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Analysis of telomere dynamics in human tissues

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A thesis submitted to Cardiff University for the degree of Doctor of Philosophy (PhD)

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September 2010

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ACKNOWLEDGEMENT

First, I would like to thank Almighty God for the wisdom and perseverance that he has bestowed upon me during this research project, and indeed, throughout my life.

I am heartily thankful to my supervisor, Dr Duncan M. Baird, for giving me the opportunity to work in his laboratory. I would also like to thank him for his valuable criticisms, guidance and support which encouraged me to work hard during this project.

I am indebted to the staff at the central biotechnology-sequencing [CBS] unit of Cardiff University for running and editing sequences throughout the project.

Words cannot express my gratitude to my family [my mum, my brothers and their wives, my sister, nieces and nephews] and friends whom their love, prayers and emotional support encouraged me through some of the most challenging episodes of the project.

Last but not least, sincere thanks to Cancer research UK, Cardiff university, School of medicine and overseas research student awards for financial support without their support this work would not have been possible.

Lastly, I offer my regards and blessings to all those who supported me in many different ways [however little it may seem] during the project.

This thesis is dedicated in loving memory of my dad, my brother and two sisters who always believed in me.

Publication List

Publications arising from this thesis:

Letsolo, B.T., Rowson, J., and Baird, D.M. (2009). Fusion of short telomeres in human cells is characterized by extensive deletion and microhomology, and can result in complex rearrangements. Nucleic Acids Res.

Contributions to other publications

Capper, R., Britt-Compton, B., Tankimanova, M., Rowson, J., **Letsolo, B.,** Man, S., Haughton, M., and Baird, D.M. (2007). The nature of telomere fusion and a definition of the critical telomere length in human cells. Genes Dev *21*, 2495-2508.

Lin, T.T., **Letsolo, B.T.**, Jones, R.E., Rowson, J., Pratt, G., Hewamana, S., Fegan, C., Pepper, C., and Baird, D.M. (2010). Telomere dysfunction and fusion during the progression of chronic lymphocytic leukaemia: evidence for a telomere crisis. Blood.

Analysis of Telomere Dynamics in Human Tissues

Boitelo Theresia Letsolo

ABSTRACT

Human telomeres are DNA-protein structures that "cap" the chromosome termini. Once uncapped, telomeres can generate chromosome instability through breakage-fusion-bridge cycles, leading to large-scale genomic rearrangements such as non-reciprocal translocations [NRTs]; that typify human cancers.

High-resolution single-molecule analysis of telomere length and fusion *in vitro* has demonstrated that telomeres are subjected to mutational changes, which generate short telomeres that are capable of fusion. This knowledge of telomere dynamics *in vitro* was translated to human tissues *in vivo*.

Initially, the single-molecule telomere fusion technology was further developed to facilitate the detection of a broader range and potentially rare telomere fusion events. Subsequently, telomere length and fusion were investigated in normal and disease tissues including normal dermis, melanocytic naevi, Barrett's metaplasia, normal oesophageal and gastric tissues. We have demonstrated that telomere dysfunction both *in vitro* and *in vivo* generated truncated telomeres that are capable of fusion. The molecular analysis of fusion events revealed that the fusion of critically short telomeres was accompanied by large deletion extending into the subtelomeric DNA up to 5.6kb; close to the limit of the assay and the presence of short patches of DNA sequence homology. Furthermore, complex fusion events that include fusion with interstitial non-telomeric loci, near sites implicated in skin cancer and other human cancers were detected.

In Barrett's oesophagus, the metaplastic tissue displayed clonal growth characterised by very short homogenous telomere profiles compared to the adjacent normal tissue. Short telomeres and fusions were also detected in normal dermis and melanocytic naevi with evidence of clonal telomere fusion events. Such events can result in clonal expansion, which could confer selective advantage for other abnormalities that may drive neoplastic progression. These data are consistent with the view that telomere dysfunction *in vivo* may drive large-scale genomic instability of the type observed in early-stage neoplasia.

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Abbreviations

A	Adenosine
ALT	Alternative lengthening of telomeres
APB	ALT-associated PML body
ARF	Alternative reading frame product of CDKN2A
A-T	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia rad3-related
BCC	Basal cell carcarcinoma
BER	Base excision repair
BFB	Breakage-fusion-bridging cycles
BLAST	Basic Local Alignment Search Tool
BLM	Bloom syndrome helicase
bp	Base pairs
BRCA1/2	Breast cancer 1/2
BS	Bloom syndrome
BSA	Bovine albumin serum
C	Cytosine
C. elegans	*Caenorhabditis elegans*
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CHK1/2	Check point kinase1/ 2
CLL	Chronic lymphocytic leukaemia
dH2O	Double distilled , deionised water
D-loop	Displacement loop
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxy-ribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
ds	Double stranded
DSB	Double-strand breaks

EcoRI	*Eschericia coli* restriction endonuclease 1
EDTA	Ethylene diamine tetraacetic acid
ERCC1	Excision repair cross complementing group 1
FCS	Foetal calf serum
FHIT	Fragile histidine triad
FISH	Fluorenscence *in situ* hybridisation
FISH	Fluorescence *in situ* hybridisation
g	Gram
G	Guanine
HCL	Hydrochloric acid
HEK293	Human embryonic kidney cell line 293
Hinf1	*Haemophilus influenzae* restriction endonuclease 1
HIV	Human Immunodeficiency Virus
HP1	Heterochromatin protein 1
HPV	Human papilloma virus
HR	Homologous recombination
hTERC	Human telomerase RNA
hTERT	Human telomerase reverse transcriptase
ICF syndrome	Immunodeficiency, Centromere instability and Facial anomalies
INK4	Inhibitor of the cyclin-dependent kinase 4
kb	kilobases
LINE	Long interspersed nuclear elements
LOH	Loss of heterozygosity
LTR	Long terminal repeat
M	Molar
M1	Mortality stage 1
M2	Mortality stage 2
min	Minutes
µg,	Microgram,
µl	Microlitre
µM	Micromolar

ml,	Millilitre
mM	Millimolar,
MMAC1	Mutated in multiple advanced cancers 1
MMEJ	Microhomology mediated end joining
MMR	Mismatch repair
MRE11	MRE11 meiotic recombination 11
Na_2HPO_4	sodium hydrogen phosphate, dibasic
$NaHPO_4$	sodium hydrogen phosphate
NaOH	sodium hydroxide
NBS1	Nijmegen breakage syndrome protein 1
NHEJ	Non-homologous end joining
Not	*Nocardia otitidis-caviarum*
NRT	Non reciprocal translocation
nt	Nucleotides
OB	Oligonucleotide binding fold
^{O}C	Degrees centigrades
8-oxo-G	8-oxo-7 8-dihydro-2'-deoxyguanosine
p16	Cell cycle protein
p53	Tumour suppressor protein
PAR 1/2	Pseudoautosomal regions
PARP1/2	Poly(ADP-ribose)polymerase 1/2
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PD	Population Doubling
PEG	Polyethylene glycol
pg	Picograms
PINX1	Pin2/TRF1 interacting factor
PML	Promyelocytic leukaemia bodies
PNA	Peptide nucleic acid
POT1	Protection of telomeres 1

pRb	Retinoblastoma protein
PRINS	Primed *in situ* labelling
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTOP/PIP/	POT1 interacting protein
pwo polymerase	*Pyrococcus woesei* polymerase
Q-PCR	Quantitative PCR
RAD50	Radiation sensitive protein 50
Rap1	Repressor activator protein 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
rpm	Revolutions per minute
Rsa1	*Rhodopseudomonas sphaeroides* restriction endonuclease 1
SCC	Squamous cell carcinoma
SCID	Severe combined immune deficiency
SDS	Sodium dodecyl sulphate
sec	Seconds
SNPs	Single nucleotide polymorphisms
SS	Single-stranded
SSA	Single-strand annealing
ssc	Saline sodium citrate
T	Thymine
TAE	Tris acetate EDTA
Tankyrase	TRF1-interacting Ankyrin Related ADP-ribose polymerase
taq polymerase	*Thermus aquaticus* polymerase
TBE	Tris borate EDTA
TE	Tris EDTA
TERRA	Telomere repeat containing RNA
TIN2	TRF1-interacting factor 2
t-loop	Telomere loop
TPE	Telomere position effect

TPP1	Tripeptidyl peptidase I
TRF	Terminal restriction fragment analysis
TRF1/2	Telomere repeat binding factor 1/2
TRIS	Tris (hydromethyl) aminomethane
T-SCE	Telomere sister chromatid exchange
TVR	Telomere variant repeat
TVR-PCR	Telomere variant repeat mapping by PCR
UV	Ultra violet
V(D)J	Variable division joining
WRN	Werner syndrome protein
WS	Werner syndrome
WWOX	WW-DOMAIN-containing oxidoreductase
XRCC4	X-ray-repair-cross-complementing defective repair in Chinese hamster mutant 4),
YAC	Yeast artificial chromosome

1 Chapter 1: Introduction: Telomeres

1.1 The structure of the thesis

This thesis describes a detailed analysis of telomere dynamics and fusion in human tissues. This work required technology development to allow the detection of rare events, a large analysis of the spectrum of mutation that accompanies telomere fusion and the application of these assays for the analysis of both normal and diseased tissues *in vivo*.

The first part of the thesis [introductory chapter 1] includes the review of literature on telomeres in the context of mammals [including humans] with little reference to other organisms as required. Initially, the history of telomere biology research will be considered, after which the structure and function of telomeres, the consequence of telomere dysfunction and processes that affect telomere homeostasis will be discussed. Since telomeres are critical for maintaining genome integrity, the telomere length measurements are important in ageing, cancer and disease research. Thus, the final sections of this chapter will discuss the methods employed to determine telomere length and telomere-induced chromosome-instability. Finally, the aims of the project will be outlined.

The second part of the thesis (chapter 2) describes the equipment, materials and experimental procedures used. Whilst the third part of the thesis describes the technological improvement of the assays used during the study [Chapter 3]; analysis of telomere instability and fusion in human cells *in vitro* [chapter 4] and *in vivo* in human skin [Chapters 5] and premalignant Barrett's oesophagus [Chapters 6]. Each result chapter is outlined such that it includes the brief introduction, the findings (results) and their implication (discussion).
Finally, chapter 7 summarises the main findings and discusses their implications. The possible limitations of the whole study are also considered and future research directions are suggested in this chapter.

1.2 Brief history of telomere Biology

The importance of the natural chromosome ends, telomeres in maintaining genome integrity was first recognised by McClintock (1941) and Muller (1938) who independently defined telomeres as the functional ends of chromosomes that are distinct from X-ray-induced double-stranded breaks [reviewed in (Olovnikov 1996; Bolzan and Bianchi 2006)].

In his experiments with fruit-fly, *Drosophila melanogaster*, Muller recognised that unlike internal double-stranded breaks, the natural chromosome ends had the protective cap against x-ray induced chromosome aberrations such as inversions, deletions and translocations [reviewed in (McKnight and Shippen 2004; Bolzan and Bianchi 2006). He later named the chromosome end "telomere" from two Greek words *telos*=end and *meros*=part meaning end part [reviewed in (Bolzan and Bianchi 2006)]. During the same period, in her experiments with corn (*Zea Mays*), Barbara McClintock discovered that unlike X-ray-induced chromosome breaks, natural chromosomes ends were concealed from fusing and forming dicentric chromosomes, and thus had "chromosome healing" properties (McClintock 1941).

About three decades later, the importance of telomeres in DNA replication and cell integrity was enhanced by the independent recognition of "end replication problem" [section 1.6] by Watson (Watson 1972) and Olovnikov (Olovnikov 1973). They hypothesised that DNA polymerases failed to replicate the 3′ ends of the chromosomes fully during semi-conservative replication. Consequently, the chromosomes shorten with each round of replication, a phenomenon called "end-replication" problem. The existence of a protective mechanism that prevents chromosome shortening was then postulated (Watson 1972). The link was also established between the "end-replication problem" and unlimited cell proliferation of cells in culture (Hayflick and Moorhead 1961; Olovnikov 1973).

However, the solution to the "end-replication problem" remained a mystery until the discovery of telomeric repeat DNA (Blackburn and Gall 1978) and reverse transcriptase, telomerase [initially named telomere terminal transferase] in *Tetrahymena thermophila* (Greider and Blackburn 1985; Greider and Blackburn 1987). The latter discovery was a major breakthrough in telomere biology and led to the trio; Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostak being awarded the Nobel Prize in medicine in 2009 (Wolinsky 2010). Telomerase has since been discovered in other organisms including humans (Morin 1989).

Telomere biology has become an area of extensive research with a principal objective of understanding the function and structure of telomeres, and their role in important biological processes such as ageing, cancer and chronic diseases.

1.3 Telomeric DNA

In most eukaryotes, telomeres consist of tandem arrays of asymmetric double stranded short GC-rich repeats [reviewed in (Zakian 1989); Table 1.1], which end with a 3′-G-strand overhang (Makarov et al. 1997; McElligott and Wellinger 1997). The exceptions to this general rule include telomeres of onion-related plants of the genus *Allium*, *Drosophila* and other dipterans. In the genus *Allium,* telomeres consist of terminal 375bp satellite (Pich and Schubert 1998). Whilst in *Drosophila* and other dipterans, telomeres are composed of multiple copies of head-to-tail terminal arrays of non-LTR retro-transposable elements HeT-A and TART [(Biessmann et al. 1990a); also reviewed in (Mason et al. 2008)].

Apart from these exceptions, terminal repeats appear to be highly conserved such that telomeres in all vertebrates consist of simple sequence repeat motif, TTAGGG (Moyzis et al. 1988; Meyne et al. 1989). In addition, similar telomeric repeats occur among unrelated organisms, for instance, filamentous fungus, *Aspergillus nidulans* maintains short vertebrate-like TTAGGG repeats

Table 1.1 illustrates the different telomere repeats in different organisms

Organism	Telomeric repeat	References
Vertebrates (e.g Human)	TTAGGG	(Moyzis et al. 1988; Meyne et al. 1989)
Trypanosoma	TTAGGG	(Blackburn and Challoner 1984)
Tetrahymena,	TTGGGG	(Blackburn and Gall 1978)
Paramecium	TTGGG(T/G)	(Baroin et al. 1987)
Oxytricha, Euplotes	TTTTGGGG	(Klobutcher et al. 1981)
Plasmodium	TTAGGG(T/C)	(Ponzi et al. 1985)
Arabidopsis thaliana	TTTAGGG	(Richards and Ausubel 1988)
Chlamydomonas	TTTTAGGG	(Petracek et al. 1990)
Schizosaccharomyces pombe	TTAC(A)(C)G(1-8)	(Matsumoto et al. 1987)
Saccharomyces cerevisiae	$(TG)_{1\text{-}6}\ TG_{2\text{-}3}$	(Shampay et al. 1984; Wang and Zakian 1990)
Filamentous fungi, slime moulds	TTAGGG	(Forney et al. 1987; Schechtman 1990)
Candida albicans	GGTGTACGGATGTCTAACTTCTT	(McEachern and Hicks 1993)
Kluyveromyces lactis	GGTGTACGGATTTGATTAGGTATGT	(McEachern and Blackburn 1994)
Caenorhabditis elegans	TTAGGC	(Wicky et al. 1996)
Bombyx mori	TTAGG	(Okazaki et al. 1993)
Ascaris lumbricoides	TTAGGC	(Muller et al. 1991)

(Bhattacharyya and Blackburn 1997) suggesting a common recent ancestry during eukaryotic evolution. However, telomeric repeats can vary in type and size, for example, telomeres of *Saccharomyces cerevisiae* consists of irregular repeats, $(TG)_{1\text{-}6}$ $TG_{2\text{-}3}$ (Wang and Zakian 1990). Furthermore, same chromosome within an individual can possess more than one type of telomere repeats as documented in *Tetrahymena thermophil*a in which micronuclear telomeres consist of internal G_4T_3 and external G_4T_2 (Kirk and Blackburn 1995).

1.3.1 Human telomeres

In human, telomeres consist of 9-25 kb of duplex non-coding TTAGGG tandem repeats in the germ-line and no more than several hundred nucleotides of single-stranded 3′ overhang [Figure 1.1; (Wright et al. 1997; Baird et al. 2006)]. The proximal region (0-3kb) is marked by interspersion of the canonical TTAGGG repeats, with telomere variants repeats such as TGAGGG (G-type), TCAGGG (C-type) and TTGGGG (J-type) among others [N-type; (Allshire et al. 1989; Varley et al. 2002)]. Whilst the distal region consists of a homogenous array of TTAGGG repeats (Allshire et al. 1989; Varley et al. 2002).

1.4 Telomeric Repeat-containing RNA (TERRA)

Until recently when two independent research groups reported that mammalian telomeres are transcribed into telomeric-repeat-containing RNA called TERRA or TelRNA (Azzalin et al. 2007; Schoeftner and Blasco 2008), telomeres were considered transcriptionally silent. Telomeres are transcribed by DNA-dependent RNA Pol II from multiple different sub-telomeric sites to generate heterogeneous lengths (100bp to 9kb) of UUAGGG repeats with transcripts containing CCCUAA expressed at very low to undetectable levels (Schoeftner and Blasco 2008) in mammals, Zebrafish (*Danio renio*) and yeast (Luke et al. 2008; Schoeftner and Blasco 2008). However, it is not yet known if TERRA molecules are translated. However, TERRA molecules are found only in nuclear fractions but not in cytoplasm suggesting that they may not be translated.

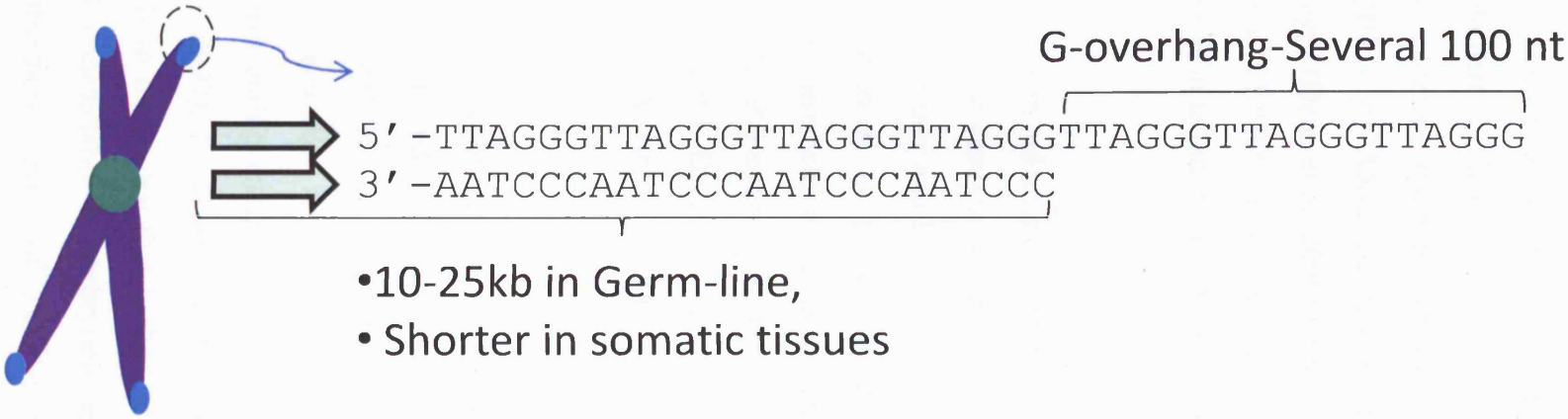


Figure 1.1 Telomere structure. An "open" linear structure of the chromosome termini. In humans, telomeres are composed of duplex TTAGGG repeats and several hundreds of TTAGGG overhang.

The role of TERRA molecules in telomere structural stability (Deng et al. 2009b), heterochromatin formation (Schoeftner and Blasco 2008) and telomerase regulation (Schoeftner and Blasco 2008) have been suggested. For instance, TERRA molecules interact with shelterin components TRF1/2 and their depletion induces telomere dysfunction characterised by increased frequency of telomere free ends, telomere doublets, telomere double minutes, chromatid duplications and 53BP1/ γH2AX-associated telomere foci without significant telomere length changes (Deng et al. 2009b), indicating loss of telomere capping function. In addition, TERRA molecules have been reported to form G-quadruplex structures with telomeric DNA (Xu et al. 2008; Randall and Griffith 2009).

In support of their role in telomerase regulation, TERRA containing oligo-nucleotides hybridise to the catalytic subunit of telomerase *in vitro* thus inhibit telomerase activity (Schoeftner and Blasco 2008). Whilst the role of TERRA in heterochromatin formation is supported by the observation that TERRA associates with several heterochromatin-associated proteins, including methyl CpG binding protein (MeCP2) and heterochromatin protein 1 (HP1) at telomeres (Schoeftner and Blasco 2008; Deng et al. 2009b). TERRA also accumulates at the vicinity of inactivated X-chromosome in female mouse cells (Schoeftner and Blasco 2008).

TERRA levels are regulated by several mechanisms, including developmental stage, cellular localisation and differentiation, telomere length and telomeric chromatin (Schoeftner and Blasco 2008; Caslini et al. 2009). For instance, TERRA localises to telomeres at different phases of the cell cycle, being detectable at telomeres during interphase and metaphase (Azzalin and Lingner 2007; Azzalin et al. 2007). In addition, differential expression of TERRA is limited to adult cells in mice such that highest levels are detected in thymus, kidney, spleen and undetectable in embryonic mouse cells (Schoeftner and Blasco 2008). Furthermore, levels of TERRA are directly correlated with

telomere length such that cells with longer telomeres express high levels of TERRA. Similarly, cells from laboratory mice exhibit higher levels of TERRA compared to human cells (Schoeftner and Blasco 2008). Likewise, diminished TERRA levels have been reported in ageing fibroblasts with shortened telomere repeat tracts (Caslini et al. 2009), further implicating direct correlation between telomere length and levels of TERRA molecules at telomeres. On the contrary, human cells derived from ICF syndrome patients exhibit elevated TERRA levels and shortened telomeres suggesting that TERRA expression cannot be simply correlated with telomere length (Yehezkel et al. 2008; Schoeftner and Blasco 2009; Deng et al. in press).

1.5 Telomere length

The length of the telomere repeat arrays is not precise and varies considerably among species (Zakian 1995), in similar organisms as well as in different cells of the same organism (Schmitt et al. 1994; Londono-Vallejo et al. 2001; Baird et al. 2003; Graakjaer et al. 2004; Londono-Vallejo 2004; Graakjaer et al. 2006b). For instance in humans, telomeres vary between 9-25kb in germ-line (Moyzis et al. 1988; Baird et al. 2006) and are much shorter in somatic cells depending on the tissue type, age and replicative history (Lindsey et al. 1991). However, in cancer cells telomeres can extend beyond 20kb depending on the telomere maintenance mechanism employed (Schmitt et al. 1994). For example, long telomeres (70.2kb) have been reported in cells maintaining their telomeres through telomerase-independent recombination-based mechanisms collectively known as alternative lengthening of telomeres (Cesare and Griffith 2004).

In contrast, laboratory mice, *Mus Musculus* have very long telomeres exceeding 50kb (Kipling and Cooke 1990) while wild-type mice have shorter telomeres (Zijlmans et al. 1997). In other organisms, telomeres are much shorter being as short as 20 base pairs in *Oxytrichia* (Klobutcher et al. 1981) and a few hundred bases long in *Saccharomyces cerevisiae* (Wang and Zakian 1990; Zakian 1995).

In addition to being species-specific, telomere lengths vary greatly among telomeres within the same cell as well as among alleles of the same telomere (Lansdorp et al. 1996; Londono-Vallejo et al. 2001; Baird et al. 2003; Londono-Vallejo 2004; Britt-Compton et al. 2006; Capper et al. 2007). However, specific telomeres display similar length profiles in different cells of the same individual but varies between individuals (de Lange et al. 1990). For instance, different studies have demonstrated that telomeres on both arms of chromosome 17 are among the shortest in human cells while 4q telomere is among the longest of human telomeres (Martens et al. 1998; Perner et al. 2003; Zou et al. 2004b).

The telomere length is paternally inherited (Nordfjall et al. 2005; Njajou et al. 2007). In support of this, it has been reported that paternal age is an important determinant of telomere length in offspring, such that offspring of older fathers have longer telomeres, consistent with the notion that telomere length of human sperm cells increases with donor age (Allsopp et al. 1992; Unryn et al. 2005; De Meyer et al. 2007). In addition, the relative telomere length is established in the zygote and maintained throughout life (Graakjaer et al. 2004; Graakjaer et al. 2006a; Graakjaer et al. 2006b) suggesting that telomere length detected in any tissue will be reflective of the length at birth, assuming similar telomere dynamics at all telomeres.

Moreover, variation in telomere length according to tissue and age of an individual has been reported (Takubo et al. 2002). For instance, the telomere length is typically 2-8kb in peripheral blood (Valdes et al. 2005) while in germ-line, it can extend beyond 20kb (Baird et al. 2006). In addition, the age-dependent decline in telomere length has been reported to occur more rapidly in peripheral blood lymphocytes (53bp/year) than in granulocytes (Hoffmann et al. 2009). The variation in telomere length among the different tissues from the same individual has been demonstrated previously (Friedrich et al. 2000; Takubo et al. 2002). For instance, shorter telomeres were reported in leukocytes (6.5kb) than in skin (7.8kb) and synovial tissues [7.9kb; (Friedrich et al. 2000)], whilst

longer telomeres (>10kb) were reported in cerebral cortex, myocardium, liver, renal cortex and spleen regardless of the age of the subject (Takubo et al. 2002).

1.6 Telomere functions

Telomeres have a number of functions, which are dependent on both the telomere length as well as the telomere structure (Blackburn 2001).

1.6.1 Provide a solution to the "end–replication problem"

The unidirectional semi-conservative replication nature and inability of the DNA polymerases to initiate replication without a need for RNA pose a problem for replication of chromosome termini during lagging strand synthesis. Due to these features, the parental lagging strand of the newly synthesised molecule is not fully replicated such that when the terminal primer is removed the gap is left which is prone to nucleolytic processing and cleavage. Hence there is a loss of few nucleotides with each round of replication (Watson 1972). This phenomenon has been termed "end-replication problem" [(Olovnikov 1973); Figure 1.2]. Eukaryotes have evolved different mechanisms to compensate for the loss of DNA upon replication, some of which are discussed below.

