (1999). The neurofibromatoses. An overview. *Ital J Neurol Sci* 20: 89-108.

Ruggieri M, Huson SM (2001). The clinical and diagnostic implications of mosaicism in the neurofibromatoses. *Neurology* 56: 1433-1443.

Ruggieri M, Pavone P, Polizzi A, Di Pietro M, Scuderi A, Gabriele A, Spalice A, Iannetti P (2004). Ophthalmological manifestations in segmental neurofibromatosis type 1. *Br J Ophthalmol* 88: 1429-1433.

Ruggieri M, Polizzi A (2000). Segmental neurofibromatosis. *J Neurosurg* 93: 530-532.

Rutkowski JL, Wu K, Gutmann DH, Boyer PJ, Legius E (2000). Multiple mechanisms of benign tumour formation in neurofibromatosis type 1. *Hum Mol Genet* 9: 1056–1066.

Sadeh M, Martinovits GM, Goldhammer Y (1989). Occurrence of both Neurofibromatosis 1 and 2 in the same individual with a rapidly progressive course. *Neurology* 39: 282-283.

Saitta SC, Harris SE, Gaeth AP, Driscoll DA, McDonald-McGinn DM, Maisenbacher MK, Yersak JM, Chakraborty PK, Hacker AM, Zackai EH, Ashley T, Emanuel BS (2004). Aberrant interchromosomal exchanges are the predominant cause of the 22q11.2 deletion. *Hum Mol Genet* 13: 417-428.

Samuelsson B, Axelsson R (1981). Neurofibromatosis. A clinical and genetic study of 96 cases in Gothenburg, Sweden. *Acta Derm Venereol* 95: 67-71.

Sanger F, Nicklen S, Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 74: 5463-5467.

Sangueza OP, Requena L (1998). Neoplasms with neural differentiation: a review. Part II. Malignant neoplasms. *Am J Dermapathol* 20 : 89-102.

Sawada S, Florell S, Purandare SM, Ota M, Stephens K, Viskochil D (1996). Identification of *NF1* mutations in both alleles of a dermal neurofibroma. *Nat Genet* 14: 110–112.

Schadt EE, Monks SA, Drake TA, Lusis AJ, Che N, Colinayo V, Ruff TG Milligan SB, Lamb JR, Cavet G, Linsley PS, Mao M, Stoughton RB, Friend SH (2003). Genetics of gene expression surveyed in maize, mouse and man. Nature 422: 297-302.

Scheffzek K, Ahmadian MR, Wiesmuller L, Kabsch W, Stege P, Schmitz F, Wittinghofer A (1998). Structural analysis of the GAP-related domain from neurofibromin and its implications. *EMBO J* 17: 4313-4327.

Scheffzek K, Lautwein A, Scherer A, Franken S, Wittinghofer A (1997). Crystallization and preliminary X-ray crystallographic study of the Ras-GTPase-activating domain of human p120GAP. *Proteins* 27: 315-318.

Schena M, Shalon D, Davis RW, Brown PO (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270: 467-470.

Schena M, Shalon D, Heller R, Chai A, Brown PO, Davis RW (1996). Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc Natl Acad Sci U S A* 93: 10614-10619.

Sermon BA, Eccleston JF, Skinner RH, Lowe PN (1996). Mechanism of inhibition by arachidonic acid of the catalytic activity of Ras GTPase-activating proteins. *J Biol Chem* 271: 1566-1572.

Sheela S, Riccardi VM, Ratner N (1990). Angiogenic and invasive properties of neurofibroma Schwann cells. *J Cell Biol* 111: 645-653.

Shen MH, Upadhyaya M (1993). A de novo nonsense mutation in exon 28 of the neurofibromatosis type 1 (NF1)gene. *Hum Genet* 92: 410-412.

Shenk TE, Rhodes C, Rigby PW, Berg P (1975).Biochemical method for mapping mutational alterations in DNA with S1 nuclease:the location of deletions and temperature-sensitive mutations in simian virus 40. *Proc Natl Acad Sci U S A* 72: 989-993.

Sherlock G (2000). Analysis of large-scale gene expression data. *Curr Opin Immunol* 12: 201-205.

Schmidt M, Michels V, Dewald G (1987). Cases of neurofibromatosis with rearrangements of chromosome 17 involving band 17q11.2. *Am J Med Genet* 28: 771-775.

Schneider J, Buness A, Huber W, Volz J, Kioschis P, Hafner M, Poustka A, Sultmann H (2004). Systematic analysis of T7 RNA polymerase based in vitro linear RNA amplification for use in microarray experiments. *BMC Genomics* 5: 29.

Schorry EK, Lovell AM, Milatovich A, Saal HM (1996). Ullrich-Turner syndrome and neurofibromatosis-1. *Am J Med Genet* 66: 423-425.

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* 30: e57.

Schull WJ, Crowe FW (1953). Neurocutaneous syndromes in the M kindred: A case of simultaneous occurrence of tuberous sclerosis and neurofibromatosis. *Neurol* 3: 904-909.

Schuppert F, Berger D, Peters H, Schroder S, Schofl C, Tischler J, Hiller WF, von zur Muhlen A (2000). [A young woman with neurofibromatosis 1 (Recklinghausen disease), abdominal tumour and hypertension] *Dtsch Med Wochenschr* 125: 1390-1394.

Seizinger BR, Martuza RL, Gusella JF (1986). Loss of genes on chromosome 22 in tumorogenesis of human acoustic neuroma. *Nature* 322: 644-647.

Seizinger BR, Rouleau GA, Ozelius LJ, Lane AH, Faryniarz AG, Chao MV, Huson S, Korf BR, Parry DM, Pericak-Vance MA (1987). Genetic linkage of von Recklinghausen neurofibromatosis to the nerve growth factor receptor gene *Cell* 49: 589-594.

Sergeyev AS (1975). On the mutation rate of neurofibromatosis. *Humangenetik* 28: 129-138.

Serra E, Ars E, Ravella A, Sánchez A, Puig S, Rosenbaum T, Estivill X and Lázaro C (2001). Somatic *NF1* mutational spectrum in benign neurofibromas: mRNAsplice defects are common among point mutations. *Hum Genet* 108:416-429.

Serra E, Puig S, Otero D, Gaona A, Kruyer H, Ars E, Estivill X, Lazaro C (1997). Confirmation of a double-hit model for the NF1 gene in benign neurofibromas. *Am J Hum Genet* 61: 512-519.

Serra E, Rosenbaum T, Winner U, Aledo R, Ars E, Estivill X, Lenard H G, Lazaro C (2000). Schwann cells harbor the somatic *NF1* mutation in neurofibromas: evidence of two different Schwann cell subpopulations. *Hum Molec Genet* 9: 3055-3064.

247

Schlessinger J (2000). Cell signaling by receptor tyrosine kinases. *Cell* 103: 211-225.

Shaffer LG, Lupski JR (2000).Molecular mechanisms for constitutional chromosomal rearrangements in humans. *Annu Rev Genet* 34: 297-329.

Shaikh, T. H., Kurahashi, H. & Emanuel, B. S (2001). Evolutionarily conserved duplications in 22q11 mediate deletions, duplications, translocations and genomic instability. *Genet Med* 3: 6–13.

Shaikh TH, Kurahashi H, Saitta SC, O'Hare AM, Hu P, Roe BA, Driscoll DA, McDonald-McGinn DM, Zackai EH, Budarf ML, Emanuel BS (2000). Chromosome 22-specific low copy repeats and the 22q11.2 deletion syndrome: genomic organization and deletion endpoint analysis. *Hum Mol Genet* 9: 489–501.

Shannon KM, O'Connell P, Martin GA, Paderanga D, Olson K, Dinndorf P, McCormick F (1994).Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. *N Engl J Med* 330: 597-601.

Sharland M, Burch M, McKenna VM, Paton MA (1992). A clinical study of Noonan syndrome. *Arch Dis Child* 67: 178-183.

Sharland M, Taylor R, Patton MA, Jeffery S (1992). Absence of linkage of Noonan syndrome to the neurofibromatosis type 1 locus. *J Med Genet* 29: 188-190.

Shaw CJ, Bi W, Lupski JR (2002). Genetic proof of unequal meiotic crossovers in reciprocal deletion and duplication of 17p11.2. *Am J Hum Genet* 71: 1072–1081.

Shaw CJ, Lupski JR (2004). Implications of human genome architecture for rearrangement-based disorders: the genomic basis of disease. *Hum Mol Genet* 13 Spec No 1:R57-64.

Sheela S, Riccardi VM, Ratner N (1990). Angiogenic and invasive properties of neurofibroma Schwann cells. *J Cell Biol* 111: 645–652.

Shen MH, Harper PS, Upadhyaya M (1993). Neurofibromatosis type 1 (NF1): the search for mutations by PCR-heteroduplex analysis on Hydrolink gels. *Hum Mol Genet* 2: 1861-1864.

Sherman LS, Atit R, Rosenbaum T, Cox AD, Ratner N (2000). Single cell Ras-GTP analysis reveals altered Ras activity in a subpopulation of neurofibroma Schwann cells but not fibroblasts. *J Biol Chem* 275: 30740-30745.

Sherr CJ (2004).Principles of tumor suppression. *Cell* 116: 235-246.

Sicilia SL, Aldamiz OH, Torres AC, Avendano HL (1968). Asociacion de esclerosis tuberosa, neurofibromatosis y rinones poliquisticos. Revista Clinica Espanola 108: 311-318.

Side LE, Emmanuel PD, Taylor B, Franklin J, Thompson P, Castleberry RP, Shannon KM (1998). Mutations of the *NF1* gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis type 1. *Blood* 92: 267-272.

Side L, Taylor B, Cayouette M, Conner E, Thompson P, Luce M, Shannon K (1997). Homozygous inactivation of the *NF1* gene in bone marrow cells from children with neurofibromatosis type 1 and malignant myeloid disorders. *N Engl J Med* 336: 1713-1720.

Silva AJ, Frankland PW, Marowitz Z, Friedman E, Laszlo GS, Cioffi D, Jacks T, Bourtchuladze R (1997). A mouse model for the learning and memory deficits associated with neurofibromatosis type 1. *Nat Genet* 15: 281–284.

Silva AJ, Simpson EM, Takahashi JS, Lipp HP, Nakanishi S, et al. (1997a). Mutant mice and neuroscience: recommendations concerning genetic background. *Neuron* 19: 755–759.

Sippel KC, Fraioli RE, Smith GD, Schalkoff ME, Sutherland J, Gallie BL, Dryja TP (1998). Frequency of somatic and germ-line mosaicism in retinoblastoma: implications for genetic counseling. *Am J Hum Genet* 62: 610-619.

Skuse GR (1998). Identification of an insertion and accompanying deletion in exon 31 of the neurofibromatosis type 1 gene. *Hum Mutat* Suppl 1: S50-52.

Skuse GR and Cappione AJ (1997). RNA processing and clinical variability in neurofibromatosis type 1 (NF1). *Hum Mol Genet* 6: 1707-1712.

Smith RW (1849). *A Treatise on the pathology, diagnosis and treatment of neurofibroma*. Hodges and Smith, Dublin, pp 414-444.

Stankiewicz P, Inoue K, Bi W, Walz K, Park SS, Kurotaki N, Shaw CJ, Fonseca P, Yan J, Lee JA, Khajavi M, Lupski JR (2003). Genomic disorders: genome architecture results in susceptibility to DNA rearrangements causing common human traits. *Cold Spring Harb Symp Quant Biol* 68: 445-454.

Stankiewicz P, Lupski JR (2002). Genome architecture, rearrangements and genomic disorders. *Trends Genet* 18:74-82.

Stankiewicz P, Lupski JR (2002). Molecular-evolutionary mechanisms for genomic disorders. *Curr Opin Genet Dev* 12: 312-319.

Stankiewicz P, Shaw CJ, Dapper JD, Wakui K, Shaffer LG, Withers M, Elizondo L, Park SS, Lupski JR (2003). Genome architecture catalyzes nonrecurrent chromosomal rearrangements. *Am J Hum Genet* 72: 1101–1116.

Stark M, Assum G, Krone W(1991). A small deletion and an adjacent base exchange in a potential stem-loop region of the neurofibromatosis 1 gene. *Hum Genet* 87: 685-687.

Stern HJ, Saal HM, Lee JS, Fain PR, Goldgar DE, Rosenbaum KN, Barker DF (1992). Clinical variability of type 1 neurofibromatosis: is there a neurofibromatosis-Noonan syndrome? *J Med Genet.* 29:184-187.

Streubel B, Latta E, Kehrer-Sawatzki H, Hoffmann GF, Fonatsch C, Rehder H (1999). Somatic mosaicism of a greater than 1.7-Mb deletion of genomic DNA involving the

entire NF1 gene as verified by FISH: further evidence for a contiguous gene syndrome in 17q11.2. *Am J Med Genet* 87: 12-16.

Stumpf DA, Alksne JF, Annegers JF, et al. (1988). Neurofibromatosis. Conference statement. National Institutes of Health Consensus Development Conference. *Arch Neurol* 45: 575-578.

Sobata E, Ohmura H, Suzuki S (1988). Cerebrovascular disorders associated with von Recklinghausen's neurofibromatosis: a case report. *Neurosurgery* 22: 544–549.

Spirio L, Joslyn G, Nelson L, Leppert M, White R (1991). A CA repeat 30-70 KB downstream from the adenomatous polyposis coli (*APC*) gene. *Nucleic Acids Res* 19: 6348.