1.6.1.1 Telomerase

Unlike in somatic cells where the telomeres erode as a function of time, in rapidly proliferating cells such as germ-line, lymphocytes, basal layer of the epidermis, some adult stem cells and most cancer cells, telomere attrition is counteracted by a reverse transcriptase telomerase, that *de novo* adds telomeric repeats to chromosome ends (Kim et al. 1994). Telomerase is fully discussed in section 1.11.

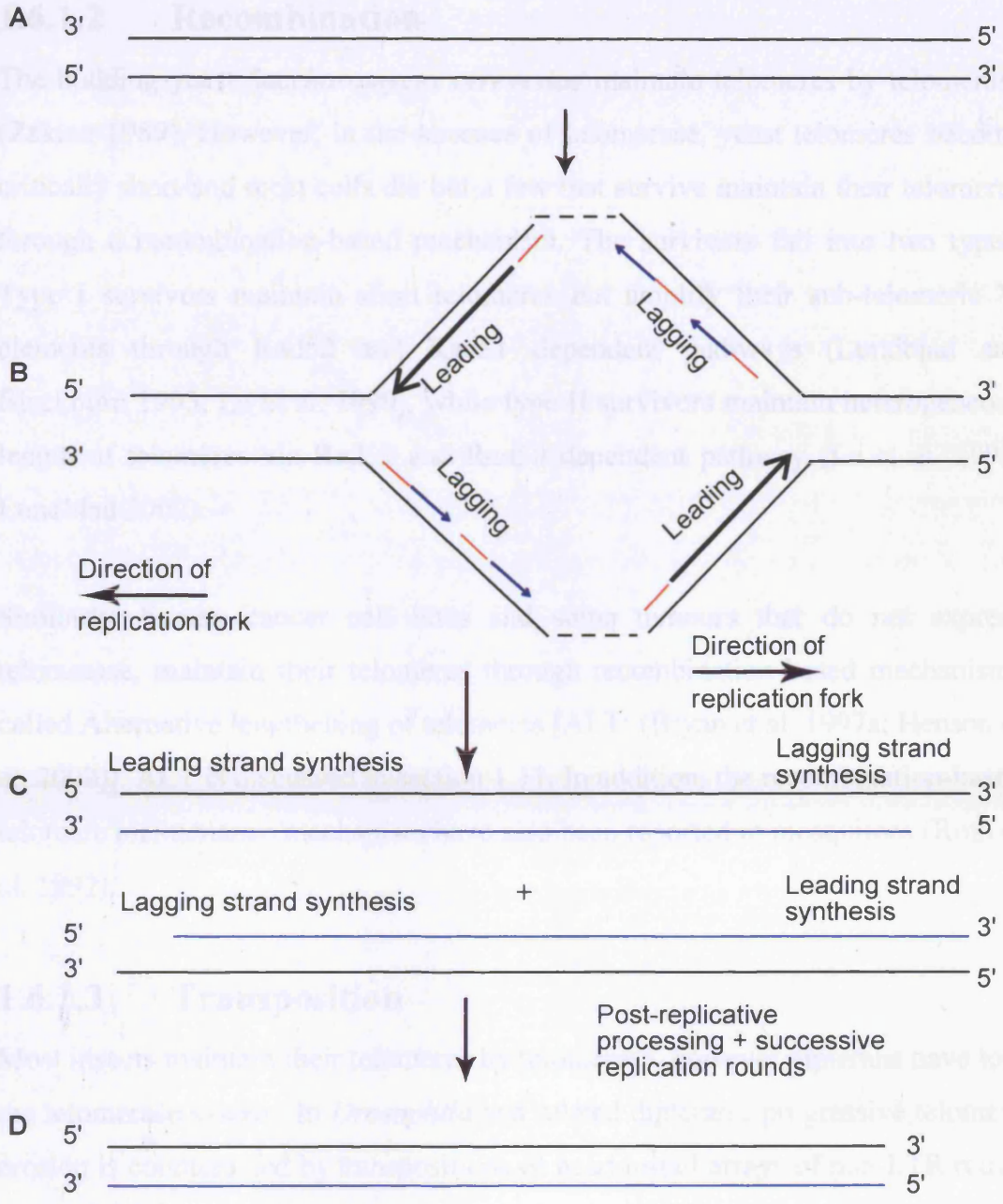


Figure 1.2. Illustrating the "end-replication" problem. DNA polymerases require an RNA primer to intiate DNA synthesis. When the 5′ terminal primer is removed from the lagging strand, a single-stranded DNA molecule is left. A. Outline of the double strand (ds) DNA prior to replication. B. Semi-conservative replication showing the direction of the replication fork, the leading strand (black arrow) and okazaki fragments of lagging strand synthesis (blue arrows). Primers are denoted by red lines at the start of newly synthesised strand. C. dsDNA with 3′ single-stranded overhang on the parental strand of the lagging strand synthesis (black) as the newly synthesised strand is shorter (blue). D. Shortened chromosome termini after replication of the newly synthesised strand.

1.6.1.2 Recombination

The budding yeast *Saccharomyces cerevesiae* maintain telomeres by telomerase (Zakian 1989). However, in the absence of telomerase, yeast telomeres become critically short and most cells die but a few that survive maintain their telomeres through a recombination-based mechanism. The survivors fall into two types; Type I survivors maintain short telomeres but amplify their sub-telomeric Y' elements through Rad52 and Rad51 dependent pathways (Lundblad and Blackburn 1993; Le et al. 1999), while type II survivors maintain heterogeneous length of telomeres via Rad52 and Rad50 dependent pathway (Le et al. 1999; Lundblad 2002).

Similarly, human cancer cell lines and some tumours that do not express telomerase, maintain their telomeres through recombination-based mechanisms called Alternative lengthening of telomeres [ALT; (Bryan et al. 1997a; Henson et al. 2002)]. ALT is discussed in section 1.11. In addition, the recombination-based telomere maintenance mechanism have also been reported in mosquitoes (Roth et al. 1997).

1.6.1.3 Transposition

Most insects maintain their telomeres by telomerase, however dipterans have lost the telomerase system. In *Drosophila* and related dipterans, progressive telomere erosion is counteracted by transpositions of head-to-tail arrays of non-LTR retro-transposable elements HeT-A and TART thereby forming a heterochromatin that protects the telomeres from degradation [(Biessmann et al. 1990b); also reviewed in (Mason et al. 2008)].

1.6.2 Telomere capping and Chromosome stability

The primary function of telomeres is to preserve the integrity of the chromosomes, prevent chromosome instability and allow cell cycle progression (McEachern et al. 2000b; Blackburn 2001). This telomere capping function

depends largely on the minimum telomere length and integrity of multi-protein complex, "Shelterin" or "telosome" which together form a scaffold in which the end is sequestered within the duplex DNA. In this higher-order conformation, telomeres (unlike internal DSBs) are protected from nucleolytic attack, end-to-end fusions and illegitimate DNA damage checkpoints and repair mechanisms such as homologous recombination and non-homologous end-joining [(Griffith et al. 1999; de Lange 2005; Denchi and de Lange 2007); reviewed in (McEachern et al. 2000a)]. Telomeres are believed to be capped at all the times except during cell division (Smith and Blackburn 1999) indicating that the integrity of the telomere cap is essential for cell survival. In support of this, defects in shelterin component, TRF2 leads to rapid loss of overhang, cell cycle arrest, apoptosis and increased frequency of end-to-end chromosome fusions (van Steensel et al. 1998).

1.6.3 Chromosome healing

As early as the 1940s, Barbara McClintock demonstrated that telomeres have a potential of preventing the Breakage-Fusion-Bridging cycles thus healing chromosomes (McClintock 1941). It was only after the discovery of telomerase, that it became apparent that telomerase was capable of *de novo* addition of telomeric repeats to broken chromosome and maintain chromosome stability, a process termed "chromosome healing" (Murnane and Yu 1993; Flint et al. 1994; Melek and Shippen 1996; Slijepcevic and Bryant 1998). Direct addition of telomere repeats to broken chromosomes has been reported in humans (Flint et al. 1994; Wong et al. 1997; Fouladi et al. 2000; Varley et al. 2000) and mouse embryonic stem cells (Sprung et al. 1999; Gao et al. 2008). For example in human diseases such α-thalassaemia (16p) and mental retardation syndrome (22q), the breakage point within the sub-telomeric DNA (about 50kb-2Mb proximal to the telomeres) is stabilised by direct addition of telomeric DNA to the breakage point (Wilkie et al. 1990; Lamb et al. 1993). Similarly, *de novo* addition of telomere repeats to sites similar to those found at terminal deletion

resulting in α-thalassaemia have been reported in extracts from human cancer cells (Morin 1991).

1.6.4 Telomeres and chromosome segregation

Telomeres have also been implicated in chromosome segregation. In support of this, telomeres cluster close to the inner nuclear envelope during meiotic prophase I in various organisms, suggesting a possible role of telomeres in facilitating homologous chromosome pairing during meiosis (de Lange 1992; Morin 1997; Harper et al. 2004). In addition, recent studies in human cells have suggested that degradation of a telomere-specific cohesion complex is necessary for sister telomere segregation during mitosis (Dynek and Smith 2004). Furthermore, the isoform of TIN2 (TIN2L), a central player in "shelterin" has been found to interact with the nuclear matrix suggesting the role of TIN2 in tethering the telomeres to nuclear matrix (Kaminker et al. 2009).

1.6.5 Telomere position effect

By virtue of being heterochromatic, telomeres have the potential to influence the transcription of genes located in their proximity by silencing them (Zakian 1995; Urquidi et al. 2000); the phenomenon termed telomere position effect (TPE). TPE is extensively documented in yeast (Tham and Zakian 2002) but it is not as dramatic in human cells (Baur et al. 2001; Baur et al. 2004). In *S.cerevisiae*, repressor activator protein 1 (Rap1) interacts with silent information regulator (Sir) 2, 3 and 4 proteins and deacetylate histone 3 and 4 thus facilitating the spread of telomeric heterochromatin. TPE is stronger closer to the telomeres but can extend extensively into the sub-telomere, such that genes located 10–40 kb upstream of telomeres are silenced in *S. cerevisiae* (Kyrion et al. 1993; Halme et al. 2004).

Consistent with being heterochromatic (Tommerup et al. 1994), human and mouse sub-telomeres are heavily methylated (Steinert et al. 2004) through the

action of the DNA methyltransferases DNMT1, DNMT3a and DNMT3b (Brock et al. 1999; Gonzalo et al. 2006). In addition, the heterochromatin proteins HP1 alpha and beta (Garcia-Cao et al. 2004) associate with telomeres. The sub-telomeric and telomeric heterochromatin is believed to be regulated by telomere length (Garcia-Cao et al. 2004); such that loss of telomeric repeats in telomerase-deficient ($Terc^{-/-}$) mice leads to continuous loss of H3K9me3, H4K20me3 and HP1, significant reduction in sub-telomeric DNA methylation as well as increase in histone H3 and H4 acetylation at telomeres and sub-telomeres (Benetti et al. 2007).

1.7 Telomere structure

At least three factors are necessary for telomere protection: minimum telomere length, the integrity of the G-strand overhang and high-order telomere structure t-loop with associated telomere binding proteins (Griffith et al. 1999; Blasco 2002).

1.7.1 Telomere-loop (t-loop)

Mammalian telomeres terminate with a 3' overhang, which is believed to loop back and invade the proximal part of the telomere to form a high order, lariat structure called the telomere loop [t-loop; Figure 1.3; (Griffith et al. 1999; Stansel et al. 2001; de Lange 2004)]. In this structure, the 3' overhang displaces the G-strand and base pairs with the C-strand thus creating a displacement loop [D-loop; Figure 1.3; (Griffith et al. 1999)]. The t-loop is stabilised by telomeric proteins especially TRF2 which binds at the t-loop junctions (Griffith et al. 1999). In addition to being a high order end-protection structure, t-loop might restrict access of telomerase to telomeres, thus prevents unnecessary telomere elongation.

The t-loops of varying size have also been observed in other organisms including trypanosomes (0.3kb), ciliates, plants (50kb in peas), *C.elegans* and some yeasts

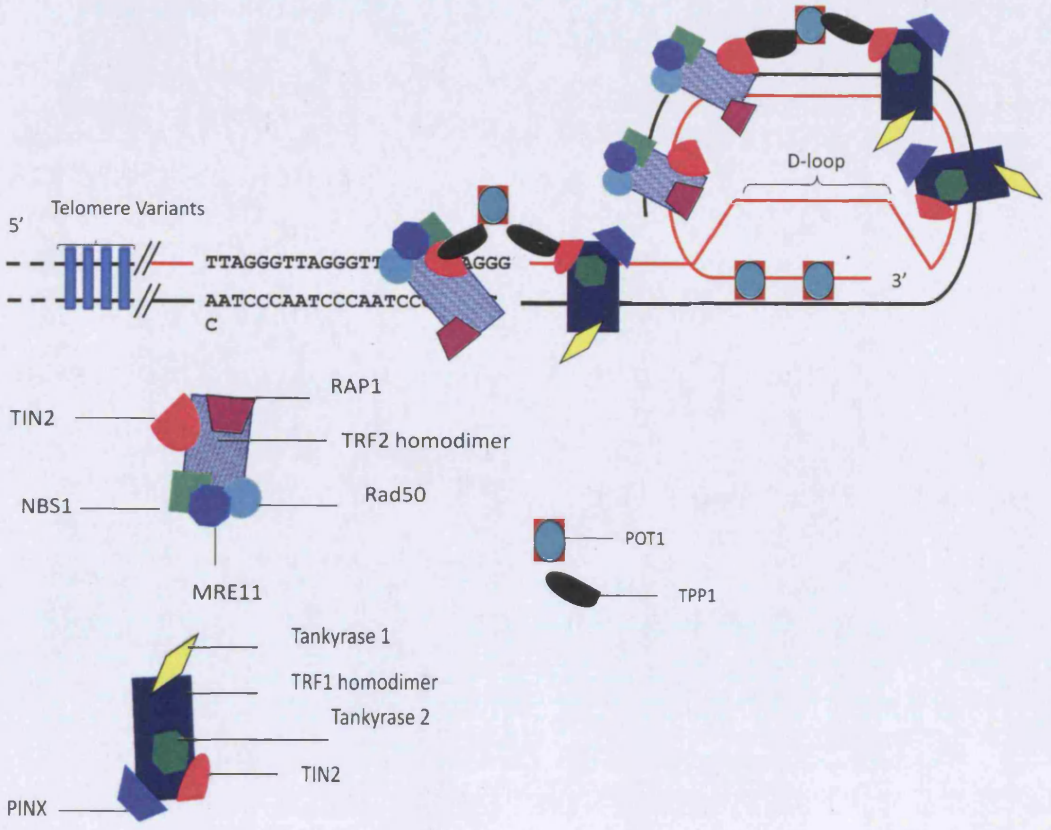


Figure 1.3 Telomere-loop. The simplified telomere structure in a t-loop "closed" conformation with telomere binding proteins bound at telomeres. The 3′ overhang has looped back and invaded the proximal telomere to form the t-loop structure. The t-loop is stabilised by a number of protein complexes. Two related factors, TRF1 and TRF2 bind duplex TTAGGG and recruit the other four subunits to the shelterin complex; single-strand binding protein, POT1 which is linked to the TRF1 and TRF2/RAP1 sub-complex by TIN2, TPP1 (de Lange 2005a; Palm and de Lange 2008)]. Other proteins that bind on TRF2 such as BLM and WRN are not shown.

[excluding *S. cerevisiae*; (Munoz-Jordan et al. 2001; Cesare et al. 2003; de Lange 2004; Cesare et al. 2008; Cesare and Reddel 2008)].

1.7.2 G-strand over hang

The 3′ overhang which is several hundred of nucleotides long plays an important role in telomere structure and protection (Wright et al. 1997). It prevents formation of end-to-end fusions by sequestering the ends of the telomeres from DNA repair machinery, through formation of a t-loop (Griffith 1999). Evidence for the importance of overhang in preventing telomere fusions comes from the increased frequency of the fusions in the presence of any mutations that lead to overhang loss such as inhibition of TRF2 (van Steensel et al. 1998).

The overhang also forms a platform for single strand telomere binding proteins; TEBP in ciliates (Price and Cech 1987), Cdc13p in *S-cerevisiae* (Lin and Zakian 1996; Chandra et al. 2001) and POT1 in human, mouse, chicken and plants (Baumann and Cech 2001; Baumann et al. 2002; Wei and Price 2004).

1.8 Telomere associated proteins (shelterin)

The telomeric DNA forms a platform for binding of a multi-protein complex called "Shelterin" (de Lange 2005) or telosome (Liu et al. 2004a). These proteins play a role in structural capping of telomeres in which they shield the chromosome ends from DNA repair machinery (Karlseder et al. 2004; Celli and de Lange 2005; Denchi and de Lange 2007). They are also involved in telomere length regulation through either direct interaction with telomerase or through remodelling telomeric chromatin (Smogorzewska and de Lange 2004).

The shelterin complex consists of six factors; three [TRF1, TRF2, POT1] bind directly to the telomere DNA and recruit three others [RAP1 TIN2, TPP1] which links the shelterin into one complex (de Lange 2005).

1.8.1 Telomere Repeat binding Factor 1 (TRF1)

TRF1 is a small dimeric protein which consists of three domains; C-terminal DNA binding (Bianchi et al. 1999; Court et al. 2005; Hanaoka et al. 2005), dimerisation/TRF homology (TRFH) and the acidic N-terminal protein interacting domains (Smith and de Lange 1997). It is considered to be a principal negative regulator of telomere length as its over-expression induces gradual telomere shortening to a new telomere length range while inhibition of TRF1 binding through expression of the dominant negative form ($TRF1^{66-439;}$ lacks DNA binding domain) results in telomerase-mediated telomere elongation (van Steensel and de Lange 1997; Smogorzewska et al. 2000). TRF1 interacts with a number of proteins (through its acidic N-terminal domain), including TIN2, tankyrases1/2 and Pinx1, all of which control its telomere length regulatory role (Smogorzewska and de Lange 2004). In addition to its telomere length regulation function, TRF1 may play a role in telomere protection by stabilising the "shelterin complex." In fact TRF1 can bend telomeric DNA (Bianchi et al. 1997; Griffith et al. 1998), and disruption of TRF1 leads to decrease in TRF2 at telomeres (Iwano et al. 2004) and an increased association of remaining TRF2 with TIN2/TPP1 (Houghtaling et al. 2004). TRF1 may also be involved in cell survival as it has been demonstrated that its deletion in mice was lethal (Karlseder et al. 2003). In addition, TRF1 is required for proper replication of telomeres (Sfeir et al. 2009).

1.8.2 Telomere Repeat binding Factor 2 (TRF2)

The duplex-binding TRF2 is a close relative of TRF1 with similar architecture except that its N-terminal domain is guanine and arginine-rich (GAR) and hence was formerly called the N-terminal basic domain (Broccoli et al. 1997). Like TRF1, it binds DNA as homo-dimers and never hetero-dimerises with TRF1. It plays an important role in telomere "capping" and thus it is often referred to as a principal telomere "capping" protein. TRF2 has several properties that can potentially enable it to remodel TTAGGG-like containing DNA into t-loops *in*

vitro (Griffith et al. 1999; Stansel et al. 2001). Its multimeric binding ability can promote unwinding and strand invasion (Amiard et al. 2007). Whilst, its GAR domain has sequence-independent affinity for DNA junctions including Holliday junctions. Evidently, the mutant TRF2 without this GAR domain results in increased frequency of telomeric t-loop size circular DNA, an observation consistent with processing of the junction (Wang et al. 2004). In addition, TRF2 has been demonstrated to remodel the telomeric DNA into Holliday junctions and protects telomeres from DNA repair Holliday junction resolving activities (Poulet et al. 2009).

In support of its role in telomere capping, loss of TRF2 function destabilises the chromosomes, induces rapid loss of 3' single-strand G-overhang, senescence or apoptosis and increased frequency of NHEJ-dependent chromosome end-to-end fusions (van Steensel et al. 1998; Karlseder et al. 1999; Smogorzewska et al. 2000; Smogorzewska et al. 2002; Zhu et al. 2003; Celli and de Lange 2005).

The role of TRF2 at telomeres is controlled by interacting factors, some of which are involved in DNA damage response and repair pathways (Zhu et al. 2000; Karlseder et al. 2004; de Lange 2005; Gomez et al. 2006; Lenain et al. 2006; van Overbeek and de Lange 2006; Buscemi et al. 2009). These factors form a sub-complex that protects the 3' overhang from degradation and DNA repair activities thus preventing end-to-end fusions (Liu et al. 2004a; Ye et al. 2004a; de Lange 2005; Bae and Baumann 2007; Sarthy et al. 2009).

1.8.3 Repressor/Activator Protein1 (Rap1)

The human Rap1 is a telomere-specific protein and a distant homologue of the budding yeast scRap1p (Longtine et al. 1989). Unlike its yeast orthologue, human Rap1 lacks a DNA binding domain and therefore its localisation and recruitment to the telomeres is TRF2–dependent (Li and de Lange 2003). In support of this, hRap1 has been isolated in a complex with TRF2 and other TRF2

interacting proteins [Rad50, MRE11, ku70/86, PARP1; (O'Connor et al. 2004)]. Recently, it has been reported that human Rap1 may protect telomeres from illegitimate non-homologous end-joining through interaction with TRF2, suggesting that the TRF2 recruitment of Rap1 may be essential for preventing the type of chromosome fusions that occur in the absence of functional TRF2 at telomeres (Bae and Baumann 2007; Sarthy et al. 2009). However, interaction of hRap1 and other TRF2-binding factors can be independent of its interaction with TRF2 (O'Connor et al. 2004).

hRap1 acts as a negative regulator of telomere length through 1:1 interaction with TRF2 (Li and de Lange 2003). Evidently, over-expression of hRap1 and its mutants [$hRap^{\Delta BR\ \Delta M}$, $hRap1^{\Delta CT}$ and $hRap1^{\Delta BR\ \Delta CT}$] results in telomere elongation (Li et al. 2000; Li and de Lange 2003).

1.8.4 TRF1 and TRF2-interacting protein 2 (TIN2)

TIN2 is a small protein that interacts with TRF1 (Kim et al. 1999), TRF2 (Liu et al. 2004a; Ye et al. 2004a) and TPP1 [(Xin et al. 2007) also called PTOP (Liu et al. 2004b), PIP1 (Ye et al. 2004b) or TINT1 (Houghtaling et al. 2004)]. It forms a bridge that brings the duplex binding and single-stranded binding components of the shelterin together (Liu et al. 2004a; Ye et al. 2004a; de Lange 2005). i.e. TIN2's interaction with TPP1, TRF2 and TRF1 brings POT1/TPP1, TRF1 and TRF2 complexes together (O'Connor et al. 2006). Although TIN2 can interact with three subunits of the shelterin, it is not known whether it is permanently bound to them or it switches between TRF1-TRF2 complex and/ or TPP1-POT1 complex (Palm and de Lange 2008).

TIN2/TRF2 interactions stimulate telomere DNA interactions *in vitro* suggesting the role of the complex in modulating a tertiary telomeric structure (Kim et al. 2003) and hence telomere capping (Kim et al. 2008). Whilst its interaction with TRF1 stabilises TRF1-tankyrase interaction and protects TRF1 from being

ribosylated by tankyrases *in vitro* (Ye and de Lange 2004). Thus TIN2 plays a role in telomere capping and regulation of telomerase-mediated elongation (Smogorzewska and de Lange 2004; Ye and de Lange 2004).

1.8.5 Protection of Telomeres 1 (POT1)

POT1 is a single-stranded DNA binding protein that was identified through its sequence similarity to ciliate TEBP subunits (Baumann and Cech 2001). It is recruited to telomeres by TRF1 complex (Ye et al. 2004b) and binds specifically to the terminal 5′-TAGGGTTAG-3′ sequence through its N-terminus folds (de Lange 2005). The binding of POT1 to single-stranded 3′ overhang *in vitro* and *in vivo* blocks telomerase access to the telomeres (Loayza and De Lange 2003; Liu et al. 2004b; Wang et al. 2007a), thus it is a regulator of telomere length. In support of this, the expression of a mutant POT1 without telomere binding site (Loayza and De Lange 2003) and the over-expression of wild-type POT1 result in telomerase-dependent telomere elongation (Armbruster et al. 2004).

POT1 can also associate with telomeres without direct binding to the telomeric DNA but through interaction with TRF2 (Yang et al. 2005) and TRF1 (Loayza and De Lange 2003) sub-complexes. The deletion of the N-terminal DNA binding domain of POT1 only affects its DNA binding ability and not its association with telomeres through interaction with TRF1 complex (Loayza and De Lange 2003). Over-expression of POT1 protects the loss of overhang generated when the dominant negative TRF2 mutant is expressed (Yang et al. 2005). Together these findings indicate that POT1 plays a role in telomere capping and telomere length regulation.

1.8.6 TPP1, TINT1, PIP1, PTOP1

It consists of three domains: centrally located POT1-interacting domain, C-terminus TIN2-interacting domain and the OB (oligonucleotide/ oligosaccharide binding) fold with which it interacts with telomerase (Liu et al. 2004b).

TIN2/TPP1's interaction with TRF1 and TRF2 recruits POT1 to telomeres (Ye et al. 2004b; Kibe et al. 2009). In addition, TPP1-POT1 interaction is important for sub-cellular localisation of POT1 as well as stabilising POT1 on telomeres (Liu et al. 2004b). Furthermore, POT1/TPP1 hetero-dimerisation regulates recruitment of POT1 to telomeres and telomere length (Liu et al. 2004b). In support of this, depletion of TPP1 through expression of TPP1 mutants that lack POT1-interacting domain results in reduction or total loss of POT1 from telomeres. Furthermore, impaired TPP1 function or disruption of TPP1/POT1 interaction leads to telomere deprotection (Liu et al. 2004b; Xin et al. 2007).

1.8.7 Shelterin associated proteins

1.8.7.1 Pin2/TRF1 interacting protein 1 (Pinx1)

Pinx1 interacts with TRF1 and its isoform Pin2 (Shen et al. 1997) but has extra-telomeric roles which include ensuring faithful chromosome segregation during mitosis, plausibly through its interaction with Pin2 (Li et al. 2009; Yuan et al. 2009). It is believed that it acts in *trans* to regulate telomerase-mediated elongation. In support of this, silencing of Pinx1 in telomerase-positive human cancer cells impaired telomere length maintenance whilst over-expression or reduction leads to telomere shortening or elongation respectively (Zhou et al. 2003; Zhang et al. 2009). Pinx1 also plays a role in telomere length regulation through mediating accumulation of TRF1 in the nucleolus and enhancing TRF1's binding affinity at the telomeres (Yoo et al. 2009). Moreover, Pinx1 interacts with (Banik and Counter 2004) and inhibit telomerase activity directly (Lin and Blackburn 2004) through sequestering the catalytic subunit of telomerase in an inactive complex without telomerase RNA subunit (Zhou and Lu 2001; Zhou et al. 2003).

1.8.7.2 ATM

ATM kinases are phosphoinositide-3-kinase related DNA signalling proteins, which are mutated in ataxia telangiecstasia (AT), a rare, autosomal recessive

inherited disease characterised by immunodeficiency, chromosomal instability, premature ageing, cancer predisposition, lack of radio-resistant DNA synthesis, cell cycle-checkpoints impairment and shortened telomeres [reviewed in (Rotman and Shiloh 1999; Kastan and Lim 2000; Barzilai et al. 2002). ATM plays a central role in the signalling pathways leading to p53 activation in response to DSBs, through direct binding and phosphorylation of p53 (Banin et al. 1998; Canman and Lim 1998). ATM and its relative ATR associate with telomeres (Takata et al. 2004; Verdun et al. 2005). Thus, ATR and ATM play a role in both telomere length maintenance and telomere capping. Evidently, ATM-defective cells show accelerated telomere shortening, high levels of end-to-end chromosome fusions and the presence of extra-chromosomal telomeric fragments and circles (Metcalfe et al. 1996; Hande et al. 2001; Pandita 2001; Pandita 2002; Karlseder et al. 2004).

1.8.7.3 Tankyrase 1 and 2 (TRF1-interacting Ankyrin Related ADP-ribose polymerase)

Tankyrases 1 and 2 are two related poly ADP-ribose polymerases (PARPS) which have the ability to form both homo and heterodimers. Like other multifunctional PARPs, they have diverse functions in different subcellular compartments such as the Golgi, nuclear pore complexes and centrosomes (Smith et al. 1998; Chi and Lodish 2000; Kaminker et al. 2001; Chang et al. 2005).

They ADP-ribosylate TRF1 *in vitro* and once ribosylated, TRF1's DNA binding ability is diminished (Smith et al. 1998; Chiang et al. 2006). Consequently, TRF1 dissociates from telomeres after which it is then ubiquinated and degraded by proteosomes (Smith et al. 1998; Smith and de Lange 2000; Cook et al. 2002; Chang et al. 2003; Ye and de Lange 2004). Once TRF1 has dissociated from the telomeres, telomerase can then elongate telomeres (Seimiya et al. 2004). Thus, tankyrases are positive regulators of telomere length as they regulate telomere length indirectly by altering levels of TRF1 bound at telomeres in telomerase-

positive cells *in vivo* and *in vitro* thereby allowing telomerase-mediated telomere elongation (Smith et al. 1998; Smith and de Lange 2000; Seimiya et al. 2004; Chiang et al. 2006). In contrast, tankyrase 1 knockdown by siRNA interference, results in telomere shortening (Donigian and de Lange 2007), thus further supporting the role of tankaryses in telomere length regulation.

Apart from TRF1-mediated telomere length regulation, PARP activity of tankyrases is required for dissolving sister chromatid cohesion at telomeres and for separating sister chromatid telomeres during mitosis, indicating the role of tankyrases in ensuring proper chromosome segregation (Dynek and Smith 2004).

1.8.7.4 PARP1 and 2

Poly (ADP-ribose) polymerase 1 and 2 (PARP-1 and 2) are proteins involved in base excision repair (BER) where they ribosylate other proteins (Ame et al. 1999; Schreiber et al. 2002). PARP-1 ribosylates TRF2 and tethers it from telomeres and thus allows access by DNA repair machinery (Gomez et al. 2006), whilst PARP-2 binds to TRF2 with high affinity (Dantzer et al. 2004) suggesting the role of both PARPs in telomere capping. Evidently, deficiency of PARP-1 in mice leads to an increased frequency of telomere fusions, abundant chromosomal breaks and telomere erosion (d'Adda di Fagagna et al. 1999). Similarly, cells from mice defective in PARP-2 display higher frequencies of individual chromosomes without detectable telomeric sequences (Dantzer et al. 2004).

1.8.7.5 Bloom syndrome protein (BLM)

BLM is a 3′-5′ RecQ DNA helicase required for maintaining genome stability and suppression of sister chromatid exchanges. It is mutated in Bloom Syndrome (BS), a rare recessive disorder associated with growth retardation, immunodeficiency and increased risk of malignancy at an early age (Ellis et al. 1995; Karow et al. 1997).

Like other RecQ proteins, BLM participates in several diverse processes such as DNA repair, replication and homologous recombination (Karow et al. 1997). At telomeres, BLM plays a role in telomere length regulation through interaction with TRF1, TRF2 and POT1, all of which regulate its DNA unwinding activity (Lillard-Wetherell et al. 2004; Opresko et al. 2005b). In ALT+ cells, BLM interacts with TRF2 and together with WRN and RPA unwind TRF2-prebound telomeric DNA (Opresko et al. 2002) thus resulting in an increased telomeric length suggesting its role in recombination-mediated telomere elongation (Stavropoulos et al. 2002; Lillard-Wetherell et al. 2004). However, in telomerase-positive cells, loss of BLM result in increased telomere length suggesting the role of BLM in inhibiting activity of telomerase at telomeres (Schawalder et al. 2003). Thus BLM may play a role in both telomerase-dependent and independent telomere elongation.

1.8.7.6 Werner syndrome protein (WRN)

WRN is a 3′-5′ RecQ helicase and 3′-5′ exonuclease (Gray et al. 1997; Huang et al. 1998; Kitao et al. 1998) which is mutated in Werner syndrome (WS); an autosomal recessive disease characterised by premature ageing, cancer-predisposition and genomic instability. Like BLM, it participates in replication, DNA repair, recombination and transcription (Bohr et al. 2002) and interacts with TRF2 and POT1 to unwind telomeric DNA (Opresko et al. 2002). WRN may also play a role in telomere capping as cells from WS patients display chromosomal abnormalities, accelerated telomere shortening and early entry into replicative senescence (Tahara et al. 1997; Opresko et al. 2002). Unlike in BS cells, expression of hTERT alone rescues cells from senescence without completely restoring the chromosomal instability observed in WS cells, indicating that other factors than telomere impairment are responsible for the elevated genomic instability in WS cells (Kruk et al. 1995; Schulz et al. 1996; Wyllie et al. 2000). Single molecule analysis of fibroblast cultures derived from Werner patients did not reveal any significant rate of telomere erosion compared

to normal fibroblasts (Baird et al. 2004). Together these indicate an indirect effect of the absence of WRN on telomere dynamics that may be influenced by the kinetics of cell growth (Wyllie et al. 2000; Baird et al. 2004).

1.8.7.7 Non-homologous end-joining (NHEJ) and Homologous Recombination (HR) proteins

NHEJ is a mechanism that involves joining of DNA ends with the use of little or no sequence homology. It employs a number of proteins, which include Ku heterodimer, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, Ligase IV, Artemis and XLF [also known as Cernunnos; (Kanaar et al. 1998; Smith and Jackson 1999; Karran 2000)]. The key components of NHEJ do not only bind telomeres but are also essential for telomere length homeostasis and chromosome end protection (d'Adda di Fagagna et al. 2004; Lenain et al. 2006; van Overbeek and de Lange 2006).

HR is a repair mechanism, in which a homologous chromatid serves as a template for repair of broken strand. HR is a predominant mechanism for DSB repair during the G_2 phase of mammalian cell cycle when the sister chromatids are available (Karran 2000). HR consists of a number of proteins, like RAD50, RAD51, RAD52, RAD54, MRE11, RPA, BRCA1, RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 and BRCA2 etc [reviewed in (Karran 2000; Li and Heyer 2008)].

1.8.7.7.1 KU heterodimer

Ku heterodimer [Ku70 and Ku80 subunits] is a DSB recognition protein that has high affinity for DNA ends [reviewed in (Fisher and Zakian 2005). It binds to telomeres (Bianchi and de Lange 1999; Hsu et al. 1999; d'Adda di Fagagna et al. 2001) and plays a role in telomere length homeostasis and chromosome end protection through interaction with TRF1 and TRF2 (Kysela et al. 2003). Ku also

play a role in telomere length regulation through inhibiting telomerase access to telomeres (Slijepcevic and Bryant 1998; Espejel et al. 2002a).

In support of its capping function, Ku86 knockout mice exhibit elevated frequencies of end-to-end chromosomal fusions with telomeric sequences clearly detectable at fusion points indicating loss of telomere cap without telomere erosion (Bailey et al. 1999; Hsu et al. 1999; Hsu et al. 2000; Samper et al. 2000; d'Adda di Fagagna et al. 2001). Whilst its role as a negative telomere length regulator is supported by the studies in human cells, in which deletion of a single Ku allele or suppression of Ku by RNA interference (RNAi) resulted in severe telomere shortening and telomeric fusions (Jaco et al. 2004; Myung et al. 2004). This role is further supported by studies in yeast where, loss of ku functions resulted in telomere erosion (Boulton and Jackson 1998).

1.8.7.7.2 DNA Pkcs

DNA-dependent protein kinase catalytic subunit (DNA PKcs) is a member of phosphoinositide-3-kinase related family, which also include ATM and ATR kinases. Similar to Ku, DNA-PKcs localises to telomeres (d'Adda di Fagagna et al. 2001) where it plays a role in telomere capping. Its deficiency in mouse cells result in accumulation of telomere fusion events without evidence of change in telomere length (Gilley et al. 2001). Whilst, cells from severe combined immunodeficiency (SCID) mice (natural DNA-PKcs mutants) display telomere fusions and abnormal telomere elongation (Hande et al. 1999; Goytisolo et al. 2001) suggesting that DNA PKcs may be involved in regulation of telomerase-mediated elongation. Evidently, mouse cells doubly deficient in DNA-PKcs and telomerase show accelerated telomere shortening (Espejel et al. 2002b).

1.8.7.7.3 MRE11 complex (MRN)

The MRE11 complex consists of evolutionary conserved MRE11 and Rad50 and less conserved NBS1 [in mammals] /Xrs2 [yeast] protein (Haber 1998;

D'Amours and Jackson 2002). MRE11 is a structure-specific nuclease, which exhibits 3'-5' double-stranded exonuclease and single-stranded/ double-stranded DNA endonuclease activities (D'Amours and Jackson 2002). The MRN complex plays a role in DNA damage detection, signalling and repair through ATM kinase pathway, HR, NHEJ (Lee and Paull 2005; Paull and Lee 2005) and micro-homology-mediated end joining [MMEJ; (Ma et al. 2003)].

At telomeres, however the complex helps to distinguish telomeres from DSBs. It binds to telomeres at different stages of cell cycle through interaction with TRF2 (Zhu et al. 2000). i.e. MRE11 and Rad50 are bound to TRF2 throughout cell cycle while NBS1 localises to telomeres only during G_2 and S-phases indicating a role in telomeric replication or t-loop formation (Zhu et al. 2000; Verdun et al. 2005).

Recently, it has been demonstrated that MRE11 plays several functions at uncapped telomeres, which include protective role in preventing NHEJ-mediated fusion of leading strand telomeric DNA and removal of the 3' telomeric overhang to promote chromosome fusions in the absence of TRF2 (Deng et al. 2009a; Zha et al. 2009). Thus, MRE11 plays an essential role in telomere capping.

The complex may also cooperate in telomerase-dependent and independent telomere maintenance. In fact, cells from NBS patients display 25% decline in telomere length and early onset of senescence, which could not be rescued by reintroduction of either hTERT or NBS1 alone. However, simultaneous expression of both hTERT and NBS1 restored proliferation capacity and increased telomere length (Ranganathan et al. 2001; Riha et al. 2006). In ALT cells, NBS1 interacts with TRF1 (Wu et al. 2000) and together with TRF2 localises with PML bodies, structures characteristic feature of ALT cells (Zhu et al. 2000; Wu et al. 2007). Furthermore, knockdown of NBS1 inhibits formation of PML bodies and thus prevent activation of ALT (Jiang et al. 2005), further suggesting the role of NBS1 in suppressing recombination at telomeres.

Deficiency of other HR components such as RAD54 (Jaco et al. 2003) and RAD51D (Tarsounas et al. 2004) result in telomere shortening and increase in frequencies of end-to-end chromosome fusions and cell death through apoptosis (in case of RAD51D) indicating loss of telomere-capping function (Tarsounas et al. 2004). Similarly, inhibition of BRCA1 localisation to telomeres results in telomere elongation in the presence and absence of telomerase activity (Ballal et al. 2009). Together these studies suggest that different HR components may play a role in telomere capping function.

1.9 Telomere evolution

In most eukaryotes, telomeres consist of TTAGGG-like repeats with the exception being budding yeasts; this is suggestive of the common evolutionary route from TTAGGG-containing recent ancestral eukaryotes [Figure 1.4; (Li et al. 2000)]. Li and colleagues have suggested a model for the evolution of the telomeric complex in which the ancestral eukaryotic telomeres were composed of TTAGGG repeats and were maintained by telomerase (Li et al. 2000). During evolution, mutations in telomerase activity could have given rise to different TTAGGG derivatives. In support of this, our recent study (Letsolo et al. 2009) and others (Allshire et al. 1989; Baird et al. 1995) analysis of the human telomeres have revealed that the proximal telomeres consists of at least 24 different repeat types ranging from penta to octa repeats all with a TTAGGG-type mortif [also chapter 4 of this thesis]. Some of these repeat types are also found in other organisms, for example in plants, telomeres consist of TT(T/A)GGG (Richards and Ausubel 1988). In their model, Li and colleagues proposed that mutations within the RNA component of telomerase could have resulted in a departure of budding yeast telomeres from the TTAGGG motif resulting in different telomeric repeats of different length and sequence (e.g., ACGGATTTGATTAGGTATGTGGTGT in *K. lactis* (McEachern and Blackburn 1994).

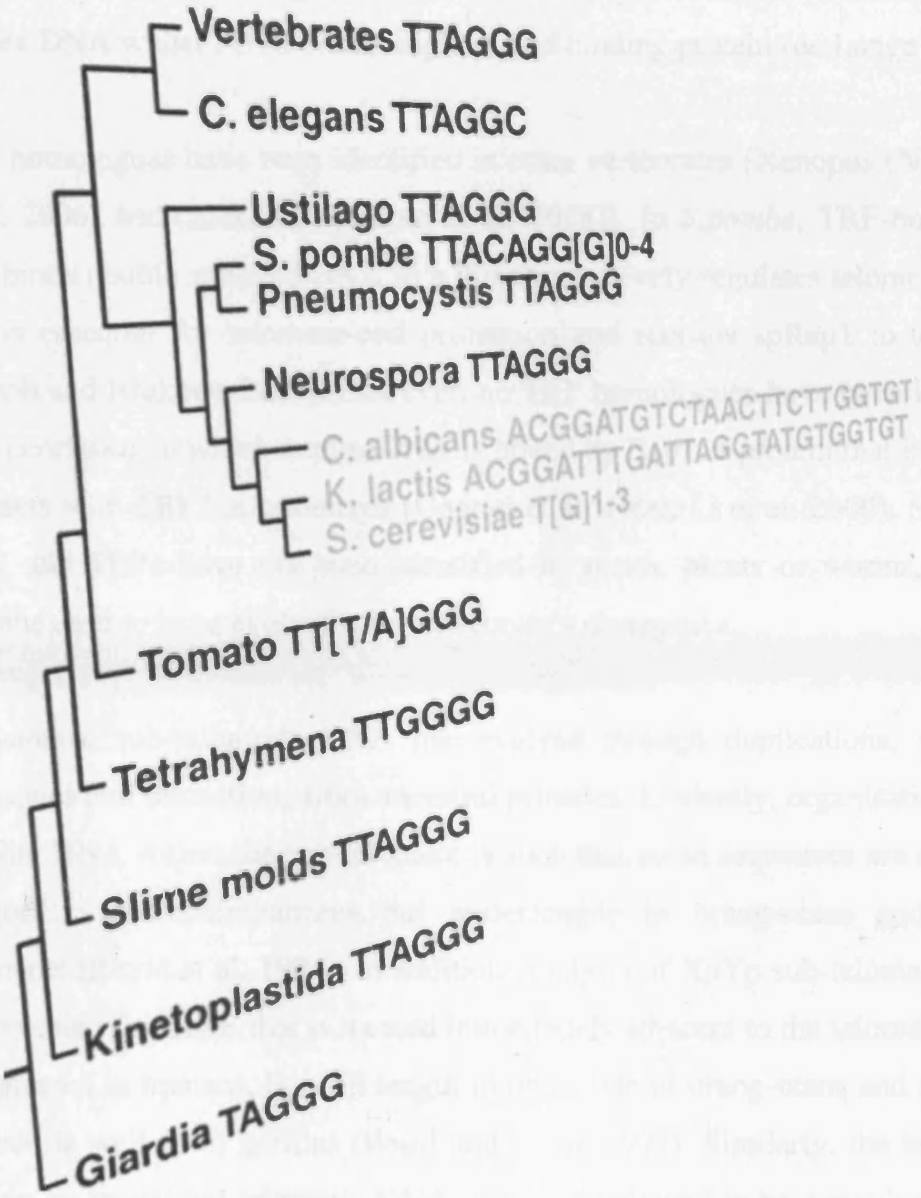


Figure 1.4. Putative model for the Evolution of Eukaryotic Telomeres. TTAGGG-like repeats predominate at telomeres of most eukaryotes with the exception of budding yeasts. For references on telomeric sequences, see (Zakian 1989). Line lengths do not represent evolutionary distances [modified from (Li et al. 2000)].

In support of the view of TTAGGG-containing ancestor, most eukaryotes have TRF-like proteins that bind the telomeres. In mammalian cells, telomeres are bound by the multi-protein complex "Shelterin" which consists of at least six core components (de Lange 2005). Two-related proteins TRF1 and TRF2 bind duplex DNA whilst POT1 binds single-strand binding protein (de Lange 2005).

TRF homologues have been identified in other vertebrates [Xenopus (Nishiyama et al. 2006) and chicken (Okamoto et al. 2008)]. In *S.pombe*, TRF-homologue taz1 binds double stranded DNA as a dimer, negatively regulates telomere length and is essential for telomere-end protection and recruits spRap1 to telomeres (Kanoh and Ishikawa 2003). However, no TRF homologues have been identified in *S. cerevisiae* in which duplex DNA is bound by Rap1, a protein that in humans interacts with TRF2 at telomeres (Conrad et al. 1990; Li et al. 2000). Similarly, TIN2 and TPP1 have not been identified in yeasts, plants or worms, and are hypothesised to have evolved after vertebrate's divergence.

In humans, sub-telomeric DNA has evolved through duplications, sequence exchanges and truncations from ancestral primates. Evidently, organisation of the satellite DNA within the sub-telomere is such that some sequences are abundant in gorillas and chimpanzees but undetectable in orang-utans and human telomeres (Royle et al. 1994). In addition, analysis of XpYp sub-telomeric DNA has revealed the SINE that is located immediately adjacent to the telomere which is truncated in humans, but full length in one allele of orang-utans and absent in another as well as in gorillas (Baird and Royle 1997). Similarly, the human 2q contain an interstitial telomeric DNA, which is believed to be a result of fusion between two arms of the ancestral chromosome 2 during evolution (JW et al. 1991; Macina et al. 1994). Thus, closely related species such as humans, chimpanzees and gorillas have distinct telomeric sequence organisations, which are subjected to rapid evolution (Royle et al. 1994; Baird and Royle 1997).

Further support for rapid evolution in human lineage comes from nucleotide substitution studies, which revealed higher frequency of base substitution within sequences immediately adjacent to the telomeres than the genome average at non-coding regions. For instance, the 850bp of the telomere-adjacent DNA of XpYp exhibits the rates of base substitution as high as 1 in 50-65bp. However, all the base substitutions are in complete linkage disequilibrium resulting in a small number of haplotypes within human populations (Baird et al. 1995; Baird et al. 2000). Linkage disequilibrium (LD) is a condition in which the haplotype frequencies in populations deviate from the values they would be under random segregation. The linkage disequilibrium coupled with high frequency of the base substitution have resulted in haplotypes that vary by 2.1% at XpYp indicating that the haplotypes are ancient and have been preserved in modern human populations (Baird et al. 1995). One of the processes that can give rise to linkage disequilibrium is the suppression of recombination. Linkage-map expansion and terminal chismata distribution indicated high rates of recombination in some terminal regions of human chromosomes (Hulten 1974). Such high rates of recombination could result in rapid evolution of the terminal regions. Indeed sub-telomeric exchanges between 4q and 10q have been reported with some people carrying a 4q/10q translocation (van Deutekom et al. 1996; Mefford and Trask 2002). In contrast, LD analysis at the telomere arrays indicated a suppression of meiotic recombination at telomeres (Baird et al. 1995; Baird et al. 2000).

1.10 Telomere replication

Semi-conservation replication is achieved through cooperation of lagging and leading strand synthesis. While replication of telomeres by leading strand synthesis of telomeres is complete and results in blunt-ended molecules, the molecules synthesised by lagging strand synthesis is incomplete (Watson 1972). There is no known mechanism of synthesising DNA when the distal primer is removed thus a gap equivalent to the size of the terminal primers may be generated. However, *in vitro* studies have shown that due to randomness of priming, the lagging strand synthesis is halted within 500bp of the end resulting in gaps of up to 500bp when the primers are removed (Ohki et al. 2001). Thus with ongoing cell division telomeres can be lost, a phenomenon called end-replication problem [Figure 1.2; section 1.6].

It has become increasingly apparent that replication of telomeres and telomerase mediated telomere elongation are highly coordinated. For example, in ciliates *Euplotes crassus* (Fan and Price 1997) and yeast *S.cerevesiae* (Diede and Gottschling 1999), inhibition of activity of DNA polymerases α and δ (C-strand synthesis) induces telomere elongation of the G-strand and inhibits G-strand polymerisation respectively, indicating that extension of 3' G-strand by telomerase and filling of gaps in C-strand by DNA primase are coordinated. It is not known whether replication is coordinated in mammalian cells, however, inactivation of the DNA polymerase in mouse cells induces elongation of the G-overhang implicating that DNA replication and telomerase elongation may be coordinated in mammals as well (Nakamura et al. 2005).

Whilst telomere replication of opposite telomeres of same chromosome is synchronised during late S-phase in yeast (Wellinger et al. 1993), mammalian telomere replication is asynchonised but timed and regulated (Zou et al. 2004a). The replication of telomeres is initiated from the sub-telomeric origin of replication (Verdun and Karlseder 2007) early in S-phase and continues

throughout the S-phase. In addition, opposite telomeres of specific chromosome within the same cell are replicated at different but specific times during the S-phase. Furthermore, some specific telomeres are replicated at early stages of S-phase (Ten Hagen et al. 1990; Wright et al. 1999; Hultdin et al. 2001; Zou et al. 2004a).

The repetitive GC-rich nature of telomeres complicates replication probably through formation of secondary structures like G-quadruplexes (Parkinson et al. 2002) and T-loop (Griffith et al. 1999). Evidently, studies have reported that TRF1 and TRF2 bound telomeric DNA stalls the replication fork *in vitro* and *in vivo* (Ohki and Ishikawa 2004). Consequently, efficient replication of telomeres requires resolution of stalled replication forks. Telomere proteins TRF1 and shelterin-associated protein WRN play an essential role in processing of stalled replication fork to allow efficient telomere replication (Crabbe et al. 2004; Sfeir et al. 2009). In support of this view, over-expression of WRN in mouse cells cause loss of telomeres generated by lagging strand synthesis in the absence of telomerase. However, expression of telomerase in these cells (expressing WRN) counteracts telomere loss (Crabbe et al. 2004).

In summary, whilst telomere replication may be problematic, telomere proteins play an essential role in regulating coordinated telomere replication and telomerase-mediated elongation.

1.11 Telomere maintenance

1.11.1 Human Telomerase

Human telomerase is a holoenzyme and ribonucleo-protein (RNP) complex consisting of two essential core components; the human telomerase RNA component [hTERC; CUAACCCUA...] and the human telomerase reverse transcriptase catalytic subunit [hTERT; (Morin 1989)]. hTERC acts as a template

for *de novo* addition of the new telomeric repeats while hTERT synthesises telomeric repeats and catalyses telomere elongation (Nakamura et al. 1997).

Telomerase adds telomeric repeats to counteract telomere erosion due to end replication problem, and other processes including nucleolytic activity (Wellinger et al. 1996) and oxidative damage (von Zglinicki 2002). It specifically recognises the 3'-OH of the G-strand at the ends of the chromosomes (Nakamura and Cech 1998; Collins and Mitchell 2002) and preferentially elongates short telomeres in human cells (Kim et al. 1994; Britt-Compton et al. 2009) thus maintaining telomeres at a stable length.

The level of telomerase activity is regulated at different levels including transcriptional regulation, mRNA splicing and assembly of the hTERT and hTERC genes. hTERC and other telomerase-associated proteins are ubiquitously expressed in all human tissues (Feng et al. 1995) thus the level of hTERT is a major determinant of telomerase activity such that the steady-state levels of hTERT are elevated in cancer cells (Avilion et al. 1996; Yi et al. 1999) but not in most somatic cells. hTERT expression is regulated by several proteins which include c-myc, Smad3 and TGFβ among others. For examples, c-myc binds on several locations on the promoter of hTERT gene to enhance transcription (Wu et al. 1999; Kyo et al. 2000) while interaction between TGFβ, Smad3 and c-myc represses hTERT activity (Li et al. 2006). Post-transcriptional regulation includes alternative splicing of hTERT into hTERTα, which results in down-regulation of hTERT (Colgin et al. 2000).