Sutter C, Gebert J, Bischoff P, Herfarth C, von Knebel Doeberitz M (1999). Molecular screening of potential HNPCC patients using a multiplex microsatellite PCR system. *Mol Cell Probes.* 13: 157-165.

Suzuki H, Ozawa N, Taga C, Kano T, Hattori M, Sakaki Y (1994). Genomic analysis of a *NF1*-related pseudogene on human chromosome 21. *Gene* 147: 277-280.

Suzuki Y, Suzuki H, Kayama T, Yoshimoto T, Shibahara S (1991). Brain tumours predominantly express the neurofibromatosis type 1 gene transcripts containing the 63 base insert in the region coding for GTPase activating protein-related domain. *Biochem Biophys Res Commun* 181: 955-961.

Szabo P, Tang SH, Rentsendorj A, Pfeifer GP, Mann JR (2000). Maternal specific footprints at putative CTCF sites in the *H19* imprinting control region give evidence for insulator function. *Curr Biol* 10: 607–610.

Szudek J, Evans DG, Friedman JM (2003). Patterns of associations of clinical features in neurofibromatosis 1 (NF1). *Hum Genet* 112: 289-297.

Szudek J, Joe H, Friedman JM (2002). Analysis of intrafamilial phenotypic variation in neurofibromatosis 1 (NF1).*Genet Epidemiol* 23: 150-164.

Tada K, Kochi M, Saya H, Kuratsu J, Shiraishi S, Kamiryo T, Shinojima N, Ushio Y (2003). Preliminary observations on genetic alterations in pilocytic astrocytomas associated with neurofibromatosis 1. *Neuro-oncol* 5: 228-234.

Takai D, Yagi Y, Habib N, Sugimura T, Ushijima T (2000). Hypomethylation of LINE1 retrotransposon in human hepatocellular carcinomas, but not in surrounding liver cirrhosis. *Jpn J Clin Oncol* 30: 306-9.

Tang Y, Lu A, Ran R, Aronow BJ, Schorry EK, Hopkin RJ, Gilbert DL, Glauser TA, Hershey AD, Richtand NW, Privitera M, Dalvi A, Sahay A, Szaflarski JP, Ficker DM, Ratner N, Sharp FR (2004a). Human blood genomics: distinct profiles for gender, age and neurofibromatosis type 1. *Brain Res Mol Brain Res* 132: 155-167.

Tang Y, Schapiro MB, Franz DN, Patterson BJ, Hickey FJ, Schorry EK, Hopkin RJ, Wylie M, Narayan T, Glauser TA, Gilbert DL, Hershey AD, Sharp FR (2004b). Blood expression profiles for tuberous sclerosis complex 2, neurofibromatosis type 1, and Down's syndrome. *Ann Neurol* 56: 808-814.

Tassabehji M, Strachan T, Sharland M, Colley A, Donnai D, Harris R, Thakker N (1993). Tandem duplication within a neurofibromatosis type 1 (*NF1*) gene exon in a family with features of Watson syndrome and Noonan syndrome. *Am J Hum Genet* 53: 90-95.

The I, Murthy AE, Hannigan GE, Jacoby LB, Menon AG, Gusella JF, Bernards A (1993). Neurofibromatosis type 1 gene mutations in neuroblastoma. *Nat Genet* 3: 62-66.

The I, Hannigan GE, Cowley GS, Reginald S, Zhong Y, Gusella JF, Hariharan IK, Bernards A (1997). Rescue of *Drosophila NF1* mutant phenotype by protein kinase A. *Science* 276: 791-794.

Tiecke F, Katzke S, Booms P, Robinson PN, Neumann L, Godfrey M, Mathews KR, Scheuner M, Hinkel GK, Brenner RE, Hovels-Gurich HH, Hagemeier C, Fuchs J,

Skovby F, Rosenberg T (2001). Classic, atypically severe and neonatal Marfan syndrome: twelve mutations and genotype-phenotype correlations in *FBN1* exons 24-40. *Eur J Hum Genet.* 9: 13-21.

Tinschert S, Naumann I, Stegmann E, Buske A, Kaufmann D, Thiel G, Jenne DE (2000). Segmental neurofibromatosis is caused by somatic mutation of the neurofibromatosis type 1 (*NF1*) gene. *Eur J Hum Genet* 8: 455–459.

Toliat MR, Erdogan F, Gewies A, Fahsold R, Buske A, Tinschert S, Nurnberg P (2000). Analysis of the NF1 gene by temperature gradient gel electrophoresis reveals a high incidence of mutations in exon 4b. *Electrophoresis* 21: 541-544.

Tong J, Hannan F, Zhu Y, Bernards A & Zhong Y (2002). Neurofibromin regulates G proteinstimulated adenylyl cyclase activity. *Nat Neurosci* 5: 95-96.

Tonsgard JH, Yelavarthi KK, Cushner S, Short MP, Lindgren V (1997). Do *NF1* gene deletions result in a characteristic phenotype? *Am J Med Genet* 73: 80–86.

Townsend JP, Cavalieri D, Hartl DL (2003).Population genetic variation in genome-wide gene expression. *Mol Biol Evol* 20: 955-963.

Trahey M, McCormick F (1987). A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. *Science* 238: 542-545.

Trask BJ, Friedman C, Martin-Gallardo A, Rowen L, Akinbami C, Blankenship J, Collins C, Giorgi D, Iadonato S, Johnson F, Kuo WL, Massa H, Morrish T, Naylor S, Nguyen OT, Rouquier S, Smith T, Wong DJ, Youngblom J, van den Engh G (1998a). Members of the olfactory receptor gene family are contained in large blocks of DNA duplicated polymorphically near the ends of human chromosomes. *Hum Mol Genet* 7: 13-26.

Trask BJ, Massa H, Brand-Arpon V, Chan K, Friedman C, Nguyen OT, Eichler E, van den Engh G, Rouquier S, Shizuya H, Giorgi D (1998b). Large multi-chromosomal duplications encompass many members of the olfactory receptor gene family in the human genome. *Hum Mol Genet* 7: 2007–2020.

Trimbath JD, Petersen GM, Erdman SH, Ferre M, Luce MC, Giardiello FM (2001). Cafe-au-lait spots and early onset colorectal neoplasia: a variant of HNPCC? *Fam Cancer* 1: 101-105.

Trofatter JA, MacCollin MM, Rutter JL, Murrell JR, Duyao MP, Parry DM, Eldridge R, Kley N, Menon AG, Pulaski K (1993). A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumour suppressor. *Cell* 72: 791-800.

Trovo' AB, Goloni-Bertollo EM, Mancini UM, Rahal P, de Azevedo Jr WF, Tajara EH (2004). Mutational analysis of the GAP-related domain of the neurofibromatosis type 1 gene in Brazilian NF1 patients. *Genetics and molecular biology* 27: 326-330.

Truta B, Allen BA, Conrad PG, Kim YS, Berk T, Gallinger S, Bapat B, Terdiman JP, Sleisenger MH (2003). Genotype and phenotype of patients with both familial adenomatous polyposis and thyroid carcinoma. *Fam Cancer.* 2: 95-99.

Underhill PA, Jin L, Lin AA, Mehdi SQ, Jenkins T, Vollrath D, Davis RW, Cavalli-Sforza LL, Oefner PJ (1997). Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. *Genome Res* 7: 996-1005.

Upadhyaya M, Cheryson A, Broadhead W, Fryer A, Shaw DJ, Huson S, Wallace MR, Andersen LB, Marchuk DA, Viskochil D, Black D, O'Connell P, Collins FS, Harper PS (1990). A 90 kb DNA deletion associated with neurofibromatosis type 1. *J Med Genet* 27: 738-741.

Upadhayaya M, Cooper DN (1998). The mutational spectrum in neurofibromatosis type 1 and its underlying mechanisms. Chap. 5 in: Upadhayaya M, Cooper DN (eds) *Neurofibromatosis Type 1: from Genotype to Phenotype.* BIOS Scientific, Oxford, pp 65-82.

Upadhyaya M, Han S, Consoli C, Majounie E, Horan M, Thomas NS, Potts C, Griffiths S, Ruggieri M, von Deimling A, Cooper DN (2004). Characterization of the somatic

mutational spectrum of the neurofibromatosis type 1 (*NF1*) gene in neurofibromatosis patients with benign and malignant tumors. *Hum Mutat* 23: 134-146.

Upadhyaya M, Majounie E, Thompson P, Han S, Consoli C, Krawczak M, Cordeiro I, Cooper DN (2003). Three different pathological lesions in the *NF1* gene originating de novo in a family with neurofibromatosis type 1. *Hum Genet* 112: 12-17.

Upadhyaya M, Maynard J, Osborn M, Harper PS (1997a). Six novel mutations in the neurofibromatosis type 1 (*NF1*) gene. *Hum Mutat* 10: 248-250.

Upadhyaya M, Maynard J, Osborn M, Huson SM, Ponder M, Ponder BA, Harper PS (1995). Characterisation of germline mutations in the neurofibromatosis type 1 (*NF1*) gene. *J Med Genet.* 32: 706-10.

Upadhyaya M, Osborn M, Maynard J, Harper P (1996a). Characterization of six mutations in exon 37 of neurofibromatosis type 1 gene. *Am J Med Genet.* 67: 421-423.

Upadhyaya M, Osborn MJ, Maynard J, Kim MR, Tamanoi F, Cooper DN (1997b). Mutational and functional analysis of the neurofibromatosis type 1 (*NF1*) gene. *Hum Genet* 99: 88-92.

Upadhyaya M, Roberts SH, Maynard J, Sorour E, Thompson PW, Vaughan M, Wilkie AO, Hughes HE (1996b). A cytogenetic deletion, del(17)(q11.22q21.1), in a patient with sporadic neurofibromatosis type 1 (NF1) associated with dysmorphism and developmental delay. *J Med Genet* 33: 148-152.

Upadhyaya M, Ruggieri M, Maynard J, Osborn M, Hartog C, Mudd S, Penttinen M, Cordeiro I, Ponder M, Ponder B.A.J , Krawczak M, Cooper D.N (1998). Gross deletions of the neurofibromatosis type 1 (*NF1*) gene are predominantly of maternal origin and commonly associated with a learning disability, dysmorphic features and developmental delay. *Hum Genet* 102: 591-597.

Upadhyaya M, Shaw DJ, Harper PS (1994). Molecular basis of neurofibromatosis type 1 (NF1): mutation analysis and polymorphisms in the *NF1* gene. *Hum Mutat.* 4: 83-101.

Upadhyaya M, Shen M, Cherryson A, Farnham J, Maynard J, Huson SM, Harper PS (1992). Analysis of mutations at the neurofibromatosis 1 (*NF1*) locus. *Hum Mol Genet* 1: 735-740.

Velculescu VE, Zhang L, Vogelstein B, Kinzler KW (1995). Serial analysis of gene expression. *Science* 270: 484-487.

Valero MC, de Luis O, Cruces J, Perez Jurado LA (2000). Fine-scale comparative mapping of the human 7q11.23 region and the orthologous region on mouse chromosome 5G: the low-copy repeats that flank the Williams-Beuren syndrome deletion arose at breakpoint sites of an evolutionary inversion(s). *Genomics* 69: 1-13.

Valero MC, Pascual-Castroviejo I, Velasco E, Moreno F, Hernandez-Chico C (1997). Identification of de novo deletions at the NF1 gene: no preferential paternal origin and phenotypic analysis of patients. *Hum Genet* 99: 720-726.

Valero MC, Velasco E, Moreno F, Hernandez-Chico C (1994). Characterization of four mutations in the neurofibromatosis type 1 gene by denaturing gradient gel electrophoresis (DGGE). *Hum Mol Genet* 3: 639-641.

van Asperen CJ, Overweg-Plandsoen WC, Cnossen MH, van Tijn DA, Hennekam RC (1998). Familial neurofibromatosis type 1 associated with an overgrowth syndrome resembling Weaver syndrome. *J Med Genet* 35: 323-327.

Vandenbroucke I, Callens T, De Paepe A, Messiaen L (2002). Complex splicing pattern generates great diversity in human NF1 transcripts. BMC *Genomics* 3: 13.

Vandenbroucke I, Vandesompele J, De Paepe A, Messiaen L (2002a). Quantification of NF1 transcripts reveals novel highly expressed splice variants. *FEBS Lett* 522: 71-76.

Vandenbroucke I, van Doorn R, Callens T, Cobben JM, Starink TM, Messiaen L (2004). Genetic and clinical mosaicism in a patient with neurofibromatosis type 1. *Hum Genet* 114: 284–290.

Venturin M, Gervasini C, Orzan F, Bentivegna A, Corrado L, Colapietro P, Friso A, Tenconi R, Upadhyaya M, Larizza L, Riva P (2004). Evidence for non-homologous end joining and non-allelic homologous recombination in atypical NF1 microdeletions. *Hum Genet* 115: 69-80.

Viskochil D (2002). Genetics of neurofibromatosis 1 and the *NF1* gene. *J Child Neurol* 17: 562–570.

Viskochil DH (2003). It takes two to tango: mast cell and Schwann cell interactions in neurofibromas. *J Clin Invest* 112: 1791-1793.

Viskochil D, Buchberg AM, Xu G, Cawthon RM, Stevens J, Wolff RK, Culver M, Carey JC, Copeland NG, Jenkins NA, White R, O'Connell P. (1990). Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell* 62: 187–192.