1.11.1.1 Telomerase expression in human tissues

In humans, telomerase expression is restricted to male and female germ-line, some adult stem cells, 80-90% of human cancer tissues and immortalised cells (Kim et al. 1994; Kolquist et al. 1998). It is also expressed in cells of self-renewal high-proliferative tissues such as haematopoietic system [e.g. CD34+ haematopoetic progenitor cells; (Broccoli et al. 1995)] and the basal layer of the

skin (Kolquist et al. 1998). Telomerase activity is also detectable in normal keratinocytes (Harle-Bachor and Boukamp 1996; Yasumoto et al. 1996); lymphocytes after mitogenic stimulation (Hiyama et al. 1995; Greider 1998; Liu et al. 1999); in normal mature T cells on entry into S-phase (Buchkovich and Greider 1996); leukocytes (Counter et al. 1995), normal human endothelial cells in culture (Hsiao et al. 1997), endometrial tissue (is correlated with proliferation during menstrual cycle), mitotically active regions of human hair follicles, proliferative zone of intestinal crypts (Ramirez et al. 1997) and normal human blood (Broccoli et al. 1995; Counter et al. 1995). In contrast, most normal human somatic tissues express undetectable to low-level telomerase activity (Kim et al. 1994; Kolquist et al. 1998).

1.11.2 Telomerase and telomere length homeostasis

In most telomerase deficient cells telomeres erode as a function of a cell division, however in immortal cells such as germ line and cancer cells, telomerase activity elongates telomeres thus maintain them at stable length (Kim et al. 1994; Kolquist et al. 1998; Meyerson 2000). In other telomerase-positive cells, telomerase activity is insufficient to counteract telomere loss implicating a regulatory mechanism of telomerase activity in such cells. In support of this, studies have reported that limiting telomerase activity contributes to telomere homeostasis (Cristofari and Lingner 2006) such that in telomerase-positive yeast, telomere length homeostasis depends on a switch between telomerase-extendible and telomerase-nonextendible states (Teixeira et al. 2004). Telomerase access to telomeres is coordinated with cell cycle progression such that telomeres are elongated during S-phase following DNA replication (Masutomi et al. 2003). However, not all telomeres are elongated at each S-phase, only a subset of short telomeres that may display open telomere structure [where the G-overhang is accessible] are elongated in yeast (Teixeira et al. 2004) and human cells (Britt-Compton et al. 2009). Regulation of accessibility depends on the amount of telomeric proteins bound at the telomeres such that longer telomeres carry more

TRF1 molecules and thus are not accessible and vice versa for short telomeres (de Lange 2005).

1.11.3 Alternative lengthening of telomeres (ALT)

ALT represents telomerase-independent mechanism(s) expressed in a subset of human cancer cells and tumours (Bryan et al. 1995; Bryan et al. 1997b; Henson et al. 2002). ALT is believed to be a recombination-based mechanism characterised by high rates of telomeric exchanges (Londono-Vallejo et al. 2004), however the exact mechanism is not known. It is prevalent in tumours of mesenchymal origin such as osteosarcoma (35-47%), soft tissue sarcomas (35%) and liposarcomas [24-33%; (Henson et al. 2005; Johnson et al. 2005; Costa et al. 2006; Johnson and Broccoli 2007; Jeyapalan et al. 2008)]. However, some exceptions to this trend have been documented. For example, Ulaner and colleagues have reported that majority of Ewing's sarcoma tumours expressed telomerase as opposed to osteosarcomas which expressed ALT (Ulaner 2004). Moreover, telomerase activity has been documented in other sarcomas with low frequency of ALT, such as synovial sarcomas (Montgomery et al. 2004) implicating that in some tumours both ALT and telomerase are methods of telomere maintenance.

ALT-expressing cells (ALT+) are characterised by lack of telomerase activity, heterogeneous length of telomeres varying from undetectable to >20kb, presence of extra-chromosomal telomeric circular DNA (Cesare and Griffith 2004) and presence of ALT-associated promyelocytic leukaemia nuclear bodies (APBs) containing telomeric DNA and binding factors, TRF1 and TRF2 (Yeager et al. 1999). However, PMLs have also been documented in the absence of either telomerase or ALT suggesting that their role in ALT cells may not be essential (Fasching et al. 2005).

1.12 Telomere dysfunction triggers DNA damage response

As illustrated in section 1.8, several DNA repair factors localise to the telomere and participate in telomere maintenance. In mammalian cells, DNA damage lesions are detected by two related phosphatidylinositol 3-kinase proteins: ATM (DSBs) and ATR [for Single-stranded DNA breaks; (Zou 2007; Cimprich and Cortez 2008)] which are auto-phosphorylated at the site of the lesion. Once activated, they phosphorylate histone γH2AX on ser139, CHK1 and CHK2 (Liu et al. 2000) and through cascades of events activate p53/p21 pathways (Wang et al. 2007b). Unlike DSBs, telomeres are protected from these pathways by shelterin (de Lange 2005).

The loss of the telomere function due to either erosion, or loss of telomeric protein function, leads to a DNA damage response. In support of this, cells without functional TRF2 display molecular markers characteristic of cells bearing DNA DSBs such as phosphorylated γH2AX, p53-binding protein 1 (TP53BP1), NBS1 (also known as nibrin), MDC1 and CHK2 at the site of the lesion, forming telomere-induced foci [TIFs; (d'Adda di Fagagna et al. 2003; Takai et al. 2003)]. This indicates that TRF2-depleted telomeres are recognised as sites of DNA damage (van Steensel et al. 1998; Karlseder et al. 1999). Similarly, loss of both mouse Pot1s results in formation of TIFs and cell cycle arrest whilst POT1-deficient cells contain phosphorylated CHK1 and CHK2 consistent with ATR signalling (Denchi and de Lange 2007). TIFs have also been detected in senescent human diploid fibroblasts (d'Adda di Fagagna et al. 2003; Takai et al. 2003), indicating that senescent cells contain dysfunctional telomeres that induces DNA damage response and cell cycle exit.

1.13 Telomere dysfunction and Genomic instability

Chromosomes that have lost the telomere function through either progressive erosion or uncapping are highly unstable. i.e. Critically short or uncapped telomeres are "sticky" and tend to fuse forming dicentric chromosomes among

others (Murnane and Sabatier 2004). With subsequent divisions when the centromeres are pulled apart during anaphase, the fused chromosomes may form a bridge and break not necessarily at the original fusion point. The broken fragments can then fuse to other uncapped chromosomes and initiate another cycle of breakage-fusion-bridge cycle and hence genomic instability [Figure 1.5; (DePinho and Wong 2003)]. The consequence of genomic instability is the loss of genome integrity, which in turn may leads to mis-regulation of genes involved in growth control and tumour suppression (Murnane and Sabatier 2004). Thus through accumulation of additional abnormalities loss of genomic stability can promote tumourgenesis (Artandi et al. 2000; Artandi and DePinho 2000; DePinho 2000).

Several studies have demonstrated that disruption of the telomere cap such as through loss of TRF2 (van Steensel et al. 1998; Celli and de Lange 2005), POT1 (Hockemeyer et al. 2005) and/ or RAP1 (Bae and Baumann 2007; Sarthy et al. 2009) functions, induce widespread NHEJ-mediated /ligase IV-dependent telomere fusions, consisting of long tracts of TTAGGG repeats. In addition, loss of POT1 (He et al. 2006; Wu et al. 2006) or TRF2 and ku increases the frequency of sister telomere exchanges, a form of homologous recombination predominant in ALT. Moreover, mutant TRF2 lacking the DNA-binding domain (ΔB) represses NHEJ but result in rapid erosion of telomeres as well as formation of t-loop sized telomere circles implicating the XRCC3-dependent processing of the Holliday junction at the t-loop (Wang et al. 2004), an intermediate stage in homologous recombination. Similarly, perturbation of the shelterin stability through loss of interacting factors such as PARP1 (d'Adda di Fagagna et al. 1999) or Ku (Samper et al. 2000) in knock-out mice exhibit elevated frequency of telomere fusions, abundant chromosomal breaks and aneuploid cells compared to the wild type mice. Taken together these studies support the view that loss of telomere function triggers DNA repair at telomeres.

[reviewed in (Campisi et al. 2001)]. Senescence can also be triggered by telomere uncapping without telomere erosion as indicated previously (Blackburn 2001; Karlseder et al. 2002). In support of this, loss of telomere cap through depletion of TRF2 *in vitro* (Karlseder et al. 1999), ablation of TIN2 (Chiang et al. 2004; Kim et al. 2008) and conditional deletion of mPot1a (Wu et al. 2006), all induce p53-dependent cell cycle arrest. Similarly, telomere dysfunction in telomerase-deficient mouse cells and human cells expressing a mutant hTERT induce apoptosis in the presence and absence of functional p53 (Chin et al. 1999; Hahn et al. 1999; Zhang et al. 1999; Guiducci et al. 2001; Kim et al. 2001); indicating that telomere dysfunction induces both p53-dependent and independent apoptosis in mammalian cells.

1.14.1 Ageing

In long-lived species such as humans, senescence whether telomere-dependent (Harley et al. 1990; Wright and Shay 1992) or telomere-independent [e.g. oncogene-induced (Serrano et al. 1997)] plays two antagonistic roles in ageing and cancer (Campisi 1997). By limiting the cell proliferation, senescence can contribute to ageing by accumulation of changes that may alter microenvironment thus leading to disruption of tissue homeostasis. These changes include secretion of extracellular matrix as well as matrix-degrading enzymes, inflammatory cytokines and growth factors [reviewed in (Campisi et al. 2001), all of which may alter tissue homeostasis. Accumulation of senescent cells may therefore impair tissue function and contribute to ageing. Indeed, senescent cells accumulate with age in human tissues and mice, especially at sites of age-related pathologies such as skin ulcers and osteoarthritic joints, as well as premalignant tumours (Dimri et al. 1995; Price et al. 2002; Collado et al. 2005). Accumulation of senescent cells has also been reported in skin of ageing baboons (Herbig et al. 2006; Jeyapalan et al. 2007).

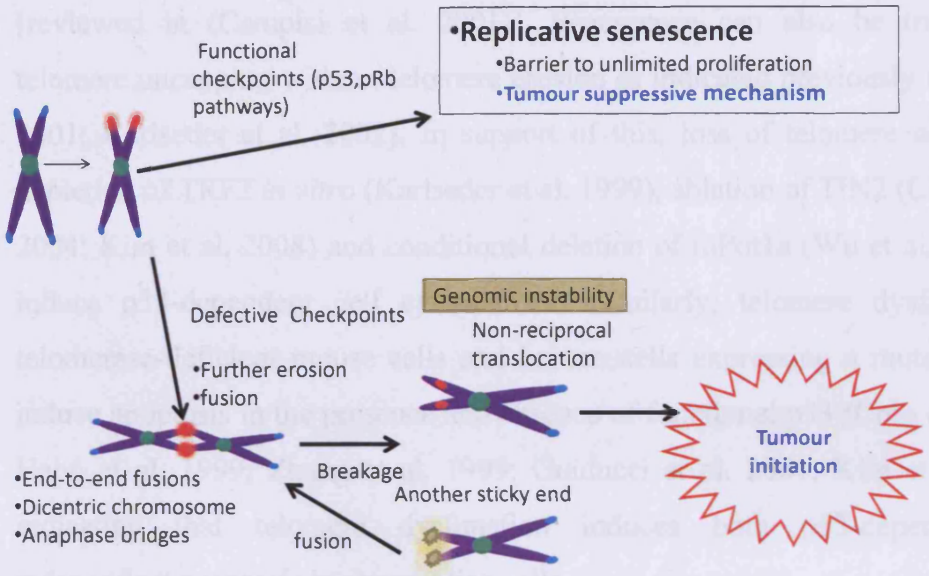


Figure 1.5. Schematic representation of dual antagonistic role of telomeres in cancer. When the telomeres become uncapped, they are detected by cell machinery as damaged DNA and triggers cell cycle exit, a mechanism that is believed to be a barrier to uncontrolled proliferation and hence tumour suppressive. However if DNA repair machinery is impaired, uncapped telomeres can fuse with each other, forming dicentric chromosomes. The fused chromosomes form a bridge when the two centromeres are pulled to opposite poles. With subsequent cell divisions, fused chromosomes could result in extensive chromosome instability, as the breakage does not occur at the original fusion site.

1.14 Telomere dysfunction may contribute to Ageing and tumourigenesis

In most somatic cells, telomeres erode as a function of age due to the "end replication" problem, insufficient telomerase activity and post replicative processing until they reach the telomere length at which they undergo replicative senescence, often termed mortality stage I [(Wright and Shay 1992; Wei and Sedivy 1999); M1; Figure 1.6). Senescence (biological ageing) is a state of permanent cell-cycle arrest in which cells stop dividing but remain viable

While it is clear that telomere dysfunction, may contribute to ageing by virtue of its ability to trigger senescence (biological ageing), the direct biological link between telomere dysfunction and organismal ageing is very tantalising, but remains to be proven. However, several studies have reported telomere erosion with age in human tissues including blood cells, lymphocytes (Weng et al. 1995; Elwood 2004), kidney epithelium (Melk et al. 2000) and intestinal epithelium (Jiang et al. 2007). It is not known whether telomere dysfunction contributes to complex process of organismal ageing.

The possible role of telomere dysfunction in organismal ageing is studied in mouse model. Laboratory mice have long telomeres (Kipling and Cooke 1990) and cellular and organismal ageing in mice is believed to be telomere-independent. Consistent with this, telomerase knockout did not induce any ageing phenotype in first generation mice, at which their telomeres have not eroded to the length at which telomeres may be dysfunctional. However late (sixth) generation telomerase knockout mice displaying short telomere profiles, also showed degeneration of proliferative lymphoid, haematopoietic and gonadal cells in generation consistent with ageing (Blasco et al. 1997; Lee et al. 1998). Whilst these mice also displayed ageing phenotype such as grey hair, alopecia, delayed healing etc, deletion of WRN protein in these mice displaying short telomeres induced phenotypes that resemble those of premature ageing human Werner syndrome such as premature death, osteoporosis, type II diabetes and cataracts (Chang et al. 2004), implicating that telomere dysfunction may play a role in ageing. Again, studies of human genetic diseases, such as premature ageing, telomerase-deficiency disorder, dyskeratosis congenital (DKC) have provided insights into the link between telomere biology and ageing. Like telomerase knockout mice, DKC pedigrees display decline in telomere length distributions from one generation to the next (Diaz de Leon et al. in press), defects in proliferative tissues such as the bone marrow and signs of premature ageing such as alopecia, grey hair and cancer predisposition [reviewed in (Armanios 2009)].

In summary, telomere dysfunction may contribute to ageing by triggering senescence. By secreting growth factors, cytokines, extracellular matrix and degradative enzymes, all of which can alter tissue microenvironments, accumulation of senescent cells may contribute to ageing. Because tissue microenvironments is crucial for suppressing growth and progression of cells carrying oncogenic mutations, changes in microenvironment may favour neoplastic progression in cells that carry oncogenic mutations (Park et al. 2000). Thus, senescence may contribute to the exponential rise in cancer that occurs with age (DePinho 2000; Krtolica et al. 2001). Senescence represents an example of a phenomenon called antagonistic pleiotropy [reviewed in (Kirkwood and Austad 2000)], in which one trait can benefit an organism's fitness early in life while at the same time can be detrimental in ageing organisms. i.e. Early in life, cell cycle arrest, senescence, prevents unlimited cell proliferation required for malignancy, i.e. senescence can benefit the organism's fitness by suppressing tumour formation. However, accumulation of senescent cells with age, may promote neoplastic progression of neighbouring cells [that carry mutations] as evident from studies by Krtolica and colleagues, in which they demonstrated that senescent human fibroblasts did stimulate growth and progression of preneoplastic, epithelial cells carrying p53 mutations but not normal keratinocytes (Krtolica et al. 2001). In summary, because mutations accumulate with age, it is possible that cells carrying mutations in growth control genes will lie in close proximity to senescent cells in ageing organisms. Thus, changes in microenvironment may drive neoplastic progression *in vivo*.

1.14.2 Tumourigenesis

As discussed in previous section, telomere dysfunction triggers p53/pRb-dependent cell cycle arrest, called replicative senescence (d'Adda di Fagagna et al. 2003; Takai et al. 2003); barrier that cells must overcome to allow the cell proliferation necessary, to progress to malignancy. Thus, senescence represents a potential tumour suppressive mechanism. In support of the role in tumour

suppression, studies in mice without telomerase activity showed that Burkitt's lymphoma mouse models (accomplished by interbreeding terc-/- mice with Eμ-myc tumour prone mice) displayed short telomeres, reduced tumour formation, increased levels of senescence-associated β-galactosidase and senescence-associated kinase inhibitors p16 and p15, indicating induction of senescence (Feldser and Greider 2007). Similarly, telomerase-deficient mice with short telomeres are resistance to tumourigenesis in the presence of functional checkpoints (Gonzalez-Suarez et al. 2000; Rudolph et al. 2001; Qi et al. 2005; Cosme-Blanco et al. 2007). Further evidence comes from mice carrying a mutant p53 ($Trp53^{515C/515C}$) which can induce senescence but not apoptosis (Liu et al. 2004c). Unlike Trp53-null mice, which succumb to early thymic lymphoma tumourigenesis (Donehower et al. 1992; Jacks 1996), these mice [carrying $Trp53^{515C/515C}$] were resistant to early tumour formation (Liu et al. 2004c) even in response to telomere dysfunction as with terc-/-, $Trp53^{515C/515C}$ mice (Cosme-Blanco et al. 2007). Together these studies implicate that senescence but not apoptosis suppresses tumourigenesis.

However, when the DNA checkpoints are defective, telomere dysfunction can result in critically short telomeres that are capable of fusion. In culture, cells displaying critically short telomeres enter the second checkpoint, which is p53-independent called "crisis" or mortality stage II (M2; Figure 1.6). M2 is characterised by increased cell mortality and chromosome instability (Wright and Shay 1992). The cells that emerge from crisis activate one or both of the telomere maintenance mechanisms; telomerase (Counter et al. 1992; Bodnar et al. 1998) and alternative lengthening of telomeres (Ducray et al. 1999; Dunham et al. 2000).

As discussed in section 1.13, telomere dysfunction can lead to loss of genomic integrity and hence promote the tumourigenic process by increasing the rate of mutation of oncogenes and tumour-suppressor genes (Maser and DePinho 2002). Supporting evidence comes from studies in which late generation $terc^{-/-}$, $p53^{+/-}$

mice with short telomeres showed a shift in tumour spectrum from lymphomas and sarcomas to accumulation of carcinomas, such as colorectal cancer with characteristic chromosome instability and complex rearrangements similar to those of human epithelial cancers (Artandi et al. 2000). Similarly, telomere dysfunction in late generations of APC^{min} $Terc^{-/-}$ led to increased onset of microscopic adenomas with the early adenoma-carcinoma transition being marked by increased frequency of anaphase bridges, surrogate markers of telomere dysfunction (Rudolph et al. 2001). In contrast, in the presence of functional p53-signaling pathway, $Terc^{-/-}$ mice with critically shortened telomeres are highly resistant to tumour development (Greenberg et al. 1999; Gonzalez-Suarez et al. 2000). This dual role of telomere biology in tumourgenesis, which is such that telomere dysfunction promote tumour initiation while at the same time tumour progression is limited by cell cycle arrest (Rudolph et al. 2001; Lechel et al. 2007) is another example of antagonistic pleiotropy. In this case, loss of telomere function in proliferating cells induces cell cycle exit in the presence of functional DNA damage machinery while progressive telomere attrition in cells with defective checkpoints can initiate chromosome instability and ultimately loss of genome integrity, which could drive malignant progression if genes involved in growth control are switched on or off.

1.15 Telomere biology in human diseases

Several genetic disorders are associated with telomere shortening through loss of telomerase function via mutations in hTERT/hTERC genes or defects in specific components of DNA repair or defects in telomere proteins. Below I discuss briefly some of these human disorders.

1.15.1 Diseases associated with telomerase insufficiency

Dyskeratosis congenital (DKC) is a rare syndrome of premature ageing characterised by the abnormalities of the oral mucosa known as leukoplakia, skin

hyper-pigmentation and nail dystrophy/ridging (Dokal and Vulliamy 2003). DKC is both an autosomal and X-linked disease. The familial Dyskeratosis congenital (DKC) is linked to mutation in dyskerin gene (DKC1), a nucleolar protein that stabilises hTERC. These mutations impair telomerase activity through either reduced ability of dyskerin to stabilise hTERC or disrupting the holoenzyme assembly (Bessler et al. 2004). The autosomal dominant form of the disease results from mutations in hTERC gene (Vulliamy et al. 2001), which frequently result in low hTERC expression in patients, compared to siblings. Affected individuals have low telomerase activity and short telomeres as well as increased chromosome end-to-end fusions, indicating telomere dysfunction (Dokal 2000). The X-linked DC patients have lower levels of telomerase and short telomeres (Heiss et al. 1998). Some cases are characterised by a functionally null allele in hTERT (Armanios et al. 2005), mutations in NOP10 and NHP2 [components of the holoenzyme; (Walne et al. 2007; Vulliamy et al. 2008)]. In addition to telomerase mutations, mutations in TIN2, a component of shelterin have been identified in severe cases of DKC (Savage et al. 2008).

Aplastic anaemia is a serious bone marrow disorder that is characterised by low blood cell count (Young 2002). Sufferers have short telomeres compared to controls probably due to insufficient telomerase activity. The patients carry heterozygous mutations in hTERT, hTERC, TRF1, TRF2 and TIN2 (Savage et al. 2006; Walne et al. 2008).

Other blood diseases such as myelodysplatic syndromes (MDS), a group of clonal diseases in which the bone marrow fails to produce blood cells and are characterised by low blood cell counts, only hTERC mutations but no mutations in hTERT or dyskerin have been identified (Yamaguchi et al. 2003). Short telomeres in MDS are associated with development of acute myeloid leukaemia [AML; (Boultwood et al. 1997)].

Similarly, mutations in telomerase components have been reported in non-blood conditions such as Cri du chat syndrome (CdCS) and idiopathic pulmonary fibrosis. Cri du chat syndrome (CdCS) is characterised by a distinct cat-like cry, microcephaly, widely spaced eyes, low set ears, a low broad nasal bridge and palmar creases in infants (Cerruti Mainardi 2006; Laczmanska et al. 2006). The characteristic cat-like cry has been linked to terminal deletion at 5p (5p15.3-5pter), encompassing the hTERT gene [5p15.33; (Zhang et al. 2003; Laczmanska et al. 2006)].

Idiopathic pulmonary fibrosis (IPF) is a rare non-neoplastic pulmonary disease that is characterised by the scarring of the lung tissues without any known cause [reviewed in (Meltzer and Noble 2008)]. Similar to other conditions already discussed, in IPF, mutations in telomerase components, hTERT and hTERC, underlie inheritance of familial IPF in 8–15% of individuals. Heterozygous carriers of all of the mutations in TERT or TERC display shorter telomeres than age-matched family members without mutations (Armanios et al. 2007; Tsakiri et al. 2007). However, short telomeres but not mutations in telomerase components are the risk factor for development of sporadic IPF (Alder et al. 2008).

1.15.2 Human cancer

One of the earliest neoplastic changes that occur in most cancers is genomic instability (Hanahan and Weinberg 2000). It is clear from discussion of *in vitro* studies and mouse models that telomere dysfunction can be the key driving force in generating genomic instability which in turn may lead to mis-regulation of genes involved in growth control, and tumour suppression and hence promotes cancer development (DePinho 2000; Maser and DePinho 2002). In this section, telomere dysfunction is discussed in premalignant conditions and human tumours.

Several human premalignant precursor lesions termed intraepithelial neoplasia that predispose to development of cancer display short telomeres, increased frequency of anaphase bridges and end-to end fusions, markers of telomere dysfunction, expression of telomerase or recombination-based telomere maintenance mechanism (Meeker et al. 2002b; Meeker et al. 2004). For instance, telomerase activity is expressed early during carcinogenesis of the gastric, oesophageal, liver, pancreas, cervix, colorectal and oral cavity etc (Meeker et al. 2004). While telomerase does not induce genomic instability, it is essential for maintaining the telomeres to ensure continued proliferation that cells require to accumulate more abnormalities that could drive neoplastic progression (Bodnar et al. 1998; Morales et al. 1999). In Barrett's oesophagus, a premalignant condition that predispose to oesophageal adenocarcinoma, telomerase activity increases gradually at all precancer stages being high in dysplasia and carcinoma *in situ* (Lord et al. 2000). Similarly, telomerase is expressed in intestinal metaplasia of the stomach which became elevated in adenomas and gastric cancers; regardless of whether nodal metastases, peritoneal metastases and recurrent gastric tumours (Tahara et al. 1995a; Maruyama et al. 1997). Similar patterns of telomerase activation, increased chromosome instability and telomere erosion have been reported in other conditions of the gastrointestinal tract such as liver cirrhosis-hepatocellular carcinoma transition (Tahara et al. 1995b); pancreatic intraepithelial neoplasia-pancreatic ductal adenocarcinoma transition (Hiyama et al. 1997) and ulcerative colitis-colorectal cancer transition (Hastie et al. 1990; Tahara et al. 1995a; Engelhardt et al. 1997).

Telomere dysfunction and chromosomal instability are believed to pose clonal advantage in the absence of functional DNA checkpoint, which through accumulation of more mutations have potential to initiate cancer. i.e. cells that have lost telomere function can dominate the culture and result in clonal expansion, and allow for accumulation of more abnormalities that may drive neoplastic progression. Clinical evidence demonstrates the clonal heterogeneity of telomeres and telomerase activity in tumour cells. For instance, telomere

length in colorectal carcinoma have been reported to be shorter than in normal controls and polyps, without any correlation between the telomere length and telomerase activity (Kim et al. 2002).

In addition, losses of p53 and p16 function are some of the early lesions in non-neoplastic epithelium of ulcerative colitis and Barrett's oesophagus with telomere dysfunction, as marked by short telomeres and increased frequency of anaphase bridges (O'Sullivan et al. 2002; Finley et al. 2006; O'Sullivan et al. 2006; Risques et al. 2008), occurring at advanced stages.

Telomere length has been suggested in prognosis of numerous cancers. For instance, telomere erosion in peripheral blood lymphocytes increases the risk of gastric cancers (Liu et al. 2009). Again, short telomeres and increased frequency of telomere fusion is associated with disease progression and poor outcome in chronic lymphocytic leukaemia (Lin et al. 2010).

1.16 Telomere length Dynamics and homeostasis

Telomere length homeostasis depends on a balance between the processes that result in telomere attrition or telomere elongation. It also depends on shelterin complex that work in *cis* to control access of telomerase to telomeres (Smogorzewska and de Lange 2004).

1.16.1 End-replication losses and post-replicative processing.

The majority of somatic cells do not express any telomere maintenance mechanism thus, their telomeres erode as a function of cell division with age (Harley et al. 1990). Following replication, the telomere created during the lagging strand synthesis is shorter than the parent telomere by the size of the terminal primer (Watson 1972). Thus the lagging strand synthesis result in telomere erosion with each round of cell division. The leading strand on the other

hand is fully replicated resulting in blunt ended molecules, which are then processed by putative nucleases to create the overhang necessary for t-loop formation (Makarov et al. 1997; Wright et al. 1997; Bailey et al. 2001; Sfeir et al. 2005). Although the nucleases that process the telomeres to generate overhangs are not known, they may include endonucleases such as FEN-1 (Liu et al. 2004d; Saharia and Stewart 2009) and ERCC/XPF1 (de Laat et al. 1998; Hoeijmakers 2001) or exonucleases such as Apollo (Lenain et al. 2006; van Overbeek and de Lange 2006), WRN (Li and Comai 2001), MRE11 (D'Amours and Jackson 2002) and EXO1 (Sharma et al. 2003; Schaetzlein et al. 2007), all of which play a direct or indirect role in telomere homeostasis. For example loss of FEN1 has been reported to induce telomere instability in ALT expressing cells (Saharia and Stewart 2009). The end-replication problem and post-replicative processing result in 50-150bp losses per cell division in human fibroblasts (Harley et al. 1990; Makarov et al. 1997).

1.16.2 Oxidative damage

By virtue of being dithymidine and triple-Guanine-containing structures, telomeres are highly sensitive to damage by oxidative stress (Henle et al. 1999), alkylation (Petersen et al. 1998) or ultraviolet (UV) irradiation (Oikawa and Kawanishi 1999; Jin and Ikushima 2004; Kawanishi and Oikawa 2004). On exposure to UV, adjacent thymines tend to form an unusual covalent bond to generate thymine dimers which when excised by base excision repair may create single-stranded breaks [SSBs] on the G-strand. Similarly, guanine has low oxidation potential, which is even lower on tri guanines. Generation of the 8-oxo guanines can be very mutagenic if unrepaired, as 8-oxoG base pairs with adenine resulting in a GC to AT transversion just after two replication cycles (Boiteux and Radicella 1999). These mutations can lower the affinity of the telomere binding proteins (Opresko et al. 2005a) and result in telomere uncapping and/ or increased rate of recombination.

Alternatively, excision of the 8-oxoG can generate the single stranded break on the G-strand and single strand DNA on the C-strand which may be cleaved and lead to telomere shortening. In support of this, studies have reported detectable SSBs in human cells (von Zglinicki et al. 2000) and mouse cells (Wang et al. 2010) under high oxygen tension. All these studies suggest that oxidative damage may result in a substantial loss of telomere repeats and thus an increase in the rate of telomere erosion (Oikawa and Kawanishi 1999; von Zglinicki 2002; Kawanishi and Oikawa 2004; Houben et al. 2008). Indeed, UVA exposure, which can generate oxidative stress via reactive oxygen species (ROS) such as H_2O_2, O_2^- and NO-, which have been implicated in accelerated telomere shortening of cultured fibroblasts (Oikawa et al. 2001; Kawanishi and Oikawa 2004). In addition, it has been shown that chronic oxidative stress compromises the telomere integrity and accelerates the onset of senescence in endothelial cells (Kurz et al. 2004). Similarly, AT cells display sensitivity to oxidative stress such that mild chronic oxidative stress increased the rate of telomere shortening in AT cells (Metcalfe et al. 1996; Tchirkov and Lansdorp 2003).

1.16.3 Telomere Capture

Telomere capture is a non-reciprocal process in which telomere repeats are added to DSB creating sub-telomeric translocations (Meltzer et al. 1993; Fouladi et al. 2000; Lo et al. 2002). The process has been observed in human cancer cells such as malignant melanoma cells, and chronic lymphocytic leukaemia cells (Meltzer et al. 1993; Fouladi et al. 2000; Amiel et al. 2005). Since it is a non-reciprocal process, it results in short telomeres on the donor chromosome and longer telomeres on recipient. Telomere capture of sequences from another chromosome has been shown to stabilise the chromosomes, following terminal deletions in human malignant and normal cells (Meltzer et al. 1993).

1.16.4 Telomeric sister-chromatid exchange

Recombination events between sister chromatid telomeres can be detected cytogenetically with a characteristic reciprocal pattern of hybridisation during CO-FISH (Bailey et al. 2004c). Elevated frequency of sister chromatid exchanges have been observed at sub-telomeres [210×10^{-9} SCE/(bp × cell generation] compared to the genome average [1.3×10^{-9} SCE/(bp × cell generation; (Rudd et al. 2007)]. In contrast, meiotic recombination is suppressed at telomeres (Baird et al. 1995; Baird et al. 2000). However, high frequency of the T-SCE has been documented in immortalised and cancer cells that maintain their telomeres by telomerase-independent methods (Bechter et al. 2004; Londono-Vallejo et al. 2004), implicating increased recombination. Furthermore, it has been shown that both human and murine cells that are telomerase-deficient and /or have impaired DNA repair proteins exhibit spontaneous T-SCE (Bailey et al. 2004a; Londono-Vallejo et al. 2004). Moreover, embryonic stem cells with critically short telomeres and lack telomerase activity have been shown to exhibit high levels of telomere sister chromatid exchanges (Wang et al. 2005a; Wang et al. 2005b); implicating that this recombination-based process can be utilised by cells to compensate for telomere loss in the absence of telomerase. In support of this view, telomere sister chromatid exchanges are only elevated after telomerase inactivation in human cells and reactivation of telomerase abolishes these events (Bechter et al. 2004). Whilst telomere sister chomatid exchanges do not result in loss of genetic material per se, unequal T-SCE can result in telomere erosion and elongation on donor and recipient respectively (Muntoni and Reddel 2005; Muntoni et al. 2009).

1.16.5 Stochastic telomere deletion events

The analysis of telomeres at single molecule level has revealed that in addition to gradual telomere erosion due to end-replication and post replicative processing, telomeres are subjected to processes that generate truncated telomeres (Baird et al. 2003; Baird et al. 2006; Baird 2008). In addition, high frequency of extra

chromosomal telomere repeat DNA, consistent with telomere breakage, has been reported in Fanconi anaemia lymphocytes (Callen et al. 2002) and ATM deficient mouse cells as well as in cells derived from AT patients (Hande et al. 2001). These events are similar to the telomere rapid deletion events described in yeast (Lustig 2003) and plants (Watson and Shippen 2007). However, in yeast and plants over-elongated telomeres are truncated to the genetically determined average telomere length through an intra-chromatid recombination process (Bucholc et al. 2001; Lustig 2003; Watson and Shippen 2007). In contrast, in human cells, the telomeres are truncated from genome-average to critically short telomeres regardless of telomerase status of the cell (Murnane et al. 1994; Baird et al. 2003; Baird et al. 2006). These telomeres have potential to fuse with other telomeres or double strand breaks and initiate chromosome instability. In fact, we have demonstrated that short telomeres below a certain threshold are capable of fusion (Capper et al. 2007; Letsolo et al. 2009). In MRC5 clone where the longer and shorter telomere alleles could be distinguished by polymorphisms in the telomere adjacent DNA, it was apparent that both alleles were involved in fusion, an observation consistent with gradual end-replication losses coupled with stochastic deletions resulting in short telomeres (Capper et al. 2007). Similarly, studies in immortal cell line (KB 319), have reported random rapid changes in telomere length which result in short telomeres that lack telomere signal (Murnane et al. 1994). However, no change in polymorphism in the sub-telomeric DNA was observed (Murnane et al. 1994), consistent with our studies, indicating that telomeres are not lost but random deletions generate short telomeres that are capable of fusion. Indeed the frequency of these shorter than average telomeres was correlated with increase in chromosomal fusions (Murnane et al. 1994).

The mechanism that generates human telomere rapid deletion events is not known. However, like in yeast, they could be a by-product of activity of the MRN complex and/or other repair proteins (Bucholc et al. 2001; Lustig 2003), which in humans could be resulting from resolution of the Holliday junctions at

the t-loop. Supporting evidence comes from the observation that t-loop size extra-chromosomal telomere repeat containing circular DNA are frequent in human cells that maintain telomeres by recombination (Cesare and Griffith 2004; Wang et al. 2004). In addition such short telomeres and circular DNA has been reported in normal fibroblasts (Vidacek et al. in press). Alternatively, these events could be generated by spontaneous chromosome breakages that may occur when the stalled replication fork (on encountering telomere proteins such as TRF1 or TRF2) is resolved (Ohki and Ishikawa 2004). Another mechanism, oxidative damage which is known to be elevated at telomeres (von Zglinicki 2002) could lead to stochastic large telomere deletions through generation of S1 nuclease-sensitive sites which could break creating single stranded breaks which are known to accelerate telomere shortening (von Zglinicki et al. 2000). In addition, oxidation may result in generation of double strand breaks. Evidently, mouse cells deficient from one of the base excision repair proteins that excise oxidised guanines (Ogg1), display moderate telomere erosion at low oxygen tension levels (3%) but accelerated telomere erosions, high frequency of SSBs and DSBs under high oxygen tension [20%; (Wang et al. 2010)]. Another possible mechanism that could generate the events is unequal sister chromatic exchanges (SCE). As explained in section 1.16.4, unequal SCE can result in both telomere elongation and truncation. Indeed both longer and shorter than genome-average telomere lengths have been detected in human cells (Britt-Compton and Baird 2006). The short telomeres are of interest because they are capable of fusion and through cycle of breakage-fusion-bridging cycles can fuel large-scale rearrangements such as non-reciprocal translocations, amplifications and deletions (Murnane 2006) . Indeed studies have shown that short telomeres fuse with other telomeres as well as with other non-telomeric interstitial loci some of which are near fragile sites (Capper et al. 2007).

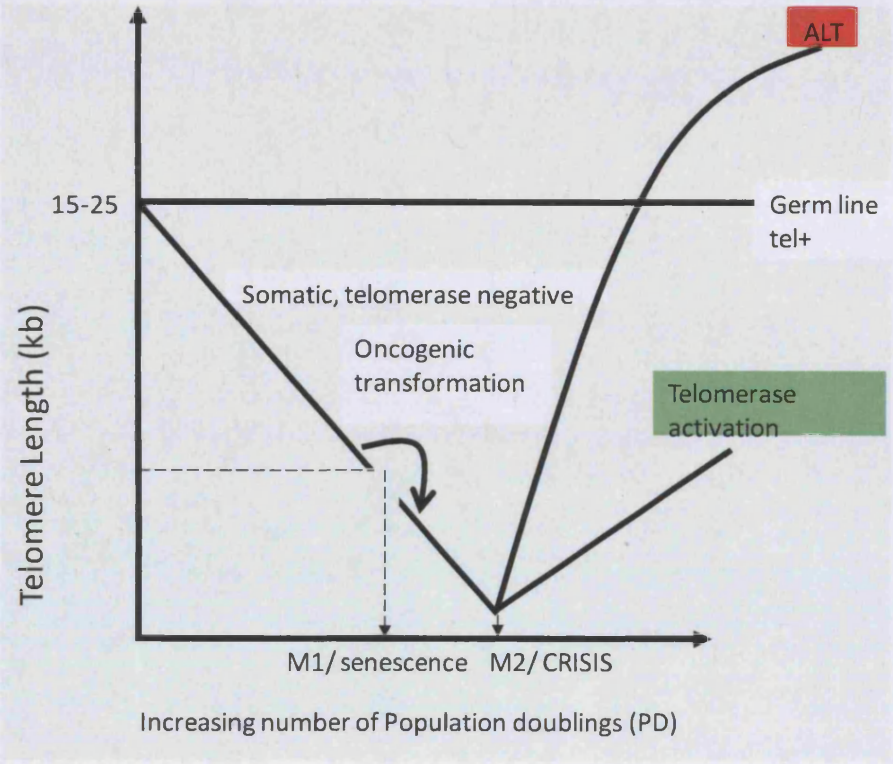


Figure 1.6. Telomere hypothesis of cell ageing. The telomere length changes as a function of number of cell division. Germ cells are telomerase positive (Tel+) and maintain their telomere length with each cell division. Unlike germ cells, somatic cells are telomerase-negative, so their telomeres shorten with each cell division. Short telomeres signal cell cycle arrest and activation of senescent phenotype (M1/Hayflick limit checkpoint). Cells can bypass M1 through oncogenic transformation events, but their telomeres continue to shorten until the chromosome function is impaired. Then cells enter crisis/M2 where most cells die. A few that emerge from crisis maintain length of telomeres by activating either telomerase or ALT and are thus immortal.

1.17 Methods for determining telomere length

Several methods are employed to determine the telomere length as well as for detecting chromosomal aberrations resulting from telomere dysfunction. Some of these methods, their advantages and limitations are discussed below.

1.17.1 Terminal restriction fragment (TRF) analysis

TRF analysis employs the principle of Southern blotting analysis, which was first described by Southern in 1974 [(Sambrook et al. 1989); reviewed in (Saldanha et al. 2003)]. In humans, the genomic DNA is usually digested with frequent-cutters, *Hinf*1 and/or *Rsa*1; which digest the majority of the genomic DNA without cutting within the telomere repeat region (Moyzis et al. 1988; Allshire et al. 1989). The restriction fragments are then resolved by agarose gel electrophoresis and transferred to the membrane where they are detected by telomere-repeat containing probe [(Sambrook et al. 1989); review in (Baird 2005; Lin and Yan 2005); Figure 1.7].

TRF analysis has advantages that make it the most popular method for telomere length analysis. For example, it utilises standard molecular biology techniques, reagents and equipment and provides genome-wide telomere-length. However, it has several drawbacks. The requirement for concentrated DNA (microgram concentrations) limits the technique use to analysis of large number of cells or tissue samples. Furthermore, the TRFs contain an unknown length of the highly variable sub-telomeric sequences and proximal regions (1-3kb) of the human telomeres (Allshire et al. 1989; Mefford and Trask 2002), thus TRFs do not represent the accurate telomere length [reviewed in (Saldanha et al. 2003; Baird 2005). Moreover, the TRF represents the mean telomere length of all chromosomes not specific chromosomes, which may not be very informative as it is the shortest chromosome that triggers senescence (Zou et al. 2004b). Importantly, the key limitation is that: because TRF analysis is a hybridisation-based method, there is a threshold length below which telomere cannot be

detected (Baird 2005). Thus, very short telomere lengths at which telomere dysfunction can occur may not be detected by TRF analysis. Despite these limitations, TRF analysis is widely used, and provides a good comprise between the accuracy and speed; thus has been successfully used in many epilogical studies (Valdes et al. 2005; Bataille et al. 2007).

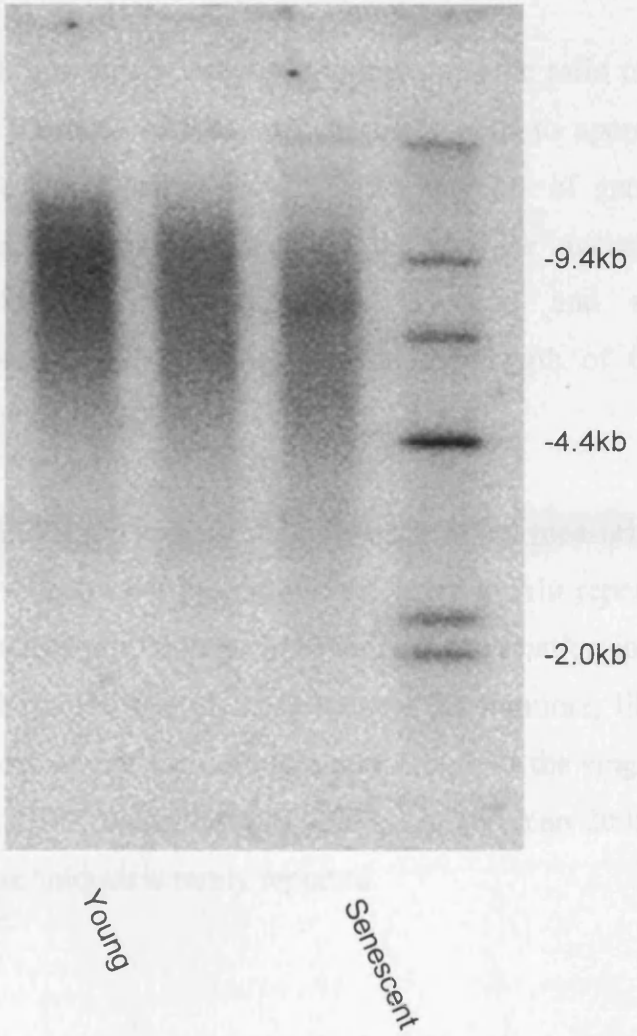


Figure 1.7. Telomere restriction analysis gel, illustrating the smears that represent telomere length range in young and senescent fibroblasts. DMB unpublished.

1.17.2 Slot blots and Hybridisation protection assay (HPA)

Unlike TRF analysis, slot blot measures the ratio of telomere to centromere content and requires the minimum of 9ng of purified DNA; thus abolishing the requirement for at least a microgram of DNA (Norwood and Dimitrov 1998).

The hybridisation protection assay (HPA) measures the ratio of telomere to *Alu* sequences up to the ratio of 0.01, which corresponds to approximately 2kb of mean TRF. Like slot blots, it requires low amounts of genomic DNA [10-3000ng; (Nakamura et al. 1999)]. In HPA, the need for unsheared pure DNA is abolished. Unlike TRF analysis, HPA is rapid and simple requiring approximately 45min and does not include the length of the sub-telomeric regions (Nakamura et al. 1999).

However, HPA and slot bots have the drawback of not measuring the actual size of the telomeres directly but the ratio of telomere to Alu repeats or centromere respectively. In addition, the large inter-individual variation in copy number of *Alu* sequences limits the use of these assays. Furthermore, like TRF analysis, HPA and slot blots cannot measure telomere length at the single-molecule level (Nakamura et al. 1999; Saldanha et al. 2003; Lin and Yan 2005). Consequently, the use of these techniques is rarely reported.

1.17.3 Fluorescence *in situ* hybridisation (FISH)

FISH has several advantages over TRF analysis, which make it suitable for telomere dynamics estimations: the requirement for isolating highly concentrated DNA is abolished as FISH uses metaphase chromosomes. It employs small size, single-stranded, synthetic telomeric-specific fluorescein isothiocyante (FITC)–conjugated or Cy3-labelled oligonucleotide (TTAGGG or CCCTAA) probes, which can easily penetrate the cells (McNeil and Ried 2000). In addition, the

signal is visualised by fluorescence microscopy, which makes the procedure time-efficient, as the need for washing off unbound radioactive probes is abolished (Lansdorp et al. 1996; McNeil and Ried 2000).

FISH can be widely used with several adaptations for detecting chromosomal aberrations such as acentric fragments using a telomere probe (Hultdin et al. 1998; Natarajan and Boei 2003).

1.17.3.1 Quantitative-FISH

The quantifying of telomere length by FISH method is called quantitative FISH [Q-FISH; (Hultdin et al. 1998; Martens et al. 2000)]. Unlike conventional FISH, Q-FISH utilises the peptide nucleic acid (PNA) oligonucleotides probes. PNAs are synthetic molecules that mimic DNA but their backbone sugar-phosphate molecule has been replaced by an uncharged flexible polyamide molecule (Pellestor and Paulasova 2004a; Pellestor and Paulasova 2004b). The PNA-FISH has the advantage of forming more stable complex than those formed by conventional DNA-DNA or DNA-RNA duplexes. In addition, the probes are more resistant to proteases and nuclease degradation. Furthermore, PNA-FISH procedure can be as short as two hours with the commercial kits, making it the preferred method for analysis of human telomeres (Lansdorp et al. 1996). In addition, PNA probes produce intense staining which improves sensitivity (Lansdorp et al. 1996). Unlike TRF analysis which require large amount of cells (>100000 cells), very few metaphase cells (<30 cells) are required for Q-FISH analysis. It can also be used to measure telomere length in cells that possesses the interstitial telomeres without overestimating telomere length as with TRF analysis. However it use metaphase spreads and thus requires cell culture and can only analyse proliferating cells (McNeil and Ried 2000).

1.17.3.2 Flow-FISH

It is an adaptation of Q-FISH, in which the interphase cells are analysed by FISH coupled with flow cytometry (Ishikawa 1998; Rufer et al. 1998; Baird 2005). It provides the genome-wide telomere length assessment for single cells thus is more informative than use of TRF analysis (Baerlocher et al. 2002; Baerlocher et al. 2006). Flow-FISH has several advantages over FISH alone: several subpopulations of cells can be analysed at the same time (Brummendorf et al. 2000; Brummendorf et al. 2001; Schmid et al. 2002). It can also be used to analyse telomeres in cells at different stages of the cell cycle without a need for metaphase spread preparation (Rufer et al. 1998; Baerlocher et al. 2002). FLOW-FISH has been employed extensively in studies of telomere length in blood cells (Rufer et al. 1998; Rufer et al. 1999; Brummendorf et al. 2000; Rufer et al. 2001).

1.17.3.3 Other FISH adaptations

Telomere/Immunostaining-FISH (TELI-FISH) combines the principles of FISH and immuno-staining techniques to measure telomere length. It requires fewer number of cells (10-15 cells) thus can be widely applied to several samples and it also gives telomere length estimates comparable to the mean TRF (Meeker et al. 2002a).

The telomere/centromere-FISH on the other hand, measures the telomere length of every single chromosome arm in metaphase cells relative to the centromere of chromosome 2. The ratio of centromere to absolute telomere length is correlated with mean TRF to determine the telomere length. Since the method still employs TRF, it suffers similar drawbacks to those described with TRF analysis (section 1.17.1). In addition, the requirement for capturing and karyotyping of metaphase images limit the application of the method to proliferating cells (Perner et al. 2003).

Finally, the fibre-FISH, a method that involves measuring telomeres on chromatin/DNA fibres is described in (Heiskanen et al. 1996). It determines telomere length of individual telomeres relative to the sub-telomeric probe of 1q [100kb; (Heiskanen et al. 1996; van de Rijke et al. 2000)].

1.17.3.4 Chromosome-orientation FISH

CO-FISH is a strand-specific hybridisation method that is used for discriminating between different types of telomere fusions and for detection of telomere sister-chromatid exchanges [T_SCE; (Bailey et al. 2004a; Bailey et al. 2004c)]. The procedure involves growing cells in 5-bromodeoxyuridine (BrdUrd) and/or bromodeoxycytidine (BrdC) for one replicative cycle. Once cultured, cells are prepared by standard cytogenetic techniques and then cells are exposed to UV light in the presence of Hoechst dye. The UV exposure generates the breaks at the sites of BrdU/BrdC incorporation, which can serve as substrates for Exonuclease III, which will in turn remove all the newly synthesised strands leaving parental strands intact. The single-stranded sister chromatids can then hybridise to the DNA or PNA probe, producing two signals, one at either end corresponding to the leading-strand [$(TTAGGG)_n$ probe] and lagging-strand synthesised [$(CCCTAA)_n$ probe] telomeres (Bailey et al. 2004c). Thus this method can be utilised to distinguish different types of telomere fusions: telomere-telomere fusions, telomere-DSB fusions and telomere sister chromatid exchanges (Bailey et al. 2004b).

1.17.4 Primed *in situ* labelling (PRINS)

It involves the *in situ* annealing of synthetic oligonucleotide $(CCCTAA)_n$ for telomere detection, followed by primer extension in the presence of fluorochrome-labelled nucleotides (Therkelsen et al. 1995; Lavoie et al. 2003). This method offers several advantages in terms of sensitivity and specificity, owing to the use of PNA primers and to the fast kinetics of annealing and elongation reactions *in situ*. This *in situ* hybridisation technology is a faster

alternative to conventional FISH for studying chromosomal aneuploidy and rearrangements, gene localisation and expression, and genomic organisation. In addition, it is fast, cost-effective, and produces intense and clearer signal (Lavoie et al. 2003; Yan et al. 2004). Furthermore, the conditions employed in PRINS prevent telomere reannealing. However, PRINS is not as appropriate for analysis of human telomeres which are short as it is, for detecting long repeat sequences such as those of mouse telomeres (Lavoie et al. 2003). However, like HPA and slot blotting, the use of this technique has not been reported extensively in the literature.

1.17.5 Quantitative polymerase chain reaction (Q-PCR).

The measurement of telomere length using Q-PCR was first described by Cawton (Cawthon 2002). Q-PCR measures the ratio of the telomere repeat copy to single copy gene number such that this ratio is proportional to average telomere length (Cawthon 2002). This method benefits from increased sensitivity and use of lower DNA concentrations compared to TRF analysis. Additionally, unlike TRF analysis, the estimated ratio does not include the variable sub-telomeric DNA [(Cawthon 2002); also reviewed in (Lin and Yan 2005)]. Furthermore, use of single gene copy avoid the problem of large inter-individual variation in copy number of *Alu* repeats, during the estimation of ratio of telomere length to *Alu* with HPA assays (Nakamura et al. 1999; Cawthon 2002).

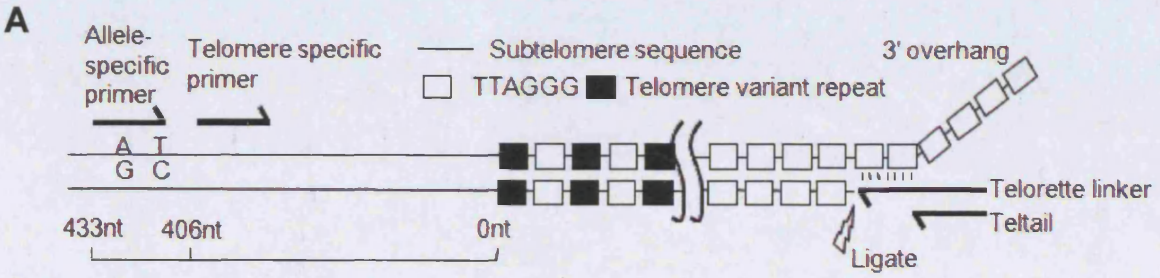
Like slot blots, Q-PCR estimates relative telomere ratio but not the actual distributions of the telomere length. Nonetheless, this technique has been widely used for large epidemiological studies (Cawthon et al. 2003; Njajou et al. 2007; Shen et al. 2007; Zhang et al. 2007).