Viskochil D, Carey JC (1994). Alternate and related forms of neurofibromatoses. Chapter 19 In *The Neurofibromatoses : A Pathogenetic and Clinical Overview*. Eds Huson S and Hughes R, Chapman & Hall Medical, p445.

Viskochil D, Carey JC (1992). Nosological considerations of the neurofibromatosis. *J Dermatol* 19: 973-880.

Viskochil DH (1999). The structure and function of the *NF1* gene: molecular pathophysiology. In: Neurofibromatosis: phenotype, natural history and pathogenesis (Friedman J, Gutmann D, MacCollin M, Riccardi VM, eds), 3rd edn. Baltimore: John Hopkins University Press, 119–141.

Viskochil D, White R, Cawthon R (1993). The neurofibromatosis type 1 gene *Annu Rev Neurosci* 16: 183-205.

Vogel KS, Klesse LJ, Velasco-Miguel S, Meyers K, Rushing EJ, Parada LF (1999). Mouse tumour model for neurofibromatosis type 1. *Science*. 286: 2176–2179.

Volkert M, Wagner M, Peters C, Waldmann H (2001). The chemical biology of Ras lipidation. *Biol Chem* 382: 1133-1145.

Von-Deimling A, Krone W, Menon AG (1995). Neurofibromatosis type 1: pathology, clinical features and molecular genetics. *Brain Pathol* 5: 153–162.

Von Recklinghausen FD (1882). Ueber die multiplen fibrome der Haut und ihre Beziehung zu den multiplen Neuromen. Hirschwald, Berlin.

Waggoner DJ, Towbin J, Gottesman G, Gutmann DH (2000). Clinic-based study of plexiform neurofibromas in neurofibromatosis 1. *Am J Med Genet* 92:132-135.

Wallace MR, Andersen LB, Saulino AM, Gregory PE, Glover TW, Collins FS (1991). A de novo Alu insertion results in neurofibromatosis type 1. *Nature* 353: 864-866.

Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, Saulino AM, Fountain JW, Brereton A, Nicholson J, Mitchell AL (1990). Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients *Science* 249: 181-186.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002). Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* 417: 941-944.

Wang Q, Lasset C, Desseigne F, Frappaz D, Bergeron C, Navarro C, Ruano E, Puisieux A (1999). Neurofibromatosis and early onset of cancers in hMLH1-deficient children. *Cancer Res* 59:294-297.

Wang Q, Montmain G, Ruano E, Upadhayaya M, Dudley S, Liskay RM, Thibodeau SN, Puisieux A (2003). Neurofibromatosis type 1 gene as a mutational target in a mismatch repair-deficient cell type. *Hum Genet.* 112: 117-23.

Warne PH, Viciana PR, Downward J (1993). Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature* 364: 352-355.

Waschek JA (2002). Multiple actions of pituitary adenylyl cyclase activating peptide in nervous system development and regeneration. *Dev Neurosci* 24: 14–23.

Watson MA, Perry A, Tihan T, Prayson RA, Guha A, Bridge J, Ferner R, Gutmann DH (2004). Gene expression profiling reveals unique molecular subtypes of Neurofibromatosis Type 1-associated and sporadic malignant peripheral nerve sheath tumours. *Brain Pathol* 14: 297-303.

Weber JL, Kwitek AE, May PE, Wallace MR, Collins FS, Ledbetter DH (1990). Dinucleotide repeat polymorphisms at the D17S250 and D17S261 loci. *Nucleic Acids Res* 18: 4640.

Wehner M, Mangold E, Sengteller M, Friedrichs N, Aretz S, Friedl W, Propping P, Pagenstecher C (2005).Hereditary nonpolyposis colorectal cancer: pitfalls in deletion screening in MSH2 and MLH1 genes. *Eur J Hum Genet* 13: 983-986.

Weiming X, Yu Q, Lizhi L, Ponder M, Wallace M, Gangfeng X, Ponder B (1992). Molecular analysis of neurofibromatosis type 1 mutations. *Hum Mutat* 1: 474-477.

Weinberg RA (1991). Tumour suppressor genes. *Science* 254:1138-1146.

Weiss WA, Aldape K, Mohapatra G, Feuerstein BG, Bishop JM (1997). Targeted expression of MYCN causes neuroblastoma in transgenic mice. *EMBO J* 16: 2985-2995.

Wheeler PG, Sadeghi-Nejad A (2005). Simultaneous Occurrence of Neurofibromatosis Type 1and Tuberous Sclerosis in a Young Girl. *Am J Med Genet* A133: 78-81.

White MB, Carvalho M, Derse D, O'Brien SJ, Dean M (1992). Detecting single base substitutions as heteroduplex polymorphisms.*Genomics* 12: 301-306.

White S, Kalf M, Liu Q, Villerius M, Engelsma D, Kriek M, Vollebregt E, Bakker

B, van Ommen GJ, Breuning MH, den Dunnen JT (2002).Comprehensive detection of genomic duplications and deletions in the DMD gene, by use of multiplex amplifiable probe hybridization. *Am J Hum Genet* 71: 365-374.

Whiteside D, McLeod R, Graham G, Steckley JL, Booth K, Somerville MJ, Andrew SE (2002). A homozygous germ-line mutation in the human MSH2 gene predisposes to hematological malignancy and multiple cafe-au-lait spots. *Cancer Res* 62: 359-362.

Wiest V, Eisenbarth I, Schmegner C, Krone W, Assum G (2003). Somatic NF1 mutation spectra in a family with neurofibromatosis type1: toward a theory of genetic modifiers. *Hum Mut* 22: 423-427.

Wimmer K, Eckart M, Meyer-Puttlitz B, Fonatsch C, Pietsch T (2002a). Mutational and expression analysis of the *NF1* gene argues against a role as tumour suppressor in sporadic pilocytic astrocytomas. *J Neuropathol Exp Neurol* 61: 896-902.

Wimmer K, Eckart M, Stadler PF, Rehder H, Fonatsch C (2000). Three different premature stop codons lead to skipping of exon 7 in neurofibromatosis type I patients. *Hum Mutat* 16: 90-91.

Wimmer K, Muhlbauer M, Eckart M, Callens T, Rehder H, Birkner T, Leroy JG, Fonatsch C, Messiaen L (2002b). A patient severely affected by spinal neurofibromas carries a recurrent splice site mutation in the *NF1* gene. *Eur J Hum Genet* 10: 334-338.

Wimmer K, Yao S, Claes K, Kehrer-Sawatzki H, Tinschert S, De Raedt T, Legius E, Callens T, Beiglbock H, Maertens O, Messiaen L (2006). Spectrum of single- and multiexon *NF1* copy number changes in a cohort of 1,100 unselected NF1 patients. *Genes Chromosomes Cancer* 45: 265-276.

Wolf U (1995). The genetic contribution to the phenotype. *Hum Genet.* 95: 127-148.

Wolf U (1997). Identical mutations and phenotypic variation. *Hum Genet.* 100: 305-321.

Wolkenstein P, Mahmoudi A, Zeller J, Revuz J (1995). More on the frequency of segmental neurofibromatosis. *Arch Dermatol* 131:1465.

Woodruff JM (1999). Pathology of tumours of the peripheral nerve sheath in type 1 neurofibromatosis. *Am J Med Genet* 89: 23-30.

Woody RC, Perrot LJ, Beck SA (1992). Neurofibromatosis cerebral vasculopathy in an infant: clinical, neuroradiographic, and neuropathologic studies. *Pediatr Pathol.* 12: 613–619.

Wu BL, Austin MA, Schneider GH, Boles RG, Korf BR (1995). Deletion of the entire NF1 gene detected by the FISH: four deletion patients associated with severe manifestations. *Am J Med Genet.* 59: 528-535.

Wu BL, Boles RG, Yaari H, Weremowicz S, Schneider GH, Korf BR (1997a). Somatic mosaicism for deletion of the entire *NF1* gene identified by FISH. *Hum Genet* 99: 209–213.

Wu BL, Schneider GH, Korf BR (1997b). Deletion of the entire *NF1* gene causing distinct manifestations in a family. *Am J Med Genet* 69: 98-101.

Wu R, Legius E, Robberecht W, Dumoulin M, Cassiman JJ, Fryns JP (1996). Neurofibromatosis type 1 gene mutation in a patient with features of LEOPARD syndrome. *Hum Mutat* 8: 51-56.

Wu R, Lopez-Correa C, Rutkowski JL, Baumbach LL, Glover TW, Legius E (1999). Germline mutations in NF1 patients with malignancies. *Genes Chromosomes Cancer* 26: 376-380.

Xiao W, Stern D, Jain M, Huber CG, Oefner PJ (2001). Multiplex capillary denaturing high-performance liquid chromatography with laser-induced fluorescence detection. Biotechniques 30: 1332-1338.

Xu H, Gutmann DH (1997). Mutations in the GAP-related domain impair the ability of neurofibromin to associate with microtubules. *Brain Res* 759: 149-152.

Xu GF, Lin B, Tanaka K, Dunn D, Wood D, Gesteland R, White R, Weiss R, Tamanoi F (1990). The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements ira mutants of *S. cerevisiae*. *Cell* 63: 835-841.

Xu W, Mulligan LM, Ponder MA, Liu L, Smith BA, Mathew CG, Ponder BA (1992). Loss of *NF1* alleles in phaeochromocytomas from patients with type 1 neurofibromatosis. *Genes Chromosomes Cancer* 4: 337-342.

Xu GF, O'Connell P, Viskochil D, Cawthon R, Robertson M, Culver M, Dunn D, Stevens J, Gesteland R, White R (1990a). The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell* 62: 599-608.

Yamauchi T, Houkin K, Tada M, Abe H (1997). Familial occurrence of moyamoya disease. *Clin Neurol Neurosurg.* 99: S162-167.

Yamauchi T, Tada M, Houkin K, Tanaka T, Nakamura Y, Kuroda S, Abe H, Inoue T, Ikezaki K,Matsushima T, Fukui M (2000). Linkage of familial Moyamoya disease (spontaneous occlusion of the Circle of Willis) to chromosome 17q25. *Stroke.*31: 930-935.

Yang FC, Ingram DA, Chen S, Hingtgen CM, Ratner N, Monk KR, Clegg T, White H, Mead L, Wenning MJ, Williams DA, Kapur R, Atkinson SJ, Clapp DW (2003). Neurofibromin-deficient Schwann cells secrete a potent migratory stimulus for $Nf1^{+/-}$ mast cells. *J Clin Invest* 112: 1851-1861.

Yazdizadeh M, Tapia JL, Baharvand M, Radfar L (2004). A case of neurofibromatosis-Noonan syndrome with a central giant cell granuloma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 98: 316-320.

Yeoh EJ, Ross ME, Shurtleff SA, Williams WK, Patel D, Mahfouz R, Behm FG, Raimondi SC, Relling MV, Patel A, Cheng C, Campana D, Wilkins D, Zhou X, Li J, Liu H, Pui CH, Evans WE, Naeve C, Wong L, Downing JR (2002). Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell* 1: 133-143.

Yla-Outinen H, Koivunen J, Nissinen M, Bjorkstrand AS, Paloniemi M, Korkiamaki T, Peltonen S, Karvonen SL, Peltonen J (2002).NF1 tumour suppressor mRNA is targeted to the cell-cell contact zone in Ca(2+)-induced keratinocyte differentiation. *Lab Invest* 82: 353-361.

Yoshiura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T, Hirohashi S (1995). Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc Natl Acad Sci USA* 92: 7416–7419.

Youil R, Kemper BW, Cotton RG (1995). Screening for mutations by enzyme mismatch cleavage with T4 endonuclease VII. *Proc Natl Acad Sci U S A* 92: 87-91.

Youssoufian H (1996). Natural gene therapy and the Darwinian legacy. *Nature Genet* 13: 255-256.

Youssoufian H, Pyeritz RE (2002). Mechanisms and consequences of somatic mosaicism in humans. *Nat Rev Genet* 3: 748-758.

Yunoue S, Tokuo H, Fukunaga K, Feng L, Ozawa T, Nishi T, Kikuchi A, Hattori S, Kuratsu J, Saya H, Araki N (2003). Neurofibromatosis type 1 tumour suppressor neurofibromin regulates neuronal differentiation via its GTPase-activating protein function toward Ras. *J Biol Chem* 278: 26958-26969.

Zanca A, Zanca A (1980). Antique illustrations of neurofibromatosis. *Int J Dermatol* 19: 55-58.

Zatkova A, Messiaen L, Vandenbroucke I, Wieser R, Fonatsch C, Krainer AR, Wimmer K (2004). Disruption of exonic splicing enhancer elements is the principal cause of

exon skipping associated with seven nonsense or missense alleles of NF1. *Hum Mutat* 24: 491-501.

Zendman AJ, Ruiter DJ, Van Muijen GN (2003). Cancer/testis-associated genes: identification, expression profile, and putative function. *J Cell Physiol* 194: 272–88.

Zhong J, Spiegel R, Boltshauser E, Schmid W (1993). Two novel mutations: 108delAG and 5816insG in the *NF1* gene detected by SSCP analysis. *Hum Mol Genet* 2: 1491-1492.

Zhou ZH, Akgun E, Jasin M (2001). Repeat expansion by homologous recombination in the mouse germ line at palindromic sequences. *Proc Natl Acad Sci U S A* 98: 8326-8333.

Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF (2002). Neurofibromas in *Nf1*: Schwann cell origin and role of tumor environment. *Science* 296: 920-922.

Zhu Y, Parada LF (2001). Neurofibromin, a tumour suppressor in the nervous system. *Exp Cell Res* 264: 19-28.

Zhu Y, Romero MI, Ghosh P, Ye Z, Charnay P, Rushing EJ, Marth JD, Parada LF (2001). Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the brain. *Genes Dev* 15: 859-876.

Zlotogora J., (1998). Germ line mosaicism. *Hum Genet* 102: 381-386.

Zori RT, Lupski JR, Heju Z, Greenberg F, Killian JM, Gray BA, Driscoll DJ, Patel PI, Zackowski JL (1993). Clinical, cytogenetic, and molecular evidence for an infant with Smith-Magenis syndrome born from a mother having a mosaic 17p11.2p12 deletion. *Am J Med Genet* 47: 504-511.

Zou M-X, Butcher DT, Sadikovic B, Groves TC, Yee S-P, Rodenhiser DI (2003). Characterization of functional elements in the neurofibromatosis (*NF1*) proximal promoter region. *Oncogene* 23: 330–339.

Appendix

The table lists all of the *NF1* mutations published to date (HGMD)

Complex rearrangements (including inversions)

Description	Reference
Complex splicing, multiple skipped exons, incl. 4b, ins 31	Park & Pivnick (1998)
Del G nt 2430, ins GTCTT nt 2427	Fahsold *et al.* (2000)
Del. 12021 bp incl. ex. 32-39, Ins. 30 bp	Lazaro *et al.* (1995)
Del. 28 bp, Ins. approx 200 bp nt. 4784	Skuse (1998)
GC-AA Q315Q L316M	Colapietro *et al.* (2003)
Translocation t(17;22) breakpoint in intr. 31 (different to Kehrer-Sawatzki)	Kurahashi *et al.* (2003)
Translocation t(17;22) breakpoint in intr. 31	Kehrer-Sawatzki *et al.* (1997a)

Gross deletions

Description	Reference
105 bp nt. 731 I5E6+1 to I5E6+105 (mutation described at cDNA level)	Heim *et al.* (1995)
11 kb 3' end (mutation described at genomic DNA level)	Viskochil *et al.* (1990)
12 kb in. 31-39 (mutation described at genomic DNA level)	Lazaro *et al.* (1994)
190 kb (mutation described at genomic DNA level)	Viskochil *et al.* (1990)
1.2 Mb incl. entire gene (mutation described at genomic DNA level)	Kehrer-Sawatzki *et al.* (2004)
1.3 Mb incl. entire gene (mutation described at genomic DNA level)	Venturin *et al.* (2004)
1.4 MB incl. entire gene (mutation described at genomic DNA level)	Kehrer-Sawatzki *et al.* (2004)
1.5 Mb incl. entire gene (mutation described at genomic DNA level)	Jenne *et al.* (2001)
2 Mb incl. entire gene (mutation described at genomic DNA level)	Kehrer-Sawatzki *et al.* (2005)
21 bp nt. -14 to +7 (mutation described at genomic DNA level)	Mattocks *et al.* (2004)
23 bp nt. 4088 (mutation described at cDNA level)	Heim *et al.* (1995)

23 bp nt. 597
(mutation described at genomic DNA level)

Mattocks *et al.* (2004)

2829 bp E37I37+91 to E38I38+1899
(mutation described at genomic DNA level)

Osborn & Upadhyaya (1999)

3 Mb incl. entire gene
(mutation described at genomic DNA level)

Venturin *et al.* (2004)

318 bp ex. 10c-12a
(mutation described at cDNA level)

Heim *et al.* (1995)

40 kb
(mutation described at genomic DNA level)

Upadhyaya *et al.* (1992)

40 kb
(mutation described at genomic DNA level)

Viskochil *et al.* (1990)

517 bp ex. 33
(mutation described at genomic DNA level)

Xu *et al.* (1992)

700 kb
(mutation described at genomic DNA level)

Kayes (1992)

74 bp ex. 14
(mutation described at cDNA level)

Osborn & Upadhyaya (1999)

80 kb
(mutation described at genomic DNA level)

Upadhyaya *et al.* (1992)

90 kb 5' end
(mutation described at genomic DNA level)

Upadhyaya *et al.* (1990)

Vandenbroucke *et al.* (2004)

99571 bp, ex. 13-28
(mutation described at genomic DNA level)

entire gene
(mutation described at genomic DNA level)

Serra *et al.* (2001)

entire gene
(mutation described at genomic DNA level)

Riva *et al.* (1996)

entire gene
(mutation described at genomic DNA level)

Rasmussen *et al.* (1998)

ex. 1 - 7
(mutation described at genomic DNA level)

Fang *et al.* (2001)

ex. 4b - 3' UTR
(mutation described at genomic DNA level)

Fang *et al.* (2001)

incl. entire gene
(mutation described at genomic DNA level)

Petek *et al.* (2003)

incl. entire gene
(mutation described at genomic DNA level)

Jenne *et al.* (2000)

incl. entire gene
(mutation described at genomic DNA level)

Dorschner *et al.* (2000)

incl. entire gene
(mutation described at genomic DNA level)

Dorschner *et al.* (2000)

incl. entire gene (mutation described at genomic DNA level)	Dorschner *et al.* (2000)
incl. ex. 3 (mutation described at genomic DNA level)	Hoffmeyer *et al.* (1994)
>200 kb ex. 2 to in. 27 (mutation described at genomic DNA level)	Leppig *et al.* (1997)
>275 kb (mutation described at genomic DNA level)	Leppig *et al.* (1996)
>30 kb ex. 28-37 (mutation described at genomic DNA level)	Lazaro *et al.* (1993)
>700 kb (mutation described at genomic DNA level)	Wu *et al.* (1997)
>700 kb (mutation described at genomic DNA level)	Wu *et al.* (1995)
>700 kb (mutation described at genomic DNA level)	Tonsgard *et al.* (1997)
>700 kb entire gene (mutation described at genomic DNA level)	Kayes *et al.* (1994)
~17.5 kbp incl. ex. 39-45 (mutation described at genomic DNA level)	Li *et al.* (2003)
~2 Mb (mutation described at genomic DNA level)	Leppig *et al.* (1997)
~700 kb incl. entire gene (mutation described at genomic DNA level)	van Asperen *et al.* (1998)

Gross insertions & duplications

Description	Reference
Insertion of 10 kb (mutation described at genomic DNA level)	Upadhyaya *et al.* (1992)
Duplication of 23 bp c.5556-5578 (mutation described at cDNA level)	De Luca *et al.* (2004)
Duplication of 42 bp cd. 1699-1713 (near perfect) (mutation described at genomic DNA level)	Tassabehji *et al.* (2003)
Insertion of 74 bp from intr. 25, nt 4247 (mutation described at genomic DNA level)	Fahsold *et al.* (2000)
Insertion of Alu seq. I5E6-44 (mutation described at genomic DNA level)	Wallace *et al.* (1991)

Small indels

Accession Number	Location/codon	Deletion	Insertion	Reference
CX011901	326	CTGTGTC^AAAcTGTGTAAAGC	tt	Serra *et al.* (2001)
CX021481	677	GAACC^CCCCCaATTTGCCGAC	cg	Origone *et al.* (2002)
CX041668	812	AAGACC^ATTGttAAGAGGCGAA	a	De Luca *et al.* (2004)
CX030372	848	GCCCTT^GGAGgagTGTGCCTCCA	a	De Luca *et al.* (2003)
CX972753	1049	AGACTGG^GTTatGGGAACATCA	g	Abernathy *et al.* (1997)
CX031927	1179	AGCTACA^TTTatggaaGTTCTGACAA	g	Ars *et al.* (2003)
CX041669	1400	TCTCA^CCGTAtgAAGCAGGGAT	a	De luca *et al.* (2003)
CX020619	1742	TGCTGTC^CAAgtaACTTCAGCAG	t	Kluwe *et al.* (2002)
CX004454	2474	CCATGG^TCCTctCCCAAAGGTT	ag	Fahsold *et al.* (2000)

Small deletions

Accession Number	Location/codon	Deletion	Reference
CD031848	29	CAGCAG^AACAcacataccaaagtCAGTACTGAG	Ars *et al.* (2003)
CD040910	42	CAAGGAA^TGTctAATCAATATT	Mattocks *et al.* (2004)
CD033206	65	AAGAAT^GTTAacaatatg_E2I2_gtgagtattTGGGTTACTG	Kluwe *et al.* (2003b)
CD000946	73	ATTTGGA^GAAgCTGCTGAAAA	Fahsold *et al.* (2000)
CD020575	76	GCTGCT^GAAAaAAATTTATAT	Kluwe *et al.* (2002)
CD020576	84	TCTCAG^TTGAttATATTGGATA	Kluwe *et al.* (2002)
CD031849	115	TGCTG^CCAGAaATCTGCCATT	Ars *et al.* (2003)
CD000947	141	CTGGG^GTTTTattttCTCTCAGCTG	Fahsold *et al.* (2000)
CD000077	148	AGCTGC^AACAaCTTCAATGCA	Ars *et al. et al.* (2000)
CD992532	164	AGGAA^TTAACtgttTGTTCAGAAG	Osborn & Upadhyaya (1999)
CD000948	165	GGAATTA^ACTgtTTGTTCAGAA	Toliat *et al. et al.* (2000)
CD000949	175	GATGTT^CATGaTATAGAATTG	Fahsold *et al.* (2000)
CD000078	176	TGTTCAT^GATatAGAATTGTTA	Ars *et al.* (2000)
CD031850	183	CAGTAT^ATCAaTGTGGATTGT	Ars *et al.* (2003)
CD000079	193	AAAACGA^CTCcTGAAGG_E4bI4b_GTAA	Ars *et al.* (2000)
CD000950	195	ACTCCTG^AAGg_E4bI4b_GTAAGTTTAA	Fahsold *et al.* (2000)

CD972345	219	TTATAG_I4cE5_^GCATtTTGGAACTGG	Abernathy *et al.* (1997)
CD031851	228	AATTAT^CCAGaTGAATTTACA	Ars *et al.* (2003)
CD000951	234	TACAAAA^CTGtaCCAGATCCCA	Fahsold *et al.* (2000)
CD000952	251	CTATTT^GACTtGGTGGATGGT	Fahsold *et al.* (2000)
CD000953	279	GTGTCCA^GAAataaTCCAGGATAT	Fahsold *et al.* (2000)
CD031852	292	GATGAA^AACAaCATGAATAAG	Ars *et al.* (2003)
CD000954	295	AACATG^AATAaG_E6I6_GTAAGGAGG	Fahsold *et al.* (2000)
CD982823	305	TACGA^AAAGCtCTTGCTGGCC	Hoffmeyer *et al.* (1998)
CD040911	305	TCTACGA^AAAgCTCTTGCTGG	Mattocks *et al.* (2004)
CD000955	318	GCTGACA^GAAagTGCTGCAATT	Fahsold *et al.* (2000)
CD972346	326	GTGTC^AAACTgtGTAAAGCAAG	Abernathy *et al.* (1997)
CD030336	332	AGCAAGT^ACTtACATCAATTG	De Luca *et al.* (2003)
CD031853	332	CAAGT^ACTTAcATCAATTGGG	Ars *et al.* (2003)
CD972347	339	GAAGAT^AACTctGTCATTTTCC	Upadhyaya *et al.* (1997a)
CD031854	340	AGATAAC^TCTgtcattttcctACTTGTTCAG	Ars *et al.* (2003)
CD041590	340	AGATAAC^TCTgtCATTTTCCTA	De Luca *et al.* (2004)
CD011872	365	GCCATTC^TCAagaggcAGTCAGCCTG	Serra *et al.* (2001)
CD041591	373	TGCAGAT^GTGgATCTAATGAT	De Luca *et al.* (2004)
CD992533	375	GTGGAT^CTAAtgatTGACTGCCTT	Osborn & Upadhyaya (1999)
CD031855	401	TTTTTCTATAg_I8E9_atctgcctggctcaG^AATTCACCT	Ars *et al.* (2003)
CD000956	418	TCGAATC^ATCaCCAAT_E9I9_GTAAG	Fahsold *et al.* (2000)
CD000957	445	TTGGT^GAAACaCTTCATAAAG	Fahsold *et al.* (2000)
CD030337	446	GTGAA^ACACTtCATAAAGCAG	De Luca *et al.* (2003)
CD031856	471	TAAAGAA^AAAgTAACAAGCCT	Ars *et al.* (2003)
CD982824	482	AAACCT^ACAGaCCTGGAGACA	Horiuchi *et al.* (1998)
CD000958	494	CTTCTC^TTGTccATGGTGAAAC	Fahsold *et al.* (2000)
CD000959	509	CTTGCTT^TGT_E10bI10b_gTAAGTATTTA	Fahsold *et al.* (2000)
CD031857	509	GCTT^TGT_E10bI10b_GTAagtaTTTATTTATG	Ars *et al.* (2003)
CD962097	513	CCAAGA^AAACagGGGCCCGAAA	Robinson *et al.* (1996)
CD020577	514	AGAAAA^CAGGgGCCCGAAACC	Kluwe *et al.* (2002)
CD000960	515	AAACAG^GGGCcCGAAACCCAA	Fahsold *et al.* (2000)
CD013859	557	TAGAT^AGCATtGATTTGTGGA	Han *et al.* (2001)
CD031858	559	TAGCATT^GATtTGTGGAATCC	Ars *et al.* (2003)
CD041592	563	GGAAT^CCTGAtGCTCCTGTAG	De Luca *et al.* (2004)
CD000080	585	AGAAA^TTAACtaGTCATCAAAT	Ars *et al.* (2000)
CD982825	585	CAAGAAA^TTAactaGTCATCAAAT	Park *et al.* (1998)
CD032129	585	CAAGAAA^TTAaCTAGTCATCA	Origone *et al.* (2003)