1.17.6 Single Telomere Length Analysis (STELA)

STELA is a single-molecule PCR-based technique that determines the full spectrum of telomere length of the double-stranded telomere repeat region of a specific telomere [(Baird et al. 2003); reviewed in (Baird 2005); (Figure 1.8)]. First, the single stranded linker oligonucleotide called telorette, that targets the 3′ overhang is ligated to the 5′ end of the telomere. Then the telomere length of a given sample is determined through this simple procedure: multiple PCR reactions containing 4-10 amplifiable (250pg) molecules each are carried out with the linker, teltail and chromosome-specific telomere-adjacent oligonucleotide primer (Figure 1.8). The molecules are detected by Southern hybridisation with the chromosome-specific telomere adjacent probes and/or TTAGGG-containing probes after resolving by gel electrophoresis (Baird et al. 2003). The STELA was originally developed for analysis of XpYp telomere (Baird et al. 2003) and has since been extended for analysis of autosomal telomeres; 2p, 11q, 12q and 17p (Britt-Compton et al. 2006) as well as *C.elegans* (Cheung et al. 2004). Recently an adaptation of the STELA technique called "universal STELA" has been reported (Bendix et al. 2010). Unlike chromosome-specific STELA, this universal method estimates the telomere length from almost all chromosomes (Bendix et al. 2010). However, the discussion in this section will be limited to chromosome-specific STELA.

STELA exhibits several advantages over TRF analysis: with STELA very short telomeres that have potential to trigger replicative senescence or initiate genomic instability can be detected. In addition, STELA requires very low concentrations of DNA (in picograms) thus can be applicable to a broad spectrum of samples (Baird et al. 2003; Baird et al. 2006; Britt-Compton et al. 2006). In addition, the telomere length of specific telomeres in base pairs can be measured instead of the overall mean for all telomeres. Furthermore, STELA does not suffer from a lower length detection threshold thus STELA show a broader range of telomere distribution. For instance, using STELA large telomere variation up to and

Figure 1.8. Single telomere length Analysis (STELA) at XpYp telomere. A. Outline of the assay with oligonucleotide primers indicated, [Reproduced from (Baird et al. 2003)]. B. STELA at XpYp in young and senescent HCA2 cells, each fragment represents a single telomere molecule (DMB unpublished). C. Allele-specific STELA in Clonal fibroblast cell line (MRC5), illustrating telomere distributions with narrow variance superimposed by sporadic short telomere molecules (indicated with red markers), modified from Baird et al 2003.


A
Allele-specific primer
Telomere specific primer
Subtelomere sequence
3' overhang
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Telomere variant repeat
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406nt
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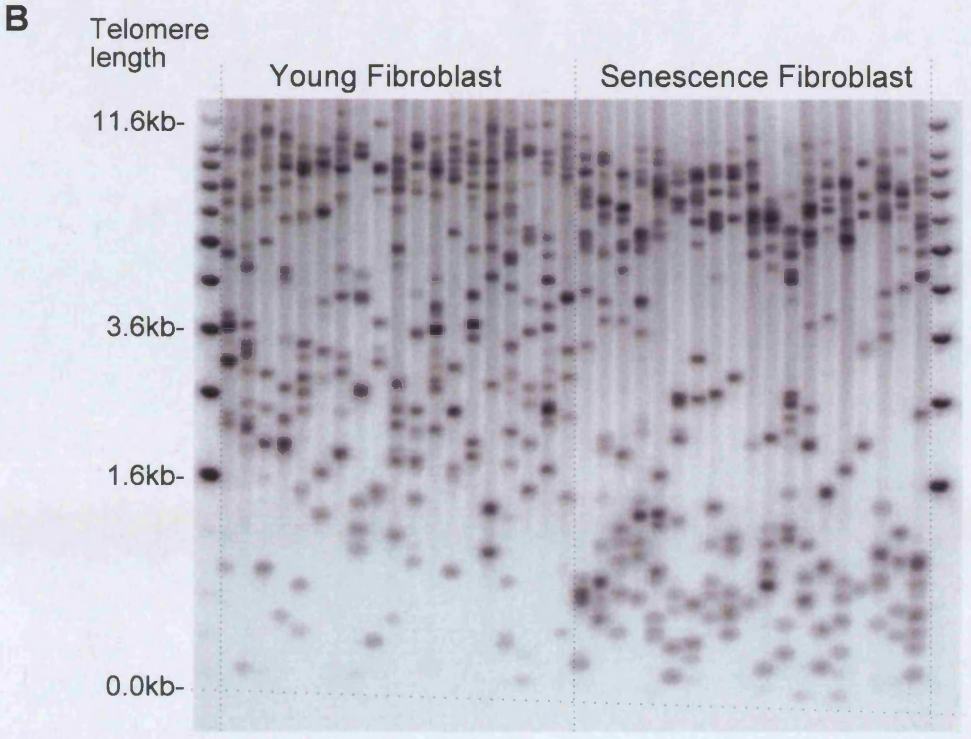
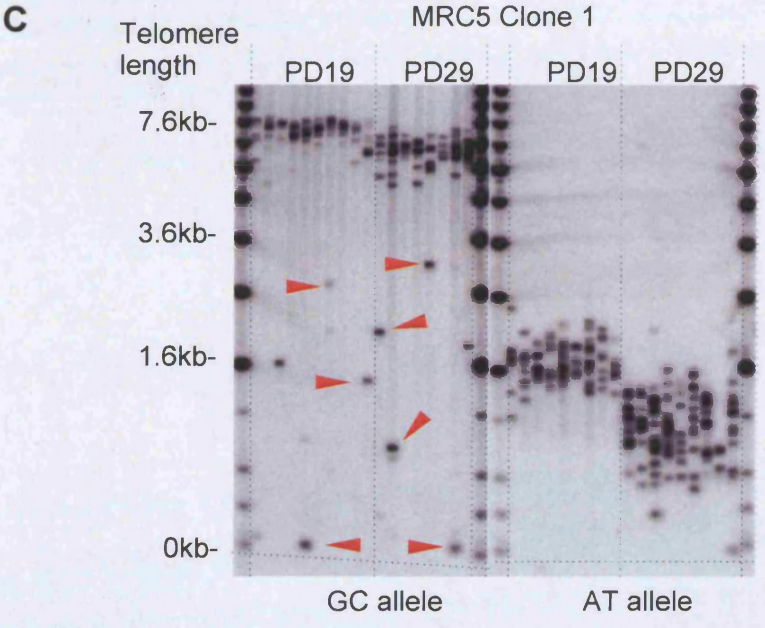

B
Telomere length
Young Fibroblast
Senescence Fibroblast
11.6kb-
3.6kb-
1.6kb-
0.0kb-


C
MRC5 Clone 1
Telomere length
PD19
PD29
PD19
PD29
7.6kb-
3.6kb-
1.6kb-
0kb-
GC allele
AT allele

beyond 20kb have been reported (Baird et al. 2006). Furthermore, by utilising the extensive sequence polymorphism within the telomere-adjacent DNA, each allele can be analysed separately thus revealing large allelic variation at the same telomere [Figures 1.8A and 1.8C; (Baird et al. 2003)]. Moreover, the length of the telomere-adjacent DNA can be compensated for by determining the distance (in nucleotides) between the oligonucleotide primer and start of telomere. Thus telomere length estimated by STELA does not include the sub-telomeric sequences (Baird et al. 2003). In addition, by combining STELA with telomere variant repeat PCR, the actual telomere lengths comprising homogeneous arrays of TTAGGG repeats can be determined after correction for the proximal variable telomere variant repeat region, which is considered to be the non-functional telomere repeat region (Baird et al. 1995; Capper et al. 2007). In addition, a single STELA experiment yields a massive dataset, thus it is more informative than TRF analysis. Again, unlike TRF analysis, it does not suffer hybridisation inefficiency as the small molecules can be detected (Baird et al. 2003).

The STELA has disadvantages of being laborious, as it requires several PCRs for each telomere. In addition, it requires good quality DNA, which limits its applicability to non-degraded and non-fixed material (Baird 2005). Furthermore, it does not give histological information and the complex hyper-variability of the sub-telomeric DNA limit the assay to a subset of telomeres (Mefford and Trask 2002; Riethman et al. 2005).

Using STELA, it has been shown that some senescent cells have telomere length distributions as small as 0.3kb (Baird et al. 2003; Baird 2005; Britt-Compton et al. 2006). In addition, STELA can detect broad range of telomeres from very short to very long telomeres, which is an important aspect for *in vivo* studies where large heterogeneity in telomere length is expected. Importantly STELA can detect telomeres within the length range at which telomere dysfunction and fusion occurs, these telomeres cannot be detected with any other assay. It is thus

a powerful technique for assessing the full extent of telomere erosion, deletion and dysfunction in human cells.

1.17.7 Telomere fusion assay

Telomere fusion assay is a PCR-based assay that allows the detection of the telomere-telomere fusion molecules between specific chromosomes (Capper et al. 2007; Letsolo et al. 2009). It can detect the fusion events including sister-chromatid-type fusion when there is a deletion in at least one of the ends creating an imperfect inverted repeats. It can also detect sister-chromatid-type fusion between heterologous chromosomes containing substantial arrays of head-to-head repeats as in cells with TRF2 knockdown (Capper et al. 2007). However, it cannot detect sister-chromatid type fusions that create perfect inverted repeats as in fusion between two homologous chromosomes without any deletion; as such events have potential to form palindromes (Capper et al. 2007; Letsolo et al. 2009).

The procedure involves DNA amplification using the oligonucleotide PCR primers designed on the sub-telomeric telomere adjacent of the specific chromosome ends that are orientated 5′-3′ towards the chromosome termini (Figure 1.9). To detect the fusion events, multiple PCR reactions containing 10-100ng of DNA are carried out. Following amplification, the products are resolved by gel electrophoresis and the products are detected by Southern hybridisation with chromosome-specific telomere-adjacent probe. The putative fusion molecules are then re-amplified in semi-nested PCR reactions with one in twenty dilutions (1 in 20) of the original fusion PCR product. Following successful re-amplification the molecular structure of the fusion events and chromosomes involved in fusion are confirmed by direct sequence analysis (Capper et al. 2007). The telomere fusion assay was originally developed for two chromosomes, XpYp and 17p (Capper et al. 2007) and has since been extended to include at least 43% of the human genome (Capper et al. 2007; Letsolo et al.

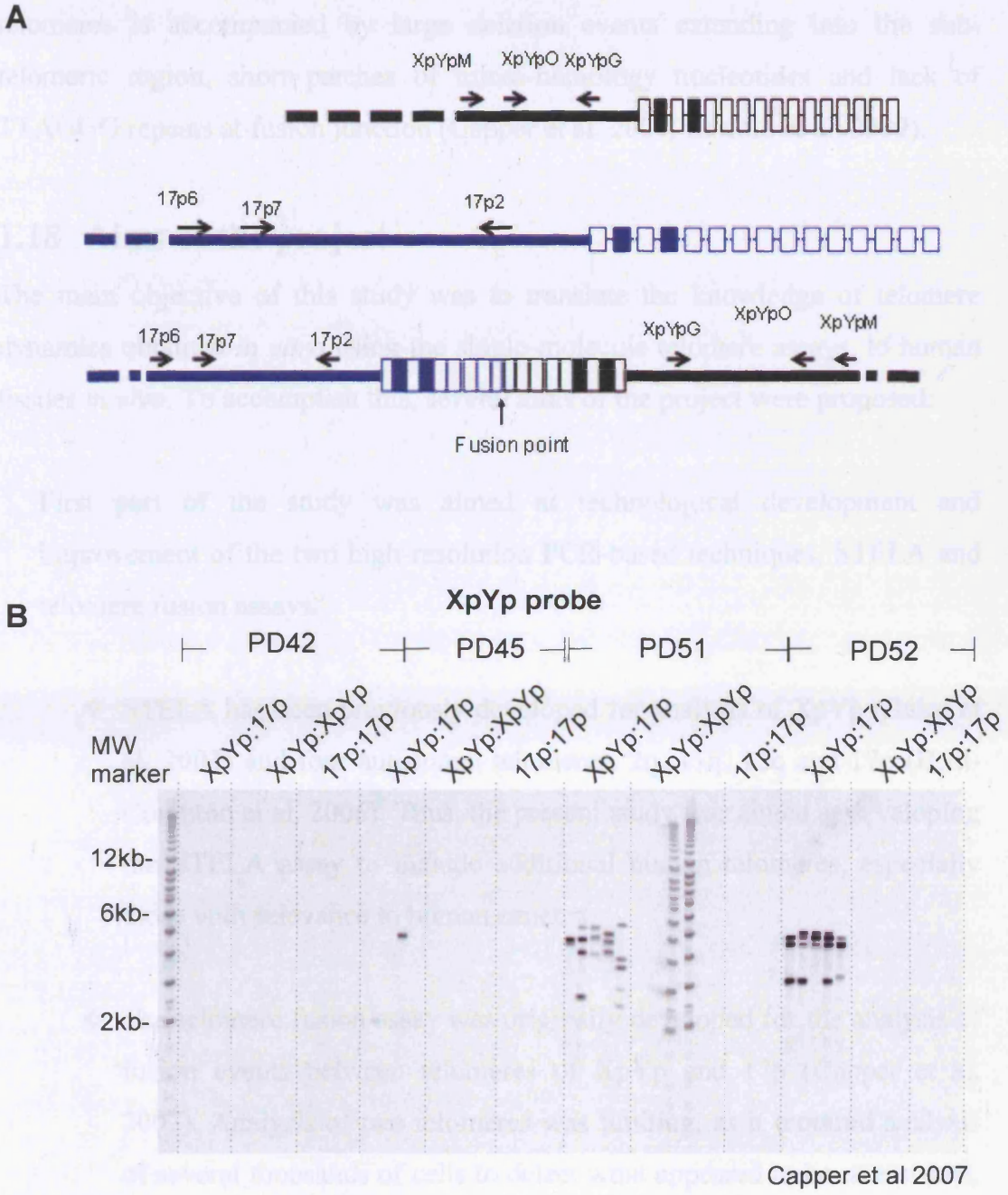


Figure 1.9. Telomere fusion assay. A. Schematic representation of the telomere fusion assay with oligonucleotide primers orientated 5′-3′ towards the telomeres for XpYp (top panel) and 17p (middle panel). The amplification can only occur when there is a fusion between the two chromosome ends (bottom panel). B. Telomere fusion gel illustrating fusion bands representing fusion events between XpYp and 17p telomeres in MRC5 cells undergoing crisis. Molecules were detected with telomere-adjacent XpYp probe. XpYp:XpYp, XpYp:17p and 17p:17p assays are shown. MW represents molecular weight while PD is population after which cells were harvested

2009). Using this assay, we have shown that fusion of short dysfunctional telomeres is accompanied by large deletion events extending into the sub-telomeric region, short patches of micro-homology nucleotides and lack of TTAGGG repeats at fusion junction (Capper et al. 2007; Letsolo et al. 2009).

1.18 Aims of the project

The main objective of this study was to translate the knowledge of telomere dynamics obtained *in vitro* using the single-molecule telomere assays, to human tissues *in vivo*. To accomplish this, several aims of the project were proposed:

i. First part of the study was aimed at technological development and improvement of the two high-resolution PCR-based techniques, STELA and telomere fusion assays.

   - ❖ STELA has been previously developed for analysis of XpYp (Baird et al. 2003) and four autosomal telomeres, 2q, 11q, 12q and 17p (Britt-Compton et al. 2006). Thus, the present study was aimed at developing the STELA assay to include additional human telomeres, especially those with relevance to human cancers.

   - ❖ The telomere fusion assay was originally developed for the analysis of fusion events between telomeres of XpYp and 17p (Capper et al. 2007). Analysis of two telomeres was limiting, as it required analysis of several thousands of cells to detect what appeared to be rare events. Given the number of different chromosomes involved in chromosome instability in cancer and other diseases, it was essential to extend the assay to include additional telomeres.

   - ❖ Using STELA and telomere fusion assay it has been demonstrated that telomeres are subjected to processes that generate short telomeres that

are capable of fusion (Capper et al. 2007). It is known that telomere dysfunction can initiate genomic instability through anaphase-bridge-breakage cycles that can result in large-scale rearrangements such as non-reciprocal translocations (Artandi et al. 2000; Feldser et al. 2003). However, the mechanisms that underlie the fusion of short dysfunctional telomeres are unknown. Thus in an attempt to understand the mechanism that underlies these events, molecular structure of fusion events was characterised using the extended and improved assays. The data set collected with this study will be used as a reference for the experimental models that are aimed at understanding the mechanistic basis of fusion.

ii. The second aim was to utilise the improved assays to investigate telomere instability and fusion in normal human tissues. Telomere dynamics were investigated in human melanocytic naevi and surrounding dermal samples. The naevi represent a good model for study of telomere dynamics in senescent cells *in vivo* as cells remain senescent and dormant for years even though the naevi predispose to the deadly melanoma in susceptible individuals (Robinson et al. 1998). Melanocytic naevi consist of functionally viable melanocytes (Bandyopadhyay et al. 2001; Bennett and Medrano 2002; Bennett 2003; Mooi and Peeper 2006) which have undergone senescence following oncogenic mutations (Michaloglou et al. 2005). They can remain dormant for many years yet they predispose to melanoma in susceptible individuals (Skender-Kalnenas et al. 1995; Bennett and Medrano 2002).

iii. One of the biological processes than may drive neoplastic progression is telomere dysfunction (Artandi et al. 2000; Rudolph et al. 2001), thus another aim of the study was to investigate telomere erosion, instability and fusion in preneoplastic condition, Barrett's oesophagus. Barrett's oesophagus is the premalignant and metaplastic condition that predisposes to oesophageal adenocarcinoma [reviewed in (Wild and Hardie 2003; Maley 2007). It is a

good model for carcinogenesis studies of solid tumours for a number of reasons: first, unlike other benign tumours such as adenomatous polyp in the colon, Barrett's oesophagus tissue is not removed upon detection because of the low number of progressions to adenocarcinoma (Jenkins et al. 2002; Maley 2007). Secondly, it is easily visualised by endoscopes. In addition, it is easily and safely biopsied to track the progression overtime. Furthermore, the genetic changes associated with progression from Barrett's metaplasia to oesophageal adenocarcinoma are universal in most human cancers, e.g. LOH at p53, p16, aneuploidy etc (Gonzalez et al. 1997; Jenkins et al. 2002; Maley 2007). Although periodic endoscopy surveillance are suggested for people with Barrett's oesophagus, the question for cost-effectiveness and time remains controversial due to low percentage of progression to adenocarcinoma (van der Burgh et al. 1996; Sampliner 2002). In addition, some patients diagnosed with the putative high-risk histological stage, dysplasia revert (Reid et al. 2000). Finally, the large inter-observer variation among pathologists and histologists when identifying the histological stage dysplasia from Barrett's oesophagus mucosa, complicates the diagnosis such that some individuals that are diagnosed as having dysplasia are already having carcinoma *in situ* (Reid et al. 1988). Thus, identification of more biomarkers that may be used for identifying patients at highest risk of progression is essential.

2 Materials and Methods

2.0 Chemicals and Molecular Biology reagents

Chemicals were obtained from Fisher Scientific (part of thermo fisher scientific, Loughborough, UK) and molecular biology reagents obtained from Gibco BRL/invitrogen Ltd (Paisley, UK), Stratagene (La Jolla, California), ABgene (Surrey, UK), Promega (Southampton, UK), New England Biolabs (UK) Ltd (Herts, UK), Roche Products Ltd (Hertfordshire, UK).

Radiochemical reagents were obtained from Amersham biosciences/GE Healthcare UK Limited (Bucks, UK) and PerkinElmer Life and Analytical Sciences (Milano, Italy). Whilst cell culture reagents were obtained from Invitrogen Ltd/Gibco (Paisley, UK), Sigma-Aldrich Company Ltd (Dorset, England) and Autogen Bioclear Ltd (Wiltshire, UK). Liquid nitrogen tanks were supplied by BOC cryospeed (Manchester, UK).

2.1 Plastic labware

Cell culture labware, all other plastics and glassware were obtained from Becton Dickson labware (Franklin, USA), Gilson (Middleton, USA), thermo electron corporation (Gormley) and Eppendorf (Cambridge, UK).

2.2 Equipment

Specialised equipment was obtained from Bio-RAD (Hertfordshire, UK), MJ Research (USA), MSE (UK) Limited (London,UK), Hybaid (Teddington, UK), Bioquell Ltd (Hampshire, UK), DJB Labcare Ltd/Heraeus (England, UK), Labcold (formerly Borolabs, Bakingstoke, UK), Patterson scientific (Cambridge, UK), GRANT instruments Ltd (Cambridge, UK), Thermo electron corporation (Gormley), Amersham biosciences (Buckinghamshire, UK), IBS integrated biosciences (Lewisberry, USA), Jencons-PLS (West Sussex, UK), Molecular dynamics (American laboratory trading, Groton), Qiagen (West Sussex,UK) and

scientific laboratory supplies Ltd (Nottingham, UK). Microscopes were supplied by Olympus UK Ltd (Hertfordshire, UK).

2.3 Oligonucleotides

The oligonucleotide primers were designed using the human DNA sequences obtained from the databases, http://www.wistar.upenn.edu/Riethman/, National Centre for Biotechnology Information (NCBI) and UCSC human genome bioinformatics. The oligos were then synthesised by MWG-Biotech AG (Ebersberg, Germany). All oligonucleotide primers used during this study are shown in Table 2.1.

Table 2.1 Oligo nucleotide primers used in the study. The distance from start of telomere tracts for STELA primers is shown in parenthesis. While * denotes first-round PCR telomere fusion primers and ** for Semi-nested PCR primers. XpYp, 11q and 17p primers have been described in (Baird et al., 2003; Britt-Compton et al., 2006; Capper et al., 2007).