CD000961	615	TAAAAAT^AAG_E12aI12a_gtaagCAAAATGACA	Fahsold et al. (2000)
CD020578	635	GATATT^CCTTctAGTGGAAATA	Kluwe et al. (2002)
CD000962	645	AATGTCC^ATGgATCATGAAGA	Fahsold et al. (2000)
CD000963	676	AGCGGA^ACCCcCCCAATTTGC	Fahsold et al. (2000)
CD031859	692	GGCCCTG^TACatGTTTCTGTGG	Ars et al. (2003)
CD040912	707	TTGCC^ATGTCctGTTTCCGCCA	Mattocks et al. (2004)
CD013860	710	CCTGT^TTCCGccACCTCTGTGA	Han et al. (2001)
CD041593	717	GAGGAA^GCAGaTATCCGGTGT	De Luca et al. (2004)
CD000964	729	CAGTG^CATAAcctctTGCCCAACTA	Fahsold et al. (2000)
CD030338	756	GCACTT^CAGAaaAGAGTGATGG	De Luca et al. (2003)
CD000965	757	TTCAG^AAAAGagTGATGGCACT	Fahsold et al. (2000)
CD031860	769	AGCAT^CCCACtgcagGAAACACTGA	Ars et al. (2003)
CD033207	832	TGACACA^GACtCCCTACAGGA	Kluwe et al. (2003b)
CD020579	860	TTCTGGC^CTGgCAACCTATAG	Kluwe et al. (2002)
CD972348	888	AAACGCA^GATaCACCTGTCAG	Maynard et al. (1997)
CD000966	888	AACGCA^GATAcACCTGTCAGC	Fahsold et al. (2000)
CD000967	891	TACACCT^GTCaGCAAATTTAT	Fahsold et al. (2000)
CD972349	919	TTAAG^GATCTgGTGGGTCTAG	Maynard et al. (1997)
CD041594	944	CAGCAAG^TTTtTTGACTCCCA	De Luca et al. (2004)
CD000968	947	TTGAC^TCCCAaGGACAG_E16I16_GTAA	Fahsold et al. (2000)
CD001517	950	AAGGA^CAG_E16I16_GTaaagtgTTCTCTTATT	Messiaen et al. (2000)
CD001518	951	TTTGTTCTTTctttAG_I16E17_^GTTTTATT	Messiaen et al. (2000)
CD000081	953	TCTTTCTTTAg_I16E17_gtttTA^TTGACTGA	Ars et al. (2000)
CD931025	989	GCATT^GAAACaatGATGTTAAAT	Shen et al. (1993)
CD031861	989	GCATT^GAAACaaTGATGTTAAA	Ars et al. (2003)
CD951796	1016	AAACTG^TGTCaattAGTTGAAGTA	Heim et al. (1995)
CD000969	1019	AATTA^GTTGAaGTAATGATGG	Fahsold et al. (2000)
CD982826	1050	CTGGGTT^ATGggAACATCAAAC	Park et al. (1998)
CD000970	1059	AGCAGAT^GATgATGTAAAATG	Fahsold et al. (2000)
CD972350	1063	GTAAAA^TGTCtTACAAG_E19aI19a_GTAA	Abernathy et al. (1997)
CD001519	1064	AAATGT^CTTAcAAG_E19aI19a_GTAAAAA	Messiaen et al. (2000)
CD031862	1071	GGACCAG^GCAagcatggaagcAGTAGTTTCA	Ars et al. (2003)
CD031863	1131	TGGCAGG^AAAcgtggcaTGTCTCGGAG	Ars et al. (2003)
CD031864	1134	GTGGC^ATGTCtcGGAGGCTGGC	Ars et al. (2003)
CD972351	1151	TGTCA^AACTTactcAATGCCAACG	Upadhyaya et al. (1997b)
CD972352	1153	AAACTTA^CTCaaTGCCAACGTA	Upadhyaya et al. (1997b)
CD031865	1169	TTAGGT^TACCaCAAGGATCTC	Ars et al. (2003)

CD000971	1174	ATCTC^CAGACaaGAGCTACATT	Fahsold *et al.* (2000)
CD040913	1175	TCCAG^ACAAGaGCTACATTTA	Mattocks *et al.* (2004)
CD040914	1181	TTATG^GAAGTtCTGACAAAAA	Mattocks *et al.* (2004)
CD000972	1214	GGTCACA^ATGatgGGTGATCAAG	Fahsold *et al.* (2000)
CD033208	1228	GCTCTG^GCCAaTGTGGTTCCT	Kluwe *et al.* (2003b)
CD972353	1242	GCTCGA^GTTCtGGTTACTCTG	Upadhyaya *et al.* (1997b)
CD040312	1243	GAGTT^CTGGTtACTCTGTTTG	Upadhyaya *et al.* (2004)
CD972354	1245	CTGGTT^ACTCtGTTTGATTCT	Upadhyaya *et al.* (1997b)
CD000973	1245	CTGGTT^ACTCtgttTGATTCTCGG	Fahsold *et al.* (2000)
CD040915	1252	CGGCAT^TTACtctacCAACTGCTCT	Mattocks *et al.* (2004)
CD000082	1252	GGCAT^TTACTctaccAACTGCTCTG	Ars *et al.* (2000)
CD000083	1273	CATGCAG^ACTctCTTCCGAGGC	Ars *et al.* (2000)
CD041595	1282	TGGCC^AGTAAaATAATGACAT	De Luca *et al.* (2004)
CD041596	1286	TAATG^ACATTcTGTTTCAAG_E22I22_G	De Luca *et al.* (2004)
CD000974	1303	CCTGGAT^CCTtTATTACGAAT	Fahsold *et al.* (2000)
CD000975	1338	CAGCGG^AACCtCCTTCAGATG	Fahsold *et al.* (2000)
CD001520	1341	CCTCCTT^CAGatGACTGAAAAG	John *et al.* (2000)
CD972355	1357	TCAGAA^TTCCcCCCTCAACTT	Upadhyaya *et al.* (1997b)
CD040916	1371	TCAACTCCTTgttTTTAG_I23E24_^GTGGT	Mattocks *et al.* (2004)
CD941731	1383	TCGGT^GCAGTaGGAAGTGCCA	Abernathy *et al.* (1994)
CD972356	1385	TGCAGTA^GGAaGTGCCATGTT	Upadhyaya *et al.* (1997b)
CD020580	1387	AGGAAGT^GCCaTGTTCCTCAG	Kluwe *et al.* (2002)
CD931026	1396	ATCCT^GCCATtGTCTCACCGT	Anglani *et al.* (1993)
CD004092	1397	TCCTGCC^ATTgTCTCACCGTA	Park (2000) *et al.*
CD000976	1436	TCACA^AAAGAagaaCATATGCGGC	Fahsold *et al.* (2000)
CD031065	1437	CACAAAA^GAAgaaCATATGCGGC	Baralle *et al.* (2003)
CD031866	1474	ATCAT^AGTCTtTCCTTCATAA	Ars *et al.* (2003)
CD000977	1476	GTCTT^TCCTTcATAAGTGACG	Fahsold *et al.* (2000)
CD030339	1493	TGGAAC^AATCagGAGAAAATTG	De Luca *et al.* (2003)
CD000978	1495	ATCAG^GAGAAaATTGGGCAGT	Fahsold *et al.* (2000)
CD951797	1524	TCTTGCA^TACcTGGGTCCTCC	Upadhyaya *et al.* (1995)
CD031867	1537	ATACA^CACTGgtCCAGCCTTAA	Ars *et al.* (2003)
CD951798	1543	CCTTAAC^CTTaCCAGTTCAAA	Upadhyaya *et al.* (1995)
CD033209	1565	ATTCAAG^GCTttGAAAACGTTAA	Kluwe *et al.* (2003b)
CD032130	1566	AGGCT^TTGAAaACGTTAAGTA	Origone *et al.* (2003a)
CD000979	1567	GCTTTG^AAAAcGTTAAGTATT	Fahsold *et al.* (2000)
CD000084	1590	TTGCA^CGGAG_E27bI27b_gtaagaaatACTATGTTTT	Ars *et al.* (2000)

CD031868	1609	TTACTG^ACTTtaaagccatattatgCAAAGCCATA	Ars et al. (2003)
CD920890	1622	ATTGTA^GTGGacCTTACCCATA	Weiming et al. (1992)
CD972357	1637	CAGAC^TTTCTctctAAGTGGTTTG	Side et al. (1997)
CD994673	1657	GCAGTC^TATAtctataACTGTAACTC	Wu et al. (1999)
CD931027	1669	AGTAC^ACCAAgTATCATGAGC	Colman et al. (1993)
CD972358	1674	CATGAG^CGGCtGCTGACTGGC	Side et al. (1997)
CD001521	1677	CTGCTG^ACTGgCCTCAAAGGT	Messiaen et al. (2000)
CD000980	1683	AGGTAGC^AAAaggcttgTTTTCATAGA	Fahsold et al. (2000)
CD931028	1692	CTGTCCT^GGGaaactggctgagcACATAGAGCA	Shen et al. (1993)
CD031869	1698	GAGCAC^ATAGagCATGAACAAC	Ars et al. (2003)
CD931029	1702	CATGAA^CAACagAAACTACCTG	Zhong et al. (1993)
CD910541	1707	CTACCT^GCTGccaccTTGGCTTTAG	Stark et al. (1991)
CD000981	1717	GGACCTG^AAGgTATTCCACAA	Fahsold et al. (2000)
CD991814	1722	CACAAT^GCTCtcAAGCTAGCTC	Peters et al. (1999b)
CD020581	1727	CTAGCT^CACAaAGACACCAAA	Kluwe et al. (2002)
CD991815	1734	TTTCT^ATTAAa_E28I28_gtaaGTTCCAGTCT	Peters et al. (1999b)
CD000982	1749	AGAGCGA^ACAaaaGTCCTAGGGC	Fahsold et al. (2000)
CD001522	1757	ATCAGTC^TTTcTAAATGACAT	John et al. (2000)
CD031870	1767	CGGAA^ATTGAaGAAATCTGCC	Ars et al. (2003)
CD972359	1795	GCACCAG^GAGtgTGAAGCCATT	Upadhyaya et al. (1997a)
CD000983	1799	GAAGCC^ATTGtCCAGTCTATC	Fahsold et al. (2000)
CD982827	1817	CCCGAC^TCTAtCCCCCAACAC	Park et al. (1998)
CD031871	1820	ATCCCC^CAACacACCAAGATTC	Ars et al. (2003)
CD000984	1827	GGCCA^AAAGAtGTCCCTGGGA	Fahsold et al. (2000)
CD001523	1855	TATAAT^CTTCtGTGTGCCTTA	Messiaen et al. (2000)
CD000985	1861	CTTAACT^TGTacCTTTAATTTA	Fahsold et al. (2000)
CD000986	1863	GTACC^TTTAAtttaaAAATCGAGGG	Harder et al. (1999)
CD000085	1872	CAGTTA^CTAGagacatcaggtttatgTATCCCTGCC	Ars et al. (2000)
CD041597	1885	AACACC^CTCTtTATTGTCTCT	De Luca et al. (2004)
CD972360	1890	TCTCT^ATTAGtaagACACTGGCAG	Abernathy et al. (1997)
CD941732	1892	TTAGT^AAGACactgGCAGCCAATG	Hatta et al. (1994)
CD031066	1929	TACATG^ACTCcATGGCTGTCA	Kluwe et al. (2003a)
CD030340	1931	GACTCCA^TGGctgTCAAATCTAG	De Luca et al. (2003)
CD001524	1932	CCATGG^CTGTcAAATCTAGTT	Messiaen et al. (2000)
CD941733	1947	GCCAAA^CGACaaAGAGTTACTG	Valero et al. (1994)
CD000987	1948	AACGA^CAAAGagTTACTGCTAT	Fahsold et al. (2000)
CD000086	1962	ATGACC^ATCAaTGAAAAACAG	Ars et al. (2000)