Primer name	17p primer Sequence
17p2	5'-GAG TCA ATG ATT CCA TTC CTA GC-3'
17p5	5'-TTT TCA TTA GGA TCC AGT TTG-3'
17p6*	5'-GGC TGA ACT ATA GCC TCT GC-3'(3078bp)
17p6B**^	5'-CTG AAC TAT AGC CTC TGC AAT G-3'
17p6C**^	5'-TGA ACT ATA GCC TCT GCA ATG GAT-3'
17p7**	5'-CCT GGC ATG GTA TTG ACA TG-3' (3011bp)
17PSEQ1	5'-CCT CAG CCT CTC AAC CTG CTT-3'
17pseq1A	5'-CCT CAG CCT CTC AAC CTC GTT-3'
17pseq1rev	5'-GAA TCC ACG GAT TGC TTT GTG TAC-3' (313bp)
17pseq2	5'-CCT CAG CCT CTC AAC CTC GTT-3'
17pseq3	5'-AGA ATC CTG TCC TCA ACA AGT-3'
Primer name	11q Primer Sequence
11q12	5'-CCC TGA TTA TTC AGG GCT GCA AAG-3'
11q13B	5'-CAG ACC TTG GAG GCA CGG CCT TCG-3' (105bp)

Primer	STELA universal primers sequences
Teltail	5'-TGC TCC GTG CAT CTG GCA TC-3'
Telorette2	5'-TGC TCC GTG CAT CTG GCA TCT AAC CCT-3'

Primer name	XpYp Primer Sequence
XpYp 433AT	5'-GGT TAT CGA CCA GGT GCT CT-3'
XpYp 433GC	5'-GGT TAT CGA CCA GGT GCT CC-3'
XpYp-427A/415T	5'-GGTTATCAACCAGGTGCTCT-3'
XpYp-427G/415C	5'-GGTTATCGACCAGGTGCTCC-3'
XpYpB2	5'-TCT GAA AGT GGA CC(A/T) ATC AG-3'
XpYpC	5'-CAG GGA CCG GGA CAA ATA GAC-3'
XpYpE	5'-TTG TCT CAG GGT CCT AGT G-3'(406bp)
XpYpG	5'-AAT TCC AGA CAC ACT AGG ACC CTG A-3'
XpYpJ	5'-CTA ATC TGC TCC CWC CCA C-3'
XpYpM*	5'-ACC AGG TTT TCC AGT GTG TT-3'
XpYpMB**^	5'-AGG TTT TCC AGT GTG TTA TC-3'
XpYpMC**^	5'-CCA GTG TGT TAT CTA TCT AC-3'
XpYpO**	5'-CCT GTA ACG CTG TTA GGT AC -3'
XpYpP	5'-ACC AGG GGC TGA TGT AAC G-3'
XpYpQ	5'-CCA TGA GAC ACA AAG GAC TC-3'

^ Duncan Baird (unpublished)

Primer name	17q Primer Sequence
17q1REV	5'-TAA ACA TCT GTT GGT GCT TGT GG-3'
17q2	5'-AGA TAA CAG ACG TCT ACA CAG CTC G-3'
17q3	5'-CAA ACT CCT GAG ATC CAT GTG TC-3'
17q4	5'-CGT GGA GAG GGT CAC TGT GCA CGT G-3'

Primer name	21q Group primer Sequence
10qASF	5'-GCT CTG ACT TTA AGT GGT GC-3'
10Q1	5'-AGG TTC CAC TCG TCT CTG CG-3'
10q1A	5'-TGC AGC CAC GAC AAT GGC AG-3'
10q2	5'-TGC AAT GTC CCT AGC TGC CAG(C)-3'
10q3	5'-AG(C)A CAC AGG ATA GTG GGC TCT G -3'
10q4rev	5'-TGC ACG CGC AGA GAC ACA CG-3'
10qA	5'-GAA ACA CCC TAG AAC TTC TCT TG-3'
10qFMS	5'-CTT AAC AAG GTC TCT TCC AGA G-3'
10qSTEL3	5'-CTA ATG CAC ACA TGA CAC CCT-3'
10qt21	5'-GTA CTG CCT GCC TTT GGG AT-3' (277bp)
19p1	5'-AGC AGA GTT CTC CTC AGG TC-3'
21Q1*	5'-CTT GGT GTC GAG AGA GGT AG-3'
21q1B**^	5'-TTG GTG TCG AGA GAG GTA GC-3'
21q1C**^	5'-GTG TCG AGA GAG GTA GCT TTT AAA TG-3'
21Q2CT	5'-AGT AGC ATC TC(CT) AGT GCT GGA G-3'
21q3	5'-CTG CAG TTG TCC TAG TCG C-3'
21q5rev	5'-GGT GTG TGC TAA AGA GAA CG-3'
21qA	5'-GTA CTG CAT GGC TTT GGG AC-3'(277bp)
21qB	5'-GTT GCT GCC CTG AAT AAT CAA GGT CAC-3'
21qBT1	5'-TTT GGG AGG CCA AGG CGG GC-3'
21qBT2	5'-TTT GGG AGG CCG AGG CGG GT-3'
21qC	5'-AGA GTT CTT CTC AGG TCA GAC CTG-3'

Primer name	21q Group primer Sequence
21qD	5'-GGC GAG GGG CTG TAT TGC AC-3'
21qE	5'-GCG AGG GGC TGT ATT GCA CG-3'
21qseq1**	5'-TGG TCT TAT ACA CTG TGT TC-3'
21qseq1rev	5'-AGC TAG CTA TCT ACT CTA ACA GAG C-3'
21qseq2	5'-TGC CCC AAT CAT CAT TCA CTC TGC-3'
21qseq2rev	5'-CTC GAT CTC CTG ACC TCA TGA TC-3'
5q1^	5'-AGC ACC TCT GGC AGT TTG-3'
6p1	5'-CTG CAC AGG ACT CCT AGG ATG-3'
6p2REV	5'-CGA CAG ACT GAC GCG ACG TG-3'
6q1^	5'-TCA TGA TGC ATG CTG TGT GTC-3'
6q2^	5'-TCT TAC ACT GTC TCT CTG GC-3'
8p1	5'-TGC ACA GGA CTC TTA GGC TG-3'
Subtel 3b	5'-GTA CGC TGT CTT CAT GGC AG-3'
Subtel1^	5'-AAG GCG GAG CAG CAT TCT TCT CAG -3'
Subtel2^	5'-GAA TCC TGC GCA CCG AGA TTC TC-3'
SUBTEL2rev	5'-GAG AAT CTC G(GC)T GC (AGC) CAG (GC)AT TC-3'
SUBTEL3REV	5'-GTG CTT AGG AAT GCT GCA TTT G-3'

^ Duncan Baird (unpublished)

Primer name	16p Group Primer Sequence
15qA	5'-ATC GGA ACG CAA ATG CAG CAG-3' (73bp)
15qSEQ1	5'-TGT CGG ATC TCT TCA ACA AGC-3'
16p1*	5'-TGG ACT TCT CAC TTC TAG GGC AG-3'
16p1B**	5'-ACT TCT CAC TTC TAG GGC AGA G-3'
16p2**	5'-CTA GAG AGG AAG CAG GGA GTA TC-3'
16p2BTL	5'-TCA CTG CTG TAT CTC CCA GTG-3'
16p5	5'-TAG CAT GTG TCT CTG CGC CTG-3'
16p6	5'-CTC CAC TCC AGT GCT CAG CTT G-3'
16pBT	5'-AGT GAA GCT TTC CTC TCT AAG C-3'
16pseq1	5'-TGC CTC ACC TCT CTG GGC CAG -3'
16pseq1rev	5'-GCT GGG TGA GCT TAG AGA GGA AAG C-3'
16pseq2	5'-CTT GCC AGA GCT CCT CTC CAG-3'
16pseq2B	5'-GCT CCA GAT GAC ATC ACA GGG -3'
16pseq3	5'-TCC AGG GCT TCA CCT GT(C) TAG -3'
16pseq4	5'-TGG GTC CTG GCA ACA CTC TG-3'
16pseq4rev	5'-CTA GGT GGG ATC TCT GAG C-3'
16pseq5rev	5'-TGC TTG TGC TCA TCT CCT TGG C-3'
1pseq1A	5'-TCA GTG ACA GAC ACA GGG TCT C-3'
1pseq2BT	5'-TGC AGC TGC CTG TCA GGA AG-3'
SUBTEL 3a	5'-GCC GTG CGA CTT TGC TCC TG-3'
9p1	5'-TGC GTT CTC GTC AGC ACA GAC CC-3'
9p2	5'-CAC ATT CCT CAT GTG CTT ACG-3'(16bp)
9p3	5'-AAA CAG GCA CTC CTA GAC ATT GG-3'
XqYqseq1	5'-TGC ACA GAT TTC G(A)G TGG TAC-3'
XqYqseq2	5'- GTT GCT CTG ACA TGG ACA CAG-3'
XqYqseq4	5'-TCT CTA GCT GTG CAG GAT GC-3'

2.4 Samples

Patient-matched oesophageal and gastric epithelial tissue samples were obtained from Dr Gareth Jenkins (University of Wales, Swansea, Table 2.2) and Professor Nicol Keith (Centre for Oncology & Applied Pharmacology, Glasgow; Table 2.3 and appendix) for collaborative work. The tissue samples obtained from Swansea were composed of squamous epithelium, Barrett's metaplasia and gastric mucosa (Table 2.2). However, no data was provided (by collaborators) regarding the composition and quality of these samples.

Tissue samples obtained from patients undergoing endoscopy at the Glasgow royal infirmary were derived 2cm above the Z-line and every 2cm below the Z-line at the distal oesophagus. These samples were taken in triplicate; one for histopathology, one for telomerase activity analysis (Going et al., 2004), the remaining material following telomerase extracts was used for STELA and fusion analysis. Additional samples were derived from the oesophago-gastric junction as well as the gastric body and antrum (Table 2.3). The presence or absence of the different mucosal types was recorded for each biopsy as outlined in (Going et al., 2004). However, that data was not supplied with the samples used for this study, thus it may be difficult to comment on the quality and purity of these samples at present.

Skin samples were obtained from Professor Tim Spector (Twin Research & Genetic Epidemiology Dept, King's College London, St Thomas' Hospital Campus; Table 2.4). The skin samples were obtained from dissected melanocytic naevi from female patients recruited for the twin study at St Thomas' Hospital Campus. The melanocytic naevi was macro-dissected and separated from the surrounding dermis with the clean scalpel. However the actual cell composition of each sample was not verified. Thus it is possible that the melanocytic naevi preparations also contained epidermal keratinocytes and some cells from the dermis and vice versa for the dermal preparations.

Ethical approval for use of human tissues was obtained as follows: for skin samples from Guy's and St Thomas NHS Trust ethics committee, oesophago-gastric samples from Glasgow Royal Infirmary Research Ethics committee (Going et al., 2004) and by Dyfed Powys LREC in Aug 2006 (ref no. 06/WMW01/28) at Singleton and Morrison Hospital, Swansea, with written permission from participating patients.

Table 2.2 Oesophageal and gastric tissue sample obtained from 14 patients undergoing periodic endoscopy for Barrett's oesophagus at Morrison hospital Swansea, UK.
Key: x-sample present, No DNA-means DNA extraction failed, None-no sample provided.

Patient ID	Squamous Mucosa	Barrett's tissue	Gastric Mucosa
144321	x	x	x
867688	x	x	x
814850	x	x	x
723792	x	x	x
753522	x	x	x
266355	x	x	x
744342	x	x	x
408975	x	no DNA	x
172729	x	x	x
345077	none	x	x
409390	none	x	x
218883	none	x	x
148058	none	x	x
21616	none	x	x

Table 2.3 Oesophageal and gastric tissue samples taken from 26 patients undergoing periodic endoscopy for Barrett's oesophagus at the Glasgow royal Infirmary, UK. Key: * Minimal dysplasia: **Severe dysplasia: ***Patient with Tumour: $ for Tumour that was not classified i.e. either from oesophagus or gastric tissue, assumed was oesophageal tumour; none=no tissue sample provided. Normal represents the normal oesophageal squamous epithelial tissue.

Patient ID	Normal	Z-line	Barrett's oesophagus segment -distance (cm) into the oesophagus																	O-G Junction	Body	Antrum
			23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40			
600426L	113	114							107		108		109		110						112	111	106
600822W	124	125		116		117		118		119		120		121							123	122	115
608163H	132	133									127		128		129						131	130	126
648384K	140	141				135		136		137											139	138	134
701142B	NONE	151			143		144		145		146		147		148						150	149	142
718557V	158	159							153		154		155								157	156	152
736499H	165	166												161		162					164	163	160
772131M	173	174											168		169		170				172	171	167
777314L	184	185	176		177		178		179		180		181								183	182	175
823098V	193	NONE	187		188		189		190		191										NONE	192	186
828803R	202	203	200						195		196		197		198						201	199	194
892847L	209	210									205		206								208	207	204
977002H	216	217															212		213		215	214	211
998755H*	227	NONE			219		220		221		222		223		224		225				228	226	218
3006544L**	237	238				230		231		232		233		234							236	235	229
41198R	336	338																333		334	337	335	332
484438X	348	349			340		341		342		343		344		345						347	346	339
312135E	57	58									52		53		54						56	55	51
454788X	64	65															60		61		63	62	59
482970M	71	72											67				68				70	69	66
492062K	81	82				74		75		76		77		78							80	79	73
507097W	NONE	NONE		84			85		86		87		88								90	89	83
591099E	97	98				92		93		94											96	95	91
594360R	104	105																100		101	103	102	99
								Tissue Adjacent to Tumour					0ESOPHAGEAL TUMOUR				GASTRIC TUMOUR						
891759L***	301	NONE											302				303				300	299	298
464583W***	316	NONE							313				317[$]								NONE	315	314

Table 2.4. The melanocytic naevi and the surrounding dermal tissue from 19 patients

* denotes same patient but two naevi samples.

Patient ID	Melanocytic naevi	Dermis
98592	x	x
95981	x	x
32811A*	x	
32811B*	x	x
20561	x	x
20562	x	x
32812	none	x
52894	x	x
17442	none	x
93941	none	x
93942	x	x
2492	x	x
2493	x	x
94041	x	x
97051	x	x
18261	x	x
93983	x	x
06651	x	x
06652	x	x
98591	x	x

2.5 Cell culture

All cell culture work was carried out in a suitable Microflow Advanced Biological Safety Cabinets category class II flow hood (Bioquell).

The cells were incubated in 5% CO_2 at 37^0C and sub-cultured when confluent using standard techniques. Human embryonic kidney cell line, HEK293 (Graham et al., 1977) and human breast cancer cell line, MCF7 (Soule et al., 1973) were cultured in 88% Dulbecco's Modified Eagle's Medium (DMEM) containing 4,500 mg/L D-glucose 110 mg/L sodium pyruvate, and non-essential amino acids but no L-glutamine (Invitrogen), supplemented with 10% (v/v) Foetal Calf Serum (FCS; Invitrogen), 1×10^5 IU/L penicillin (Sigma), 100 mg/L streptomycin (Sigma) and 2 mM glutamine (Sigma). The human fibroblast MRC5 (mixed population; Rebecca Capper, personal comm.) and MRC5 (Jacobs et al., 1970) cells expressing the human papilloma viral oncoproteins E6E7 were cultured in Eagle's minimum essential medium supplemented with Eagle's salts containing 2x nonessential amino acids (Sigma), G418 (0.4mg/ml), HEPES (Sigma), sodium bicarbonate (7.5% solution, Gibco), 15% Foetal Calf serum, 1×10^5 IU/L penicillin (Sigma), 100 mg/L streptomycin (Sigma) and 2 mM glutamine (Sigma).

The cells were fed regularly and reseeded until confluent. For re-plating, cells were passed by removing the medium with the aspirator. The cells were then washed with 1-2ml of trypsin (0.05% trypsin and 0.02% EDTA (Autogen Bioclear, biomedia). Once washed, cells were then trypsinised at 37^0C with 1-2ml (1 for T25 and 2 for T75) of fresh trypsin until cells were detached. Trypsinisation was stopped by addition of medium to make the final volume of 10ml after which the cells were counted on haemocytometer.

Depending on the number of cells, a fraction of cells was resuspended in medium and replated.

2.5.1 Cells freezing and thawing

Some cells were frozen in liquid nitrogen to enable long-term storage for future experiments. To minimise ice damage, cells to be freezed were resuspended in equal amounts of medium and freezing mix (1:4 dimethyl sulphoxide (DMSO): foetal calf serum) prior to transferring to the ampoule. The ampoules were then stored in isopropanol at -80^{0}C prior to transferring into liquid nitrogen.

Whenever required, frozen cells were quickly thawed at 37^{0}C and transferred to centrifuge tube, into which 9ml of medium was added drop-wise to minimise osmotic shock. The cell suspensions were then centrifuged at 1000 rpm for 5 minutes and the supernatant removed. Fresh medium was added prior to re-plating.

2.6 DNA extraction and quantification.

2.6.1 Cells

When confluent, cells were treated with trypsin and washed in 1x phosphate-buffered saline (PBS). The cells were then pelleted by centrifuging at 1000 rpm for 5 minutes. The cell pellet was resuspended in 1.5ml of fresh 1x PBS and pelleted again by centrifuging at 6000 rpm for 5 minutes. The pellet was then stored at -20^{0}C until required for DNA extraction.

The DNA was extracted from the cells using phenol/chloroform extraction method (Sambrook et al., 1989). The procedure involved lysing the cells with 300-500µl lysis buffer (10mM Tris-HCl pH8.0, 100mM NaCl, 5mM EDTA pH 8.0, 0.5% SDS). The lysis buffer and cells were mixed thoroughly by flicking until the solution was clear. The RNA was degraded by the addition of 3µl RNase A (Sigma; stock, 10mg/ml) and the proteins were digested using 60µg proteinase K (Sigma: stock, 20mg/ml). The cell solution was then incubated at 45^{0}C overnight after which DNA was extracted by addition of 300-500µl of

phenol: chloroform: isoamyl alcohol (25:24:1) to the cell solution mixing thoroughly but gently by rotating solution on tube rotator for atleast 2 x 30 minutes. The cell debris and proteins were separated from DNA by centrifugation at 13000 rpm for 5 minutes on Heraeus Biofuge Pico microcentrofuge (DJB labcare). The aqueous layer containing DNA was then removed and DNA was ethanol-precipitated using $1/10^{th}$ 3M sodium acetate (pH5.3; Sigma) and 4x the volume of iced-cold absolute ethanol (Fisher) at -20^0C for atleast an hour. The DNA pellet was desalted by quick wash with 70% ice-cold ethanol and air-dried in the micro-flow hood. The air-dried DNA was either resuspended in 10 mM Tris-HCl, pH 8.0 (Sigma) or in 1x *Ecor*I or *Not*I buffer (Promega).

The high molecular weight DNA was solubilised by digesting with either EcoRI or *Not*I (Promega) for atleast 8 hours at 37^0C, followed by enzyme inactivation at 65^0C for 10 (*Ecor*I) to 15 (*Not*I) minutes, or serially diluted in 10mM Tris prior to quantifying.

2.6.2 Tissues

Frozen tissues were disrupted with a tissue ruptor homogeniser (Qiagen) in lysis buffer and genomic DNA was extracted by standard proteinase K, RNase A and phenol/chloroform protocols (Sambrook et al., 1989) as outlined in section 2.6.1 except that the lysis time was reduced to 6 hours at 45^0C.
High molecular weight DNA was solubilised as outlined in Section 2.6.1.

2.6.3 DNA Quantification

The DNA concentration was estimated in triplicate using Hoechst 33258 fluorometry (BioRad) and / or Nanodrop ND-1000 system (Thermo scientific).

2.7 DNA Amplification and Enzymatic manipulation

All genomic DNA manipulation was carried out in either microflow category II advanced biosafety class II hood (Bio-quell) or in PCR-clean environment designated for that work to minimise contamination risk. All PCR reactions were cycled on MJ PTC-225 thermocycler (MJ Research).

2.7.1 Single TElomere Length Analysis (STELA)

The genomic DNA for telomere length analysis was first diluted to 10ng/µl with 10mM Tris (pH8.0, Sigma). The diluted DNA was then diluted to 250pg/µl containing 0.25µM telorrette 2 (Tel 2) primer.

Replicates/ Multiple (typically 6-8) PCR reactions containing 1x Taq reaction buffer (75mM Tris-HCl (pH 8.8), 20mM $(NH_4)_2SO_4$, 0.01 % Tween-20:Abgene), 2.0mM $MgCl_2$, 250pg DNA-tel2 mix, 0.5 µM telomere adjacent primer, 0.5 µM teltail primer, 1.2mM dNTPs, and 1 U Taq/Pwo (Abgene or CRuk/Roche) at the unit ratio of 10:1 were carried out as follows: 20sec at 94^0C, 30sec at 65^0C (XpYpE2, XpYp-427G/-415C, XpYp-427A/-415T (Baird et al., 2003), 59^0C [17pseq1rev or 17p7; (Britt-Compton et al., 2006)], 61^0C (9p2), 67^0C (10q21T and 21qD), 63^0C (15qA) and 66^0C [11q13B; (Britt-Compton et al., 2006)] and 8 minutes at 68^0C for 22 (XpYpE2& 17pseq1rev) or 24 (9p2 & 11q13B) cycles.

2.7.2 Telomere Fusion Assay

Multiple 10 µl PCR reactions (typically 7-18 per sample) containing: 10-100ng genomic DNA, 1x Taq reaction buffer (ABgene), 2.0mM $MgCl_2$, 0.5 µM each of XpYpM, 17p6 and 16p1 or 21q1, 1.2mM dNTPs, and 0.5 U Taq/Pwo (ABgene or CRuk/Roche) at the unit ratio of 10:1 were carried out as follows: denaturation for 20 sec at 94^0C, annealing for 30sec at 59^0C (Britt-Compton et al., 2006) and extension for 8minutes at 68^0C for 25 cycles.

First-round PCR reactions containing putative fusion molecules were diluted 1:20 with double distilled water. Then 3μL of the diluted PCR reaction was used in a 30μL reaction containing 0.5 μM nested primers (XpYpO, 17p7, 21qseq1, 21q2CT, 16p2, 16pseq1, 16p1B, 21q1B, 17p6B, 17p6C, XpYpMB, XpYpMC), in the buffering system similar to above. The reactions were cycled as above but for 33cycles.

2.7.3 Telomere Variant Repeat-PCR (TVR-PCR)

Separate PCR reactions containing: 100ng genomic DNA, 1x Taq reaction buffer (ABgene), 3.0mM $MgCl_2$, 1.0 μM telomere adjacent primer (XpYpE2, 17pseq1rev, 9p2, 10q21T, 21qA), 1.0 μM TVR primers Tel-X, Tel-W, or Tel-Y, 1.2mM dNTPs, and 0.5 U Taq/Pwo (ABgene or Cancer Research UK/Roche) at the unit ratio of 10:1 were carried out as follows: 20 cycles of 20sec at 96^0C, 30sec at 67^0C and 3 minutes at 70^0C.

2.7.4 Probe synthesis

The 50μL PCR reactions containing 200ng genomic DNA (MRC5, MCF7 or HEK293), 1x Taq reaction buffer (ABgene), 3.mM $MgCl_2$, 1.0 μM primer, 1.2mM dNTPs, and 0.5 U Taq/Pwo (ABgene or CRuk/Roche) at the unit ratio of 10:1 were carried out as follows: 33 cycles of 96^0C for 20 sec, 67^0C for 30 sec and 3 minutes at 70^0C.

Telomere adjacent probes for detection of telomere fusion products were made by PCR amplifications with the following oligonucleotide primers: XpYpO & XpYpG for XpYp; 17p7&17p2/17pseq3 for 17p; 21q1/21qseq1&21qseq1rev for 21q group; and 16p2&16pseq1rev for 16p group. While telomere adjacent probes for detection of STELA products were generated by amplification with the following oligonucleotides: XpYpE2 & XpYpB2 for XpYp (Baird et al., 2003);

17pseq1rev &17pseq1 for 17p; and 11q13B&11q12 for 11q (Britt-Compton et al., 2006); 10q21T &subtel2(3) rev for 10q and 21qA & subtel2(3) rev for 21q.

2.8 GEL electrophoresis

Amplified products were resolved by submarine agarose gel electrophoresis using 1x tris acetate buffer (TAE), unless stated otherwise.

2.8.1 STELA, TVR-PCR and telomere fusion assay products

The amplified products (4-5µL) containing 1x Ficoll based loading buffer (5% bromophenol, 5% Xylene and 15% Ficoll) were loaded on the 40cm long gel alongside 0.5µl of each 1.0kb (Stratagene) and 2.5kb (Biorad) ladder. The amplified products were resolved by 0.5% (STELA products) or 0.75% (first-round telomere fusion products) Seakem Gold agarose (Cambrex) or multiabgarose (ABgene) at 120-140 volts for 14-18 hours (STELA & TVR-PCR products) or 50-65volts for 16 hours (telomere Fusion products): under the circulating, cooling system at 4^0C.

2.8.2 Gel electropheresis for other PCR products

The amplified probe products containing 1x ficoll-based loading dye were resolved on 0.8-1.0% electrophoresis grade agarose (Invitrogen) in 1x trisborate buffer (TBE; probes) for 1-2 hours at 120 volts or 1x TAE (nested-PCR products) overnight at 50 volts.

2.9 Southern blotting and Hybridisation

2.9.1 Alkaline Southern Transfer of DNA

The resolved products (STELA, TVR and telomere fusions) were depurinated by washing the gel in 0.25 M hydrochloric acid (HCl) for 2 x 6 minutes. Following depurination, the gel was denatured in 1.5M NaCl/0.5M NaOH buffer for 15

minutes. The DNA was then transferred to positively-charged membrane (Hybond XL; Amersham) by alkaline Southern blotting with denaturation buffer for 4-6 hours, after which the membrane was hybridised to the ^{33}P α dCTP-labelled probe.

2.9.2 Probe labelling and Southern hybridisations

25ng of the probe and ladder (1:1 of 1.0kb: 2.5kb) in TE buffer (Tris-HCl and 1mM EDTA) was labelled in rediprime II reaction (contains exonuclease-free Klenow fragment and buffered ddNTPs; Amersham) by the random oligo-labelling method (Feinberg and Vogelstein, 1984), that involved use of klenow fragment of DNA polymerase I to incorporate radioactive $^{\alpha}$dCTP into synthesised DNA for at least 30 minutes.

After Southern blotting, the membranes were rinsed in water and left to air-dry for 5-10 minutes. The membranes were then prehybridised in 15ml modified church buffer [0.5M, pH 7.2 sodium phosphate buffer (1M disodium hydrogen phosphate and 1M sodium dihydrogen phosphate), 1mM EDTA, 1% BSA and 7% SDS] at 60^0C for 30 minutes. The hybridisations were then carried out with same buffer at 60^0C.

2.9.3 Removal of unbound probes

The southern hybridisation washes to remove unbound probe were carried out with 0.1x sodium chloride sodium citrate (SSC)/0.1% Sodium Dodecyl Sulphate (SDS) at 60^0C as follows: three quick washes followed by two thirty minutes washes. The washed blots were dried in the hybridisation oven at 60^0C for 20 minutes. The dried blots were placed against the molecular dynamics phosphoimager screen (Amersham) for at least 24 hours, after which blots were then stripped with boiling 0.1% SDS prior to reprobing with the second probe.

2.9.4 Visualisation

The gel-resolved fragments were visualised by UV-transluminator (UVP Biodoc-IT™system) at 256nm and the picture taken with video graphic printer UP 895MD (Sony). The fragments were then cut with clean scalpel and gel extracted using Alquick gel extraction kit (Qiagen) according to manufacturer's instructions.

2.10 PCR product recovery and DNA sequencing

The PCR product to be sequenced was purified from the agarose gel slices using QIAquick gel extraction kit (Qiagen). The purified DNA was then resuspended in Qiagen's elution buffer (Qiagen). The sequencing reaction was set up with 4.4µl of recovered DNA, 0.16 µM sequencing primer, and 4.0µl BigDye® Terminator v3.1 Cycle Sequencing mix or dGTP BigDye® v1.0 Terminator Cycle Sequencing mix (Applied Biosystems) for G-rich sequence. The reactions were cycled for 25 cycles as follows: 96^0C for 30 seconds, 50^0C for 15 seconds and 60^0C for 4 minutes.

The amplified products were purified by Dye Ex™ 2.0 spin kit (Qiagen) according to manufacturer's instructions prior to direct sequence analysis. The sequencing gels were run and data obtained by staff in Central Biotechnology Service (CBS), School of Medicine, Cardiff University.

The sequences were analysed and edited in sequence scanner v1.0 (Applied biosystems). The edited sequences were then blasted in NCBI blastN and reference sequence(s) with the highest match (99-100%) was (were) considered significant.

2.11 Computer and Statistical analysis

The STELA and telomere fusions image signal was detected by phosphor-autoradiography using Molecular Dynamics Storm 860 Scanner (GE health). The images were then analysed in Molecular dynamics Image Quant 5.0 (GE Health).

The molecular weight of each fragment on the STELA gel was determined using Phoretix 1D quantifier in TotalLab TL120 v 2006 (Nonlinear dynamics) such that each fragment represented a single telomere molecule. Fragment size data was exported to excel, and the actual telomere length represented by each fragment was determined by subtracting the distance between the primer site on telomere-adjacent DNA and the start of the telomere repeat arrays.

The mean telomere length and accompanying descriptive statistics which included standard deviation (measure of how widely the values are dispersed), standard error of the mean and 95% confidence intervals within which the mean can be obtained, were calculated from telomere length estimated for all fragments in all experiments for each sample (4-8 reactions, repeated until representative molecules was obtained) in Microsoft Excel 2003/2007 (Microsoft Corporation), SPSS14.0 and PRISM TRIAL 5.0 (Graphpad). The mode, the highest frequency of measurements (lots of deletions or telomere length) was estimated from the histogram for telomere length or calculated by counting the deletion values or number of TTAGGG repeats or number of micro-homology nucleotides. The histograms were drawn after binning values against telomere length in 0.1kb intervals from 0 to 20kb. The telomere fusion frequency was estimated as described in box 2.1 in the appendix.