CD040917	1965	ATGAA^AAACAgaTGTACCCATC	Mattocks *et al.* (2004)
CD941734	1982	ACTAG_I31E32_^ATTACaGATCTGCTTG	Hatta *et al.* (1994)
CD040313	2038	TAATT^GACAAgACATGCTTAT	Upadhyaya *et al.* (2004)
CD040918	2060	TGCTATT^TTAgcacgctaCATGCTGATG	Mattocks *et al.* (2004)
CD000988	2073	CAATTCC^CTTgATGTGGCAGC	Fahsold *et al.* (2000)
CD000087	2131	AGACTC^AGTCtgacagagttCTCATTACCC	Ars *et al.* (2000)
CD000989	2155	CTGTC^ATTGCcTTCCGTTCCA	Fahsold *et al.* (2000)
CD000990	2156	GTCATT^GCCTtCCGTTCCAGT	Fahsold *et al.* (2000)
CD000991	2157	CATTGCC^TTCcGTTCCAGTTA	Fahsold *et al.* (2000)
CD972361	2172	TCCTAT^GAGAgaGAGACTTTTG	Hudson *et al.* (1997)
CD001525	2192	GGAGATC^ATGgag_E34I34_gtaTAGAAGCCAA	Messiaen *et al.* (2000)
CD000992	2201	TATTCCA^ACGtGCAAGTGGCT	Fahsold *et al.* (2000)
CD000993	2213	AGCT^CAAAG_E35I35_GtATGTCCTAAA	Fahsold *et al.* (2000)
CD040919	2213	TAGCT^CAAAG_E35I35_gTATGTCCTAA	Mattocks *et al.* (2004)
CD031872	2216	AG_I35E36_ATTT^GCATtCCAATATAAT	Ars *et al.* (2003)
CD031067	2254	TTCCTAAAAG_I36E37_gCA^CTTGAGAG	Kluwe *et al.* (2003a)
CD021434	2257	GAGAGT^TGCTtAAAAGGACCT	Origine *et al.* (2002)
CD951799	2262	GACCT^GACACttacAACAGTCAAG	Robinson *et al.* (1995)
CD000994	2265	ACTTAC^AACAgtCAAGTTCTGA	Fahsold *et al.* (2000)
CD972362	2269	AAGTT^CTGATagaagCTACAGTAAT	Abernathy *et al.* (1997)
CD962098	2279	CTAACC^AAATtacAGCCACTTCT	Upadhyaya *et al.* (1996)
CD000995	2364	AACTCT^AACTtTAACTTTGCA	Fahsold *et al.* (2000)
CD941735	2365	CTCTAAC^TTTaactttGCATTGGTTG	Abernathy *et al.* (1994)
CD972363	2372	GTTGGA^CACCttttaAAAG_E39I39_GTAAAA	Rodenheiser *et al.* (1997)
CD013861	2396	TACACTA^CTAaCTCTGGTTAA	Han *et al.* (2001)
CD021435	2397	ACTACTA^ACTctGGTTAACAAA	Origone *et al.* (2002)
CD031873	2401	TTAAC^AAACAcaGAAATTGTGA	Ars *et al.* (2003)
CD000996	2402	AACAAA^CACAgaAATTGTGACA	Fahsold *et al.* (2000)
CD031068	2418	CCTAC^TTAGCag_E40I40_GTAAAAACAC	Kluwe *et al.* (2003°)
CD951800	2422	CCTTTTAG_I40E41_CTtTA^CTTACAGT	Heim *et al.* (1995)
CD000997	2422	G_I40E41_CTTTA^CTTAcaGTGTCTGAAG	Fahsold *et al.* (2000)
CD000998	2428	TGAAGAA^GTTcGAAGTCGCTG	Fahsold *et al.* (2000)
CD021436	2443	CTTCTT^ACTGaTATTTCAATG	Origone *et al.* (2002)
CD000088	2445	ACTGAT^ATTTcAATGGAAAAT	Ars *et al.* (2000)
CD000999	2455	GATACA^TATCccATTCATCATG	Fahsold *et al.* (2000)
CD011873	2457	ATATCCC^ATTcATCATGGTGA	Serra *et al.* (2001)
CD992534	2485	TTGCA^GCCACcTATCCAACTG	Osborn & Upadhyaya (1999)

CD001000	2522	AGGAAA^AGTTtTGATCACTTG	Fahsold *et al.* (2000)
CD001526	2542	ATCAGGG^ATCacAACACCCCCC	John *et al.* (2000)
CD041598	2561	AG_I43E44_AAACT^CAGagGATTTCCTCA	De Luca *et al.* (2004)
CD031874	2573	CATTTA^CGTAaAGTTTCAGTG	Ars *et al.* (2003)
CD931030	2581	GAATCA^AATGttctcttggaTGAAGAAGTA	Shen *et al.* (1993)
CD001527	2627	CCAGT^GTTGTgtTTCCCAAAGT	Messiaen *et al.* (2000)
CD020582	2632	CCCAAA^GTCTtTCCTGTTGT_E45I45_G	Kluwe *et al.* (2002)
CD041599	2633	AAAGTC^TTTCcTGTTGT_E45I45_GTAA	De Luca *et al.* (2004)
CD001528	2671	ACCAT^GAAGAaTCCCCACCAC	Messiaen *et al.* (2000)
CD001001	2674	GAATCC^CCACcACAATACCAA	Fahsold *et al.* (2000)
CD031875	2680	CCAAACA^TCTtACCTGCAAA_E46I46_G	Ars *et al.* (2003)
CD992535	2693	TGGCGG^TTTGcAGGACCGTTT	Osborn & Upadhyaya (1999)
CD000089	2711	GCTTATT^GTTaaGTTTCTTGAT	Ars *et al.* (2000)

Small insertions

Accession Number	Nucleotide	Codon	Insertion	Reference
CI951961	2027		C	Heim *et al.* (1995)
CI001012	2850		TT	Fahsold *et al.* (2000)
CI972651	4873		A	Colman *et al.* (1997)
CI972652	5289		AA	Colman *et al.* (1997)
CI920945	5446		T	Upadhyaya *et al.* (1992)
CI920946	5449		C	Upadhyaya *et al.* (1992)
CI920947	5662		C	Upadhyaya *et al.* (1992)
CI920948	5678		T	Upadhyaya *et al.* (1992)
CI931095	5816		G	Zhong *et al.* (1993)
CI931096	5852		TT	Ainsworth *et al.* (1993)
CI941893	6519		G	Purandare *et al.* (1994)
CI972653	6709		C	Colman *et al.* (1997)
CI972654	6790		TT	Boddrich *et al.* (1997)
CI962317	6791		A	Upadhayaya *et al.* (1996a)
CI941894	6922		GATGAGGTCA	Legius *et al.* (1994)
CI941895	7486		GG	Purandare *et al.* (1994)
CI032769	E3I3+4		G	Ars *et al.* (2003)

CI002562	E8I8+3		TAAA	Fahsold (2000)
CI031905	E15I15+2		T	Ars et al. (2003)
CI031906	E17I17+2		T	Ars et al. (2003)
CI032770	E10bI10b+5		A	Ars et al. (2003)
CI001013	227	76	A	Fahsold et al. (2000)
CI031907	413	139	CT	Ars et al. (2003)
CI003802	540	179	A	Toliat et al. (2000)
CI972869	752	180	A	Daschner et al. (1997)
CI000103	717	240	TCCCACAG	Ars et al. (2000)
CI000104	723	242	A	Ars et al. (2000)
CI001580	987	329	A	Messiaen et al. (2000)
CI031908	989	330	C	Ars et al. (2003)
CI031909	998	333	A	Ars et al. (2003)
CI032771	1019	340	T	Ars et al. (2003)
CI021463	1021	341	TT	Origone et al. (2002)
CI001014	1111	371	T	Fahsold et al. (2000)
CI030367	1149	383	G	De Luca et al. (2003)
CI001015	1398	466	T	Fahsold et al. (2000)
CI031910	1399	467	A	Ars et al. (2003)
CI001016	1436	480	A	Fahsold et al. (2000)
CI000105	1465	489	T	Ars et al. (2000)
CI001581	1465	489	C	Messiaen et al. (2000)
CI001017	1519	507	T	Fahsold et al. (2000)
CI001018	1817	606	T	Fahsold et al. (2000)
CI951962	1998	667	CCTCT	Boddrich et al. (1995)
CI001582		677	C	Messiaen et al. (2000)
CI031911	2076	693	TGTAC	Ars et al. (2003)
CI031912	2173	725	T	Ars et al. (2003)
CI983167	2320	774	A	Park et al. (1998)
CI992569	2537	845	TG	Osborn & Upadhyaya (1999)
CI001019	2590	864	TATA	Fahsold et al. (2000)
CI972655	2779	927	CCTGCTC	Maynard et al. (1997)
CI001020	2845	949	T	Fahsold et al. (2000)
CI011891	2943	981	GCTCTGA	Serra et al. (2001)
CI001021	2972	991	T	Fahsold (2000)
CI020607	3047	1016	GT	Kluwe et al. (2002)

275

CI983168	3193	1065	A	Klose *et al.* (1998)
CI013990	3198	1066	T	Han *et al.* (2001)
CI031913	3239	1080	T	Ars *et al.* (2003)
CI001022	3394	1132	AG	Fahsold *et al.* (2000)
CI013991	3599	1200	G	Han *et al.* (2001)
CI004100	3808	1270	TGGA	Park *et al.* (2000)
CI021464	3920	1307	T	Origone *et al.* (2002)
CI040952	3982	1327	A	Mattocks *et al.* (2004)
CI040953	4045	1349	T	Mattocks *et al.* (2004)
CI031102	4095	1365	TG	Baralle *et al.* (2003)
CI021465	4104	1369	TA	Origone *et al.* (2002)
CI013992	4323	1442	GC	Han *et al.* (2001)
CI002563	4374	1459	T	Fahsold *et al.* (2000)
CI001023	4497	1499	G	Fahsold *et al.* (2000)
CI031914	4568	1523	C	Ars *et al.* (2003)
CI001024	4649	1550	G	Fahsold *et al.* (2000)
CI013993	4798	1600	C	Han *et al.* (2001)
CI031103	4905	1636	AGCCTTTCTTA	Kluwe *et al.* (2003b)
CI002564	4936	1646	T	Fahsold *et al.* (2000)
CI040954	4950	1650	A	Mattocks *et al.* (2004)
CI992057	5055	1685	T	Peters *et al.* (1999b)
CI032772	5194	1731	A	Ars *et al.* (2003)
CI032156	5267	1756	T	Origone *et al.* (2003a)
CI031915	5351	1785	C	Ars *et al.* (2003)
CI030178	5409	1803	T	Upadhyaya *et al.* (2003)
CI002565	5448	1817	G	Girodon-Boulandet *et al.* (2000)
CI031916	5486	1830	C	Ars *et al.* (2003)
CI032773	5738	1913	AT	Ars *et al.* (2003)
CI972656	5843	1948	A	Hatta *et al.* (1995)
CI033294	6027	2010	CTGAGGTG	Kluwe *et al.* (2003a)
CI992570	6487	2163	A	Osborn *et al.* (1999)
CI031917	6579	2195	GCAT	Ars *et al.* (2003)
CI000106	6593	2198	T	Ars *et al.* (2000)
CI013994	6704	2236	C	Han *et al.* (2001)
CI972657	6711	2237	C	Abernathy *et al.* (1997)
CI041651	6792	2264	A	De Luca *et al.* (2004)

CI001025	6792	2265	A	Fahsold *et al.* (2000)
CI013995	7032	2345	G	Han *et al.* (2001)
CI001026	7080	2361	A	Fahsold *et al.* (2000)
CI040955	7096	2366	A	Mattocks *et al.* (2004)
CI013996	7132	2378	C	Han *et al.* (2001)
CI021466	7149	2384	C	Origone *et al.* (2002)
CI020608	7246	2416	G	Kluwe *et al.* (2002)
CI992571	7267	2423	A	Osborn & Upadhyaya (1999)
CI032774	7308	2436	A	Ars *et al.* (2003)
CI992572	7313	2438	A	Osborn & Upadhyaya (1999)
CI040956	7427	2475	TC	Mattocks *et al.* (2004)
CI032157	7464	2489	TCCA	Origone *et al.* (2003a)
CI001027	7528	2510	GGGGCAACCTTCTC	Fahsold *et al.* (2000)
CI002566	7544	2515	GA	Fahsold *et al.* (2000)
CI001028	7633	2546	C	Fahsold *et al.* (2000)
CI031918	7719	2574	A	Ars *et al.* (2003)
CI001029	7926	2643	T	Fahsold *et al.* (2000)
CI983169	8042	2681	A	Ars *et al.* (1998)
CI001030	8092	2697	TT	Fahsold *et al.* (2000)
CI040957	3113+3	E18I18+3	A	Mattocks *et al.* (2004)

Nucleotide substitutions (missense / nonsense)

Accession Number	Codon	Nucleotide	Amino acid	Reference
CM000768	1	ATG-ACG	Met-Thr	Fahsold *et al.* (2000)
CM000769	9	TGG-TAG	Trp-Term	Fahsold *et al.* (2000)
CM043304	11	cCAG-TAG	Gln-Term	Horan *et al.* (2004)
CM000770	19	cGAG-TAG	Glu-Term	Fahsold *et al.* (2000)
CM040769	31	CAT-CGT	His-Arg	Mattocks *et al.* (2004)
CM000771	49	TACa-TAG	Tyr-Term	Fahsold *et al.* (2000)
CM020462	82	TCT-TTT	Ser-Phe	Kluwe *et al.* (2002)
CM992365	83	tCAG-TAG	Gln-Term	Osborn & Upadhyaya (1999)
CM042439	91	gGAA-TAA	Glu-Term	Mattocks *et al.* (2004)
CM001252	93	TGT-TAT	Cys-Tyr	Messiaen *et al.* (2000)

CM040770	104	TTA-TAA	Leu-Term	Mattocks *et al.* (2004)
CM000023	117	ATC-AGC	Ile-Ser	Ars *et al.* (2000)
CM040771	145	CTC-CCC	Leu-Pro	Mattocks *et al.* (2004)
CM041401	157	ATT-AAT	Ile-Asn	De Luca *et al.* (2004)
CM000772	161	TTA-TAA	Leu-Term	Fahsold *et al.* (2000)
CM000773	180	TTA-TAA	Leu-Term	Toliat *et al.* (2000)
CM043551	186	GAT-GTT	Asp-Val	Zatkova *et al.* (2004)
CM001253	187	TGT-TAT	Cys-Tyr	Messiaen *et al.* (2000)
CM000774	192	aCGA-TGA	Arg-Term	Toliat *et al.* (2000)
CM000775	216	CTG-CCG	Leu-Pro	Fahsold *et al.* (2000)
CM013953	230	tGAA-TAA	Glu-Term	Han *et al.* (2001)
CM961023	239	aCAG-TAG	Gln-Term	Horn *et al.* (1996)
CM040773	263	tAAA-TAA	Lys-Term	Mattocks *et al.* (2004)
CM961024	267	TGGc-TGA	Trp-Term	Gasparini *et al.* (1996)
CM961025	282	cCAG-TAG	Gln-Term	Gasparini *et al.* (1996)
CM011801	304	aCGA-TGA	Arg-Term	Wimmer *et al.* (2000)
CM003945	315	gCAG-TAG	Gln-Term	Wimmer *et al.* (2000)
CM040774	324	cTGT-CGT	Cys-Arg	Mattocks *et al.* (2004)
CM003946	336	TGG-TAG	Trp-Term	Wimmer *et al.* (2000)
CM040775	337	GAA-GTA	Glu-Val	Mattocks *et al.* (2004)
CM971039	338	GAT-GGT	Asp-Gly	Upadhyaya *et al.* (1997)
CM000776	354	tAAG-TAG	Lys-Term	Fahsold *et al.* (2000)
CM000777	357	CTT-CCT	Leu-Pro	Fahsold *et al.* (2000)
CM900171	366	aAGA-TGA	Arg-Term	Cawthon *et al.* (1990)
CM000778	408	TATg-TAG	Tyr-Term	Fahsold *et al.* (2000)
CM992366	416	tCGA-TGA	Arg-Term	Osborn & Upadhyaya (1999)
CM000779	425	TGGt-TGA	Trp-Term	Fahsold *et al.* (2000)
CM042440	425	TGG-TAG	Trp-Term	Mattocks *et al.* (2004)
CM950845	440	tCGA-TGA	Arg-Term	Heim *et al.* (1995)
CM000780	461	aCGA-TGA	Arg-Term	Fahsold *et al.* (2000)
CM032612	471	aAAA-TAA	Lys-Term	Ars *et al.* (2003)
CM000781	491	TAT-TGT	Tyr-Cys	Fahsold *et al.* (2000)
CM001254	524	aGAA-TAA	Glu-Term	Messiaen *et al.* (2000)
CM040777	532	CTG-CCG	Leu-Pro	Mattocks *et al.* (2004)
CM001255	536	TCA-TAA	Ser-Term	Messiaen *et al.* (2000)
CM020463	543	tCAG-TAG	Gln-Term	Kluwe *et al.* (2002)
CM000782	549	CTG-CCG	Leu-Pro	Fahsold *et al.* (2000)

CM030911	578	CTT-CGT	Leu-Arg	Kluwe *et al.* (2003b)
CM000783	581	ATC-ACC	Ile-Thr	Fahsold *et al.* (2000)
CM000784	583	AAG-AGG	Lys-Arg	Fahsold *et al.* (2000)
CM000024	599	TGGt-TGA	Trp-Term	Ars *et al.* (2000)
CM961026	629	cGGG-AGG	Gly-Arg	Gasparini *et al.* (1996)
CM000785	665	TCC-TTC	Ser-Phe	Fahsold *et al.* (2000)
CM000025	681	cCGA-TGA	Arg-Term	Ars *et al.* (2000)
CM981380	682	aCAA-TAA	Gln-Term	Horiuchi *et al.* (1998)
CM000786	692	TACa-TAG	Tyr-Term	Fahsold *et al.* (2000)
CM000787	695	CTG-CCG	Leu-Pro	Fahsold *et al.* (2000)
CM030255	725	tGAA-TAA	Glu-Term	De Luca *et al.* (2003)
CM040778	727	TCA-TGA	Ser-Term	Horan *et al.* (2004)
CM000788	763	CTG-CCG	Leu-Pro	Fahsold *et al.* (2000)
CM000789	777	TGG-TCG	Trp-Ser	Fahsold *et al.* (2000)
CM000790	780	ACA-AAA	Thr-Lys	Fahsold *et al.* (2000)
CM000791	781	CAT-CCT	His-Pro	Fahsold *et al.* (2000)
CM041402	783	aAAA-TAA	Lys-Term	De Luca *et al.* (2004)
CM020464	784	aTGG-CGG	Trp-Arg	Kluwe *et al.* (2002)
CM013773	784	TGGg-TGC	Trp-Cys	Han *et al.* (2001)
CM000792	786	aCAA-TAA	Gln-Term	Fahsold *et al.* (2000)
CM021310	810	cAAG-TAG	Lys-Term	Origone *et al.* (2002)
CM971040	816	gCGA-TGA	Arg-Term	Maynard *et al.* (1997)
CM040779	838	ATCa-ATG	Ile-Met	Mattocks *et al.* (2004)
CM971041	844	CTT-CGT	Leu-Arg	Maynard *et al.* (1997)
CM002379	844	cCTT-TTT	Leu-Phe	Boulandet *et al.* (2000)
CM040780	844	CTT-CCT	Leu-Pro	Mattocks *et al.* (2004)
CM000793	847	CTT-CCT	Leu-Pro	Fahsold *et al.* (2000)
CM030256	848	GGA-GAA	Gly-Glu	De Luca *et al.* (2003)
CM971042	898	CTG-CCG	Leu-Pro	Maynard *et al.* (1997)
CM971043	908	gAAA-TAA	Lys-Term	Maynard *et al.* (1997)
CM030912	920	CTG-CCG	Leu-Pro	Kluwe *et al.* (2003b)
CM971044	930	TATc-TAG	Tyr-Term	Maynard *et al.* (1997)
CM000794	948	cCAA-TAA	Gln-Term	Fahsold *et al.* (2000)
CM001256	959	tCAA-TAA	Gln-Term	Messiaen *et al.* (2000)
CM001257	963	aCAA-TAA	Gln-Term	Messiaen *et al.* (2000)
CM030257	968	ATG-AGG	Met-Arg	De Luca *et al.* (2003)
CM000795	998	TATg-TAA	Tyr-Term	Fahsold *et al.* (2000)

CM032968	1015	CTG-CCG	Leu-Pro	Kluwe *et al.* (2003a)
CM950846	1017	tCAA-TAA	Gln-Term	Heim *et al.* (1995)
CM961027	1035	ATG-AGG	Met-Arg	Wu *et al.* (1996)
CM040782	1055	cCAA-TAA	Gln-Term	Mattocks *et al.* (2004)
CM040783	1073	cATG-GTG	Met-Val	Mattocks *et al.* (2004)
CM001258	1123	tGAA-TAA	Glu-Term	Messiaen *et al.* (2000)
CM000026	1140	TCA-TGA	Ser-Term	Ars *et al.* (2000)
CM013774	1147	CTT-CCT	Leu-Pro	Han *et al.* (2001)
CM000796	1156	AAC-AGC	Asn-Ser	Fahsold *et al.* (2000)
CM941091	1166	GGC-GAC	Gly-Asp	Purandare *et al.* (1994)
CM001259	1174	cCAG-TAG	Gln-Term	Messiaen *et al.* (2000)
CM042755	1189	aCAA-TAA	Gln-Term	Trovo *et al.* (2004)
CM013775	1193	TTT-TGT	Phe-Cys	Han *et al.* (2001)
CM040784	1196	CTT-CGT	Leu-Arg	Mattocks *et al.* (2004)
CM973234	1204	tCGG-GGG	Arg-Gly	Krkljus *et al.* (1997)
CM000027	1204	tCGG-TGG	Arg-Trp	Ars *et al.* (2000)
CM000797	1210	gGAA-TAA	Glu-Term	Fahsold *et al.* (2000)
CM041403	1235	tCAG-TAG	Gln-Term	De Luca *et al.* (2004)
CM000798	1236	TGG-TAG	Trp-Term	Fahsold *et al.* (2000)
CM000799	1241	tCGA-TGA	Arg-Term	Fahsold *et al.* (2000)
CM043031	1243	CTG-CCG	Leu-Pro	Ferner *et al.* (2004)
CM000800	1250	CGG-CCG	Arg-Pro	Fahsold *et al.* (2000)
CM000801	1258	TGG-TAG	Trp-Term	Fahsold *et al.* (2000)
CM000802	1276	CGA-CAA	Arg-Gln	Fahsold *et al.* (2000)
CM040785	1276	cCGA-GGA	Arg-Gly	Mattocks *et al.* (2004)
CM983421	1276	CGA-CCA	Arg-Pro	Klose *et al.* (1998)
CM950847	1276	cCGA-TGA	Arg-Term	Heim *et al.* (1995)
CM032012	1298	aCAA-TAA	Gln-Term	Origone *et al.* (2003)
CM981381	1306	aCGA-TGA	Arg-Term	Park *et al.* (1998)
CM971045	1314	TGGc-TGA	Trp-Term	Upadhyaya *et al.* (1997)
CM000803	1336	cCAG-TAG	Gln-Term	Fahsold *et al.* (2000)
CM042756	1356	GAA-GGA	Glu-Gly	Trovo *et al.* (2004)
CM971046	1362	tCGA-TGA	Arg-Term	Upadhyaya *et al.* (1997)
CM020465	1365	TGCc-TGA	Cys-Term	Kluwe *et al.* (2002)
CM971047	1391	AGAt-AGT	Arg-Ser	Upadhyaya *et al.* (1997)
CM000804	1415	cGAA-TAA	Glu-Term	Fahsold *et al.* (2000)
CM941092	1419	AAG-AGG	Lys-Arg	Purandare *et al.* (1994)

CM971048	1419	gAAG-CAG	Lys-Gln	Upadhyaya *et al.* (1997)
CM040786	1422	TCA-TAA	Ser-Term	Mattocks *et al.* (2004)
CM013776	1423	AAG-AGG	Lys-Arg	Han *et al.* (2001)
CM030258	1423	AAGg-AAT	Lys-Asn	De Luca *et al.* (2003)
CM920506	1423	aAAG-GAG	Lys-Glu	Li *et al.* (1992)
CM990929	1425	CTT-CCT	Leu-Pro	Peters *et al.* (1999a)
CM971049	1468	aAGT-GGT	Ser-Gly	Upadhyaya *et al.* (1997)
CM000805	1491	TGGa-TGA	Trp-Term	Fahsold *et al.* (2000)
CM031712	1498	GGG-GAG	Gly-Glu	Ars *et al.* (2003)
CM941093	1513	aCGA-TGA	Arg-Term	Side *et al.* (1997)
CM000806	1538	TGGt-TGA	Trp-Term	Fahsold *et al.* (2000)
CM971050	1569	TTA-TGA	Leu-Term	Upadhyaya *et al.* (1997)
CM000807	1573	TACc-TAG	Tyr-Term	Fahsold *et al.* (2000)
CM000808	1584	tATT-GTT	Ile-Val	Fahsold *et al.* (2000)
CM971051	1590	aCGG-TGG	Arg-Trp	Upadhyaya *et al.* (1997)
CM990930	1613	TATt-TAG	Tyr-Term	Peters *et al.* (1999b)
CM013777	1742	cCAA-TAA	Gln-Term	Han *et al.* (2001)
CM941094	1748	gCGA-TGA	Arg-Term	Valero *et al.* (1994)
CM941095	1754	gCAA-TAA	Gln-Term	Valero *et al.* (1994)
CM001260	1755	TCA-TGA	Ser-Term	Messiaen *et al.* (2000)
CM000809	1762	TATt-TAG	Tyr-Term	Fahsold *et al.* (2000)
CM013778	1764	tGCT-TCT	Ala-Ser	Han *et al.* (2001)
CM000810	1777	cCAG-TAG	Gln-Term	Fahsold *et al.* (2000)
CM000811	1780	TTA-TAA	Leu-Term	Fahsold *et al.* (2000)
CM000812	1785	cCAG-TAG	Gln-Term	Fahsold *et al.* (2000)
CM950848	1794	cCAG-TAG	Gln-Term	Heim *et al.* (1995)
CM002380	1810	TGG-TAG	Trp-Term	Boulandet *et al.* (2000)
CM990931	1820	cCAA-TAA	Gln-Term	Peters *et al.* (1999b)
CM043552	1907	gGAA-TAA	Glu-Term	Zatkova *et al.* (2004)
CM971052	1931	aTGG-CGG	Trp-Arg	Hudson *et al.* (1997)
CM900172	1932	CTG-CCG	Leu-Pro	Cawthon *et al.* (1990)
CM900173	1947	aCGA-TGA	Arg-Term	Cawthon *et al.* (1990)
CM030259	1965	aAAA-TAA	Lys-Term	De Luca *et al.* (2003)
CM001261	1966	aCAG-TAG	Gln-Term	Messiaen *et al.* (2000)
CM030260	1980	tGGG-AGG	Gly-Arg	De Luca *et al.* (2003)
CM013779	1991	aGAC-AAC	Asp-Asn	Han *et al.* (2001)
CM013780	2067	CTG-CCG	Leu-Pro	Kaufmann *et al.* (2001)

CM950849	2113	TGTa-TGA	Cys-Term	Heim *et al.* (1995)
CM920507	2143	gCTG-ATG	Leu-Met	Upadhyaya *et al.* (1992)
CM031713	2161	TACc-TAG	Tyr-Term	Ars *et al.* (2003)
CM920508	2171	cTAT-GAT	Tyr-Asp	Upadhyaya *et al.* (1992)
CM000813	2189	TTG-TAG	Leu-Term	Fahsold *et al.* (2000)
CM030913	2200	tCCA-GCA	Pro-Ala	Kluwe *et al.* (2003b)
CM950850	2208	TGGa-TGA	Trp-Term	Heim *et al.* (1995)
CM000814	2210	aGAA-TAA	Glu-Term	Fahsold *et al.* (2000)
CM992367	2214	aAGA-TGA	Arg-Term	Osborn *et al.* (1999)
CM020466	2236	cAAA-TAA	Lys-Term	Kluwe *et al.* (2002)
CM000815	2237	aCGA-TGA	Arg-Term	Fahsold *et al.* (2000)
CM031714	2242	gCAG-TAG	Gln-Term	Ars *et al.* (2003)
CM972796	2264	TACa-TAG	Tyr-Term	Messiaen *et al.* (1997)
CM981382	2264	TACa-TAA	Tyr-Term	Robinson *et al.* (1995)
CM000816	2280	TTA-TGA	Leu-Term	Fahsold *et al.* (2000)
CM020467	2280	TTA-TAA	Leu-Term	Kluwe *et al.* (2002)
CM961028	2281	aCAG-TAG	Gln-Term	Upadhyaya *et al.* (1996)
CM994667	2317	CTT-CCT	Leu-Pro	Wu *et al.* (1999)
CM013781	2336	aGAG-AAG	Glu-Lys	Han *et al.* (2001)
CM001262	2401	cAAA-TAA	Lys-Term	Messiaen *et al.* (2000)
CM000817	2413	aCAG-TAG	Gln-Term	Fahsold *et al.* (2000)
CM000818	2429	tCGA-TGA	Arg-Term	Fahsold *et al.* (2000)
CM030914	2432	TGCa-TGA	Cys-Term	Kluwe *et al.* (2003b)
CM032013	2471	tCAG-TAG	Gln-Term	Origone *et al.* (2003)
CM000819	2486	ACC-ATC	Thr-Ile	Fahsold *et al.* (2000)
CM941096	2496	cCGA-TGA	Arg-Term	Purandare *et al.* (1994)
CM950851	2518	tGGA-TGA	Gly-Term	Heim *et al.* (1995)
CM000820	2567	aCAA-TAA	Gln-Term	Fahsold *et al.* (2000)
CM000821	2568	aCAG-TAG	Gln-Term	Fahsold *et al.* (2000)
CM021311	2595	cCAG-TAG	Gln-Term	Origone *et al.* (2002)
CM950852	2610	cACA-GCA	Thr-Ala	Upadhyaya *et al.* (1995)
CM950853	2616	aCGA-TGA	Arg-Term	Upadhyaya *et al.* (1995)
CM031715	2683	gCAA-TAA	Gln-Term	Ars *et al.* (2003)
CM020468	2698	TCA-TGA	Ser-Term	Kluwe *et al.* (2002)

Nucleotide substitutions (splicing)

Accession Number	IVS	Donor/ Acceptor	Relative location	Substitution	Reference
CS000866	2	as	-1	G-A	Fahsold et al. (2000)
CS031782	2	ds	+1	G-A	Ars et al. (2003)
CS000867	2	ds	+1	G-T	Fahsold et al. (2000)
CS000868	2	ds	+2	T-G	Fahsold et al. (2000)
CS000049	3	ds	+1	G-A	John et al. (2000)
CS034311	3	ds	+1	G-T	Lin et al. (2003)
CS030542	3	ds	+5	G-C	Baralle et al. (2003)
CS020536	4	as	-2	A-G	Kluwe et al. (2002)
CS030990	5	as	-2	A-G	Kluwe et al. (2003b)
CS991467	6	as	-2	A-G	Klose et al. (1999)
CS000869	6	ds	+1	G-A	Fahsold et al. (2000)
CS040847	6	ds	-43	G-A	Mattocks et al. (2004)
CS983483	7	as	+22	C-T	Hoffmeyer et al. (1998)
CS001839	7	as	+55	C-T	Wimmer et al. (2000)
CS000870	7	as	-13	G-A	Fahsold et al. (2000)
CS001840	7	as	+119	G-A	Wimmer et al. (2000)
CS031783	7	ds	+1	G-A	Ars et al. (2003)
CS971822	7	ds	+1	G-C	Upadhyaya et al. (1997a)
CS000871	7	ds	-1	G-A	Fahsold et al. (2000)
CS000872	7	ds	+67	T-C	Fahsold et al. (2000)
CS000050	8	ds	+1	G-A	Ars et al. (2000)
CS961632	8	ds	+1	G-T	Horn et al. (1996)
CS004529	8	ds	-1	G-C	Schuppert et al. (2000)
CS031784	9	as	-2	A-C	Ars et al. (2003)
CS031785	9	ds	+5	G-C	Ars et al. (2003)
CS971823	10	as	-8	A-G	Side et al. (1997)
CS040848	11	as	+1	C-A	Mattocks et al. (2004)
CS000051	11	as	-3	C-G	Ars et al. (2000)
CS030306	11	ds	+2	T-G	De Luca et al. (2003)
CS941514	11	ds	+3	A-G	Purandare et al. (1994)
CS000873	11	ds	-1	G-A	Fahsold et al. (2000)
CS000874	11	ds	-1	G-C	Fahsold et al. (2000)
CS040849	12	as	+39	C-T	Mattocks et al. (2004)

CS032078	13	as	-2	A-C	Origone *et al.* (2003a)
CS032079	13	ds	+1	G-A	Origone *et al.* (2003a)
CS011839	13	ds	+2	T-C	Serra *et al.* (2001)
CS030307	14	as	-2	A-G	De Luca *et al.* (2003)
CS971824	14	ds	+1	G-A	Maynard *et al.* (1997)
CS030991	14	ds	+2	T-C	Origone *et al.* (2003b)
CS992454	15	as	-1	G-A	Osborn & Upadhyaya (1999)
CS031786	15	as	-12	T-G	Ars *et al.* (2003)
CS031787	15	as	-15	A-G	Ars *et al.* (2003)
CS000052	15	as	-16	A-G	Ars *et al.* (2000)
CS040850	16	as	-2	A-G	Mattocks *et al.* (2004)
CS031788	16	ds	+1	G-A	Ars *et al.* (2003)
CS992455	16	ds	-62	A-G	Osborn & Upadhyaya (1999)
CS000053	16	ds	-87	G-A	Ars *et al.* (2000)
CS961633	17	as	-1	G-A	Perrin *et al.* (1996)
CS000875	17	as	-1	G-C	Fahsold *et al.* (2000)
CS992456	17	as	-2	A-G	Osborn & Upadhyaya (1999)
CS013969	17	ds	+7	A-G	Han *et al.* (2001)
CS951480	18	ds	+1	G-A	Purandare *et al.* (1995)
CS031789	18	ds	+1	G-T	Ars *et al.* (2003)
CS031790	18	ds	+5	G-C	Ars *et al.* (2003)
CS982280	20	ds	+2	T-C	Klose *et al.* (1998)
CS031791	20	ds	-70	C-T	Ars *et al.* (2003)
CS971825	22	as	-2	A-G	Upadhyaya *et al.* (1997b)
CS012213	22	ds	+1	G-C	Serra *et al.* (2001)
CS011840	22	ds	+1	G-T	Serra *et al.* (2001)
CS000876	25	as	-1	G-T	Fahsold *et al.* (2000)
CS031792	25	ds	+1	G-A	Ars *et al.* (2003)
CS040851	26	as	-1	G-A	Mattocks *et al.* (2004)
CS000877	26	as	-2	A-G	Fahsold *et al.* (2000)
CS001441	26	as	-2	A-T	Messiaen *et al.* (2000)
CS000878	26	ds	+1	G-A	Fahsold *et al.* (2000)
CS002460	28	as	-2	A-G	Boulandet *et al.* (2000)
CS001442	28	as	+89	C-A	Messiaen *et al.* (2000)
CS000054	28	ds	+1	G-A	Ars *et al.* (2000)
CS031793	28	ds	+5	G-A	Ars *et al.* (2003)
CS040852	28	ds	-34	G-A	Mattocks *et al.* (2004)

CS000879	29	ds	+1	G-A	Fahsold *et al.* (2000)
CS992457	29	ds	+1	G-C	Osborn & Upadhyaya (1999)
CS000880	29	ds	+2	T-G	Fahsold *et al.* (2000)
CS000055	29	ds	-1	G-A	Ars *et al.* (2000)
CS040853	29	ds	-120	C-T	Mattocks *et al.* (2004)
CS941515	30	ds	+2	T-G	Purandare *et al.* (1994)
CS961634	30	ds	+332	A-G	Perrin *et al.* (1996)
CS992458	30	ds	+4046	A-G	Osborn & Upadhyaya (1999)
CS040854	31	as	-1	G-C	Mattocks *et al.* (2004)
CS000881	31	as	-2	A-G	Fahsold *et al.* (2000)
CS941516	31	as	-5	A-G	Ainsworth *et al.* (1994)
CS000882	31	ds	+1	G-A	Fahsold *et al.* (2000)
CS040855	31	ds	+1	G-T	Mattocks *et al.* (2004)
CS041538	32	ds	+1	G-A	De Luca *et al.* (2004)
CS992459	33	ds	+2	T-G	Osborn & Upadhyaya (1999)
CS941517	33	ds	+4	A-G	Hutter *et al.* (1994)
CS000883	34	ds	+2	T-G	Fahsold *et al.* (2000)
CS000884	34	ds	+45	T-A	Fahsold *et al.* (2000)
CS000885	34	ds	+87	G-A	Fahsold *et al.* (2000)
CS000886	35	as	-1	G-T	Fahsold *et al.* (2000)
CS041539	35	ds	+1	G-A	De Luca *et al.* (2004)
CS982281	35	ds	+1	G-T	Park *et al.* (1998)
CS031794	35	ds	+2	T-A	Ars *et al.* (2003)
CS000887	36	ds	+1	G-A	Fahsold *et al.* (2000)
CS030308	36	ds	+2	T-A	Heim *et al.* (1995)
CS031795	36	ds	+3	A-G	Ars *et al.* (2003)
CS013970	37	ds	+1	G-A	Han *et al.* (2001)
CS020537	37	ds	+1	G-C	Messiaen *et al.* (2000)
CS000056	37	ds	+2	T-G	Gasparini *et al.* (1996)
CS001443	37	ds	-1	G-C	Messiaen *et al.* (2000)
CS001444	39	as	-12	T-A	Messiaen *et al.* (2000)
CS030309	39	ds	+3	A-C	Messiaen *et al.* (2003)
CS000888	40	as	-17	C-T	Fahsold *et al.* (2000)
CS030310	40	ds	+1	G-T	Heim *et al.* (1995)
CS000889	40	ds	-1	G-C	Fahsold *et al.* (2000)
CS000890	41	as	-1	G-A	Fahsold *et al.* (2000)
CS992460	43	as	-2	A-G	Maynard *et al.* (1997)

CS031796	44	ds	+1	G-T	Ars *et al.* (2003)
CS020538	44	ds	+2	T-A	Messiaen *et al.* (2000)
CS000891	45	as	-2	A-G	Fahsold *et al.* (2000)
CS000892	45	ds	+1	G-A	Fahsold *et al.* (2000)
CS041540	45	ds	+5	G-A	Park *et al.* (1998)
CS031797	45	ds	+790	C-G	Ars *et al.* (2003)
CS982282	4b	as	-3	C-A	Li *et al.* (1992)
CS000893	4b	ds	+1	G-A	Fahsold *et al.* (2000)
CS000057	4b	ds	+5	G-A	Ars *et al.* (2000)
CS961635	4c	as	-1	G-A	Wimmer *et al.* (2000)
CS000894	4c	as	-2	A-T	Fahsold *et al.* (2000)
CS000058	10a	as	-9	T-A	Ars *et al.* (2000)
CS041541	10a	ds	+1	G-A	Park *et al.* (1998)
CS020539	10a	ds	+2	T-A	Messiaen *et al.* (2000)
CS993383	10b	ds	+1	G-A	Ars *et al.* (1999)
CS040856	10b	ds	+1	G-C	Mattocks *et al.* (2004)
CS982283	10b	ds	-15	A-G	Li *et al.* (1992)
CS000059	10b	ds	-62	A-G	Ars *et al.* (2000)
CS031798	10c	as	-2	A-G	Ars *et al.* (2003)
CS011841	10c	as	-5	A-T	Horiuchi *et al.* (1998)
CS000895	10c	as	-8	A-G	Fahsold *et al.* (2000)
CS000896	10c	ds	+1	G-T	Fahsold *et al.* (2000)
CS013460	12a	ds	+1	G-A	Fang *et al.* (2001)
CS971827	12a	ds	+1	G-T	Abernathy *et al.* (1997)
CS000060	19a	ds	+1	G-A	Ars *et al.* (2000)
CS001445	19b	as	-3	C-G	Messiaen *et al.* (2000)
CS001446	19b	ds	-38	G-A	Messiaen *et al.* (2000)
CS001447	27b	as	-2	A-T	Messiaen *et al.* (2000)
CS000897	23.1	as	-2	A-G	Fahsold *et al.* (2000)
CS013827	23.1	ds	+1	G-C	Han *et al.* (2001)
CS042824	23.2	as	-1	G-A	Trovo *et al.* (2004)
CS971828	23.2	as	-2	A-G	Upadhyaya *et al.* (1997b)
CS971829	23.2	ds	+1	G-A	Upadhyaya *et al.* (1997b)
CS000898	23.2	ds	+1	G-C	Fahsold *et al.* (2000)