The D'Agostino-Pearson omnibus normality test was undertaken to determine if the distribution of telomere length follow Gaussian distribution. For those that passed the normality test the parametric ANOVA with Tukey-Kramer Multiple Comparisons Test and Student t-test were used. Whilst for those that failed the normality test, the Kruskal-Wallis Test (Nonparametric ANOVA) with Dunn's comparison test was used to determine if the differences in telomere distributions were due to chance. The Bartlett statistic was used to test if the standard deviations were significantly different. The chi-square test was used to determine if any bias in the GC/AT micro-homology content was due to chance. Further statistical analysis for each dataset is described in relevant chapters.

3 Chapter 3: Technological improvement of telomere fusion analysis and Single Telomere Length Analysis [STELA]

3.0 Summary

The aim of the work described in this chapter was to extend telomere fusion assay by encompassing more chromosome ends, with the expectation that this would improve the sensitivity of the assays as well as the scope of the events that could be detected. An additional aim was to develop STELA at additional human telomeres.

The telomere fusion assay was originally developed to detect fusion events between the telomeres of XpYp and 17p (Capper et al. 2007); and has revealed that, unlike deprotected telomeres in the absence of TRF2, the fusion of short dysfunctional telomeres is accompanied by a distinct mutational spectrum. However, this assay could not be used across multiple chromosome ends and was thus limiting. Improving the assay will determine whether the events observed between telomeres of XpYp and 17p are a reflection of the mutational spectrum underlying fusion of short telomeres. This would also allow further study into the mechanistic basis of fusion events between dysfunctional telomeres. Importantly the increased sensitivity of the fusion may allow the detection of rare telomere fusion events in normal and diseased tissues. Using the sub-telomeric repeat sequence families shared amongst multiple chromosomes, the telomere fusion assay was extended to cover at least 43% of telomeres in the human genome.

STELA determines the length at specific telomeres, extending the STELA assay to additional chromosome ends could facilitate an examination of the role that specific telomeres may play in driving genomic instability during the progression to malignancy. STELA was developed successfully at 9p.

3.1 Introduction

3.1.1 Human sub-telomeric DNA

The human sub-telomeres are hyper-variable, dynamic and rearrangement-prone regions near the chromosome ends. They are hotspots for DNA breaks and repair (Mefford and Trask 2002; Riethman et al. 2003; Linardopoulou et al. 2005; Riethman et al. 2005) and consist of repetitive sequences, large inter-chromosomal segmental duplications and segments that vary in size and copy number. The sub-telomeric DNA accounts for over 40% of inter-chromosomal duplication since the divergent evolution of human from great apes (Linardopoulou et al. 2005). Furthermore, higher rates of recombination have been reported within the sub-telomeric DNA. For example, 17% of all sister chromatid exchanges (SCE) occurs in the terminal 0.1% of the chromosome (Rudd et al. 2007). Such sub-telomeric dynamics are important as they can disrupt the gene function through amplifications and deletions. Indeed, the sub-telomeric regions contain multiple gene families with varying copy number and chromosome distribution. These gene families include, amongst others, the cytokine receptors, zinc finger homeo-domain proteins, secretoglobins, immunoglobulin heavy chain and olfactory receptor genes (Trask et al. 1998a; Trask et al. 1998b; Linardopoulou et al. 2005; Riethman et al. 2005).

The TTAGGG-adjacent sequence is related to the families of telomere-associated repeat (TAR) sequences defined by the half-YAC clones originally described by Brown and colleagues (Brown et al. 1990). The sequences of the TAR vary among different chromosomes, allowing PCR-based techniques such as allele-specific STELA and telomere fusion assay at different chromosomes (Riethman et al. 2003; Riethman et al. 2004; Riethman et al. 2005).

The discovery that human telomeres could be cloned by complementation in yeast artificial chromosomes (YACs) enabled the molecular characterisation of sub-telomeric repetitive regions (Brown 1989; Brown et al. 1990). Using the YACs containing different restriction fragments, Brown and colleagues isolated

four telomere-associated sequences of which two mapped to single, different telomeres while the other two (Telbam 3.4 and Telbam11) hybridised to multiple telomeres (Brown 1989; Brown et al. 1990). In order to expand the telomere fusion assay, I took advantage of the sub-telomeric repeat sequences to design primers that will amplify multiple chromosome ends simultaneously. Two families (Telbam 3.4 and Telbam11) of related telomeres, which share between 90 to 99% sequence homology were identified (Riethman et al. 2003; Riethman et al. 2005; Riethman 2008). Based on this variation, I designed primers for the telomere fusion assay to target Telbam 3.4 and Telbam11 families of telomeres which I renamed 16p and 21q families respectively.

3.1.1.1 16p family

The members of this family share the sub-telomeric repeat block that hybridise to TelBam3.4 cosmid (Brown et al. 1990; Riethman et al. 2005), these include 16p, XqYq, 9p, 1p, 12p, 15q and the interstitial 2q13-14 (Brown et al. 1990; Riethman et al. 2004). They carry the most terminally located class of newly discovered subclass of the Wiskott-Aldrich syndrome Protein (Linardopoulou et al. 2005; Linardopoulou et al. 2007) gene family, called Wiskott-Aldrich Syndrome Protein and SCAR homologues (previously named MGC52000, WASH). The truncated copy of the WASH gene (MGC52000) was first annotated on pseudo-autosomal region of XqYqtel (Ciccodicola et al. 2000). These genes are conserved from *Entamoeba* to human (Linardopoulou et al. 2007) and terminate within less than 5kb of the start of telomere tracts (Riethman 2008). Like their sister subclasses, Wiskott-Aldrich syndrome Protein (WASP) and suppressor of c-AMP receptors (SCAR), WASH genes recognise and co-localise with actin at the cell periphery and promote actin polymerisation *in vitro.*

Several members of the 16p family have been implicated in human diseases especially cancer. Thus in the next sections, the telomere-related chromosomal abnormalities involving some members of the family in different human cancers and/or disease will be discussed.

3.1.1.1.1 Short arm of chromosome 1

The loss of heterozygosity (LOH) at short arm of chromosome 1 (1p) has been suggested to be one of the adverse prognostic features in Wilm's tumour (Grundy et al. 1994) and endometrial cancer (Arlt et al. 1996). Furthermore, frequent deletion of 1p is an early step in the progressive loss of chromosomal material in histological atypical and malignant meningiomas (Sawyer et al. 2000). These losses are probably a consequence of defective or impaired telomere function as 1p has been found to be involved in recurring telomere fusions and forms dicentric chromosomes with 11p, 19q and 22q (Sawyer et al. 2003).

3.1.1.1.2 Short arm of chromosome 9

The locus, 9p21 carries two very important cell control cycle genes INK4b (p15/CDKN2B) and INK4a/ARF (p16/CDKN2A and p14/ARF). CDKN2A is part of the G_1-S cell cycle checkpoint mechanism that involves the retinoblastoma-susceptibility tumour suppressor protein [pRb; (Weinberg 1995)]. Germ-line mutations at the INK4a/ARF predisposes to familial melanoma, a very deadly form of skin cancer (Hussussian et al. 1994; Hewitt et al. 2002). Whilst, frequent LOH at this locus is documented in most human cancers including oesophageal squamous carcinoma (Hu et al. 2006), lung cancers (Merlo et al. 1994) and melanoma, among others (Coleman et al. 1994). Moreover, expansion of clones with LOH at 9p has been implicated as one of the early events in Barrett's oesophagus, a premalignant condition known to predispose to oesophageal adenocarcinoma (Galipeau et al. 1999; Jenkins et al. 2002).

3.1.1.1.3 Long arm of chromosome 15

Losses of 15q have been reported in oesophageal squamous carcinoma and exocrine pancreatic cancers (Hu et al. 2006). Additionally, Zheng and colleagues have reported a significant association between telomere erosion in lymphocytes from oesophageal squamous cell carcinoma patients and chromosome instability at 15q (Zheng et al. 2009). It is however not yet known if there are any tumour

suppressor genes located on 15q. Interestingly all cases of mental/growth retardation arising from the terminal deletions of insulin-like growth factor receptor (IGF1R) gene (15q26.3) also involve the 15q26.2→qter deletion (Walenkamp et al. 2008).

3.1.1.2 21q family

The members of this family of telomeres constitute a subset of the chromosomes that share the sub-telomeric repeat region that hybridised to the TelBam 11 cosmid. The TelBam 11 class is a low-copy repeat sequence that is found in 10-20 telomeres and 2q12-14 interstitial loci; which share 90-98% nucleotide identity (Brown et al. 1990; Riethman et al. 2004; Riethman et al. 2005). Of these TelBam 11 members, only those chromosomes which further have the region that hybridise to HC_1208 probe were targeted (Brown et al. 1990; Riethman et al. 2004). These include 1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q, 22q and interstitial 2q13.

Below I give examples of the different events involving some members of 21q family in different diseases. For example, several chromosomal losses at members of 21q family such as 13q, 21q, 6q, and 17p have been reported in exocrine pancreatic cancers with high frequency LOH in oesophageal squamous carcinoma documented at 3p, 4p, 4q, **5q**, 9q, **13q** (Hu et al. 2006).

3.1.1.2.1 Long arm of chromosome 1

One of the chromosome abnormalities that involves telomeres is the jumping translocation (Hatakeyama et al. 1998). The jumping translocation is a very rare chromosome abnormality in which the segment of the specific chromosome translocates to various chromosomes. One of the most common jumping translocations is that involving the distal region of the long arm of chromosome 1 (1q), which has been described in acute lymphoblastic leukaemia (Pelz et al. 2005). Furthermore, fusion events involving 1q21-ter and the telomeres of a variety of chromosomes (3q, 4p, 5q, 7p, 8q, 9q, 13q, 21q, 7p and 22q) have been

described in acute myelomonocytic leukaemia (Hatakeyama et al. 1998). Frequent gains of chromosome 1q is a typical feature in many solid tumours including breast carcinoma (Tsukamoto et al. 1999), cervical cancers (Kloth et al. 2007) and endometrial cancers among others (Suzuki et al. 1997). Interestingly, in a subset of breast tumours (25%), lack of telomerase is correlated with amplification of the 1q31 loci, suggesting the presence of putative telomerase repressors in the region (Loveday et al. 1999). Thus chromosome instability at 1q extending internally could result in telomerase misregulation and hence telomere instability at other telomeres.

3.1.1.2.2 Long arm of chromosome 5

The chromosome arm 5q carries the APC/MCC gene (5q21), that is often mutated or deleted in colorectal and lung cancers (Ashton-Rickardt et al. 1991). The germ-line mutations in APC gene result in familial adenomatous polyposis, a dominantly inherited colorectal tumour predisposition syndrome (Konishi et al. 1996). The site 5q34-qter is one of the most commonly deleted in testicular germ-cell cancer (Peng et al. 1999).

3.1.1.2.3 Short arm of chromosome 8

The chromosomal changes on the short arm of chromosome 8 (8p) are associated with different malignancies. For example, chromosomal deletion of 8p is a prognosis marker in papillary bladder carcinoma and 8p deletions are strongly correlated with invasive tumour growth and papillary growth pattern in patients with invasive bladder cancer (Stoehr et al. 2004; Stoehr et al. 2005). In addition, dicentric chromosomes with 8p12-11q13 fusion (8qter>8pter::11q13>11qter), a potential consequence of telomere dysfunction, has been reported in a subset (5-7%) of breast cancer (Bautista and Theillet 1998; Birnbaum et al. 2003).

3.1.1.2.4 Long arm of chromosome 10

The long arm of chromosome 10 carries a tumour suppressor gene that encodes for a phosphatase PTEN/MMAC1 (10q23); a protein of which its deletion in mice results in cancer-prone phenotypes (Di Cristofano et al. 1998). PTEN is often mutated or deleted in several cancers including acute leukaemia, endometrial, brain, breast and prostate cancer (Li et al. 1997; Tashiro et al. 1997; Dahia et al. 1999). It is a cell cycle protein that is involved in modulating G_1 cell cycle progression through interaction with the cyclin-dependent kinase inhibitor p27(KIP1) thus negatively regulating the PI3K/Akt signaling pathway (Li and Sun 1998). Interestingly, studies with p53/ATM/telomerase triple null mice with dysfunctional telomeres revealed that murine lymphomas possessed complex abnormalities similar to those of human carcinoma such as deletion of the PTEN locus (Deng and Chang 2007). The authors suggested that telomere dysfunction may impact upon pathways that would allow stepwise carcinogenesis (Deng and Chang 2007). In fact, inactivation of PTEN in mice activates a p53-dependent growth arrest and promotes development of prostate cancers (Chen et al. 2005).

3.1.1.2.5 Long arm of chromosome 13

The long arm of the largest acrocentric chromosome 13 (13q), carries the genes involved in cancer and other human diseases; breast cancer type 2 (BRCA2) and tumour suppressor gene retinoblastoma (RB1) genes (Dunham et al. 2004). In patients with a history of familial upper gastrointestinal cancer, the predominant LOH is at 13q, with the gene that may predispose to upper gastrointestinal cancers being mapped to 13q14 (Champaigne et al. 2009). It is however not known whether such locus is same as the pRb gene (13q13). In addition, telomere length in carcinoma-associated fibroblasts and lymphocytes from oesophageal squamous cell carcinoma is associated with increased chromosome instability on 4q and 13q (Zheng et al. 2009). Moreover, LOH at 13q is frequent in human prostrate carcinomas (Cooney et al. 1996; Melamed et al. 1997) and lymphoid malignancies, with different loci often deleted or re-amplified. For example, homozygous deletion of the 13q14.3 is found in 10-44% of B-cell

chronic lymphocytic leukaemia, 25% of non-Hodgkin's lymphoma and 28% of acute lymphocytic leukaemia [ALL; (Liu et al. 1995; Dunham et al. 2004)].

3.1.1.2.6 Long arm of acrocentric chromosome 21

The loss of heterozygosity (LOH) on the long arm of chromosome 21 (21q) is observed in several human malignancies such as squamous cell carcinomas. LOH is often associated with presence of tumour suppressor genes. The chromosome arm 21q, carries a putative tumour suppressor gene, ANA gene which is associated with oral squamous cell carcinomas [OSCCs; (Yamamoto et al. 2001)]. In children, acute lymphoblastic leukaemia is associated with intra-chromosomal amplifications of 21q and deletions of 21q telomere have been reported in a subset of individuals (Kuchinskaya et al. 2007). Other studies have shown that the dinucleotide repeat, which is interspersed by a LINE very close to the telomere of 21q, renders the terminal very recombinogenic, with higher recombination rates observed in males than females (Blouin et al. 1995; Lynn et al. 2000).

Recently, it has been shown that the overall telomere length from peripheral blood of older mothers is linked to types of chromosome 21 non-disjunction and subsequent Down syndrome births at an advanced maternal age (Ghosh et al. 2010). This finding is in contrast to the notion that telomere length is paternally inherited (Nordfjall et al. 2005; Njajou et al. 2007).

3.1.1.2.7 Long arm of the acrocentric chromosome 22

Unbalanced translocations such as 46, XX, der(21)t(21;22) (p13;q13.2), 46, XY, der(21)t(21;22)(p10;q13.3) resulting in 22qter duplications have been associated with birth defects (Feenstra et al. 2006). Additionally, a complex unbalanced chromosomal event arising from the transposition of the distal part of 6p and 20q to 22q telomere have been reported in children with birth defects. It is however not yet known if such event is a result of telomere dysfunction (Bonaglia et al. 2003; Bonaglia et al. 2008). Telomeric deletions of 22q on the other hand are

associated with hypotonia, developmental delay, and absence of speech in affected infants (Precht et al. 1998) and ovarian endometriomas [22q12.3-qter; (Gogusev et al. 1999)].

3.1.1.3 Chromosome 17

Telomeres on both arms of the chromosome 17 have been implicated as some of the short telomeres in human cells (Martens et al. 1998; Perner et al. 2003; Zou et al. 2004). The short arm of chromosome 17 is often lost in many cancers. It carries a gene (at 17p13) that encodes for the tumour suppressor p53; which plays a crucial role in DNA damage response and apoptosis (Lane 1992; Levine 1997). The region 17p13.3-ter (distal to p53) is often deleted in sporadic breast cancer (Liscia et al. 1999; Johnson et al. 2002). Furthermore, LOH at 17p is believed to convey growth advantage and hence clonal expansion in Barrett's oesophagus (FitzGerald et al. 1996; Galipeau et al. 1999).

Rashid-Kolvear and colleagues have reported a greater erosion rate at 17q telomere relative to other telomeres (Rashid-Kolvear et al. 2007). Interestingly, 17q carries BRCA1, a breast cancer gene which when mutated pose the life time risk of familial breast and ovarian cancers in women carriers (Simard et al. 1994). In oesophageal squamous carcinoma, LOH at both arms of chromosome 17 is a frequent event (Hu et al. 2006). Interestingly the loss of 17q23-qter is frequent in carcinomas of the kidney, ovary, head and neck, and testicular germ cell (Mertens et al. 1997).

3.2 This study

As indicated in the previous sections, human chromosomal deletions or amplifications are frequent in human cancers. Although some are intra-chromosomal deletion/amplifications, it has been suggested that they might be a result of breakage-fusion-breakage cycles; a consequence of telomere dysfunction (Sabatier et al. 2005). Indeed, Kuchinskaya and colleagues have shown that frequent 21q amplification associated with acute lymphoblastic

leukaemia in children are linked to 21q telomere deletion in some individuals (Kuchinskaya et al. 2007).

The aim of the work described in this chapter was to improve the telomere fusion assay and expand the telomere length analysis (STELA) to include additional telomeres. Telomere fusion assay is a PCR-based assay that allows the detection of the telomere fusion molecules between specific chromosomes. It was originally developed for two chromosomes, XpYp and 17p. The sub-telomeric sequence of these chromosomes is sufficiently unique to allow telomere-specific STELA and telomere fusion assays (Capper et al. 2007; Letsolo et al. 2009). However, analysis of two telomeres was limiting as it required the analysis of several million cells to detect rare fusion events in normal cells, thus increasing the scope may improve sensitivity. The improved assay will also allow the detection of broader range fusions and indicate whether the type of fusions observed between XpYp and 17p was a reflection of the chromosome instability generated by eroded telomeres. This may provide further mechanistic insights into the fusion of short dysfunctional telomeres. Furthermore, the extended assay will also be important when determining the telomere fusion events in human tissues derived from normal and disease contexts (Capper et al. 2007; Letsolo et al. 2009).

STELA has several key strengths over other telomere length analysis assays; with STELA, the full spectrum telomere lengths ranging from short to longer than 20kb can be detected (Baird et al. 2006). In addition, STELA requires very low concentrations of DNA thus can be applicable to a broad spectrum of samples (Baird et al. 2003; Baird et al. 2006; Britt-Compton et al. 2006). STELA was originally developed for analysis of XpYp telomere (Baird et al. 2003) and then later for analysis of autosomal telomeres; 2p, 11q 12q, and 17p (Britt-Compton et al. 2006) and *C.elegans* (Cheung et al. 2004). Recently an adaptation of the STELA technique called "universal STELA" has been described (Bendix et al. 2010). Unlike chromosome-specific STELA, this "universal" method

estimates the telomere length from almost all chromosomes (Bendix et al. 2010). However, it has a disadvantage compared to chromosome-specific STELA of low amplification efficiency for molecules with telomere length greater than 7kb, presence of unknown length of subtelomeric DNA and non-linear correlation with TRFs. Thus for this study, only chromosome-specific STELA which can detect the full spectrum of human telomere length range (0-25kb) was considered (Baird et al. 2003; Baird et al. 2006; Britt-Compton et al. 2006). Since it is the shortest telomere not the average length of all telomeres that triggers senescence and fusion (Baird et al. 2003; Baird et al. 2006; Britt-Compton et al. 2006), developing STELA at additional telomeres, especially those of chromosomes carrying the tumour suppressor genes, will allow the examination of whether such ends have potential to drive genomic instability in human disease.

Therefore, extending the assays to include additional ends will enable further understanding of the role of telomere erosion, instability and fusion in human cells and tissues derived from normal and disease context.

3.3 Sample preparation, Data manipulation and Statistical analysis

HEK293, MCF7 and MRC5 cells were cultured until confluent (Chapter 2) and DNA obtained by standard methods (Sambrook et al. 1989). HEK293 is the human embryonic kidney cell line that has been transformed by Adenovirus type 5 which its oncoproteins E1A and E1B inactivate pRb and p53 respectively (Graham et al. 1977; Yew and Berk 1992; Bischoff et al. 1996). MCF7 is a cell line that was first isolated in 1970 from the breast tissue of a 69-year old Caucasian woman (Soule et al. 1973). Both HEK293 and MCF7 cells exhibit telomerase activity (Bryan et al. 1998) while MRC5, a human fibroblast derived from normal lung tissue of a 14-week-old male foetus, is telomerase-negative (Jacobs et al. 1970).

The telomere lengths were estimated by fragment analysis in TotalLab from STELA blots of all experiments for each sample (6-8 reactions). The mean telomere length and accompanying statistics [including the arithmetic mean, the mode (highest frequency at which a specific variable occurs), standard deviation (extend of the dispersion of values from the mean), median (middle value when data points are arrange in other from smallest to largest) and confidence intervals (interval range within which the mean value can be found)] were calculated in Microsoft excel 2007. To accurately determine the telomere length, the distance between the telomere-adjacent primer and the start of the telomere repeat array was deducted from estimated lengths; XpYp [406bp; (Baird et al. 2003)]; allele-specific XpYp [433bp; (Baird et al. 2003)]; 17p [311bp; (Britt-Compton and Baird 2006)] and 9p (0bp).

The telomere fusion frequency were estimated from the number of amplifiable input molecules based on the concentration of single diploid human DNA molecule (6 picograms) relative to the amplified molecules (box 2.1; in chapter 2).

For pair-wise comparison, it was essential to determine whether the data was normally distributed, thus the D'Agostino-Pearson omnibus test (Graphpad 5.0) was carried out to acertain this. The unpaired two-tailed student's t-test and the Bartlett's F-test determined the statistical significance of the difference in the telomere length means and variances respectively. While the Pearson's chi-square test (Microsoft Excel 2007) was used to investigate the significance of the deviation from the expected data. For all tests, the differences were considered significant enough (i.e. not due to chance) at $P<0.05$, while $P<0.001$ was considered very significantly different.

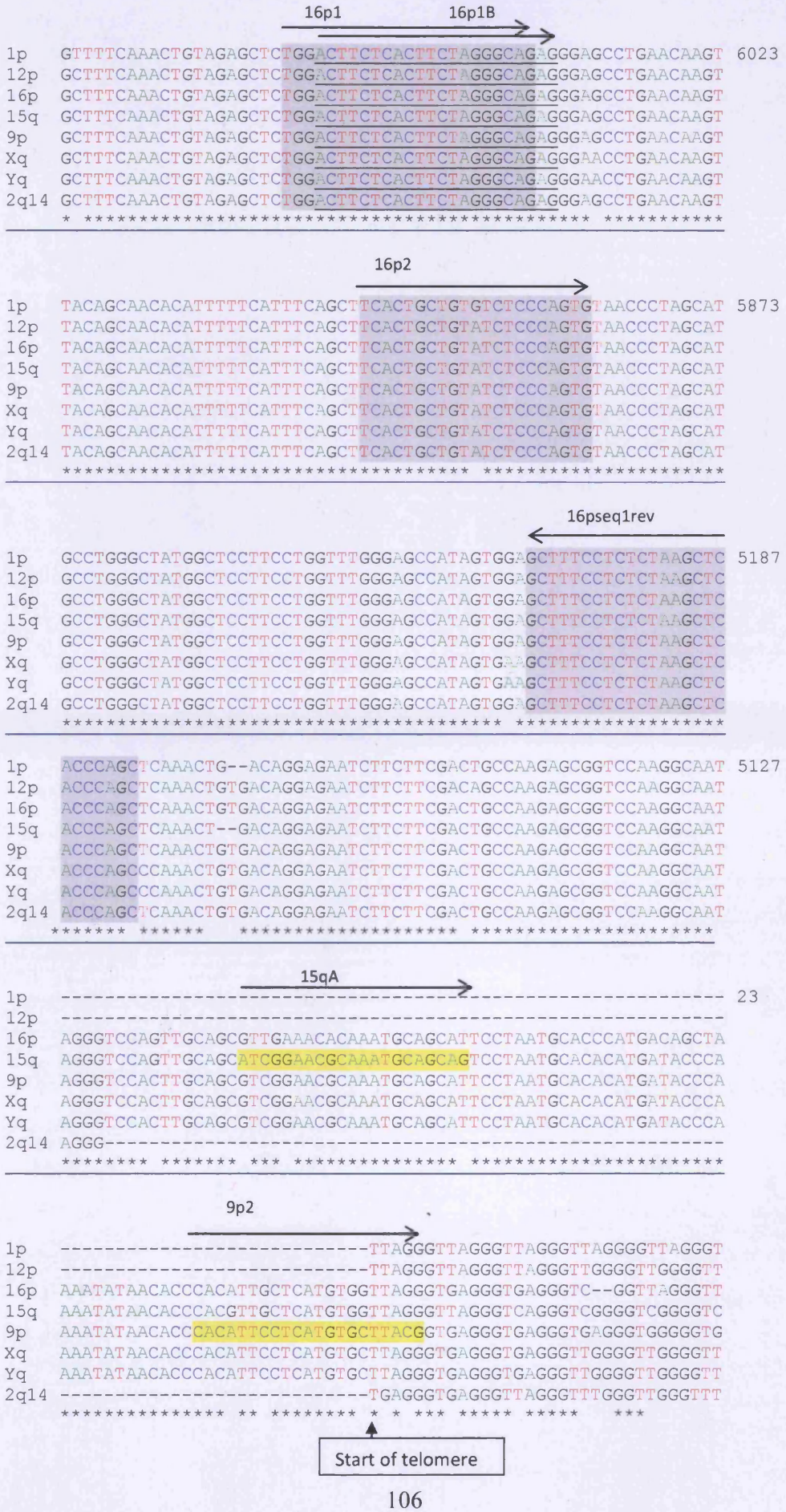
3.4 Results

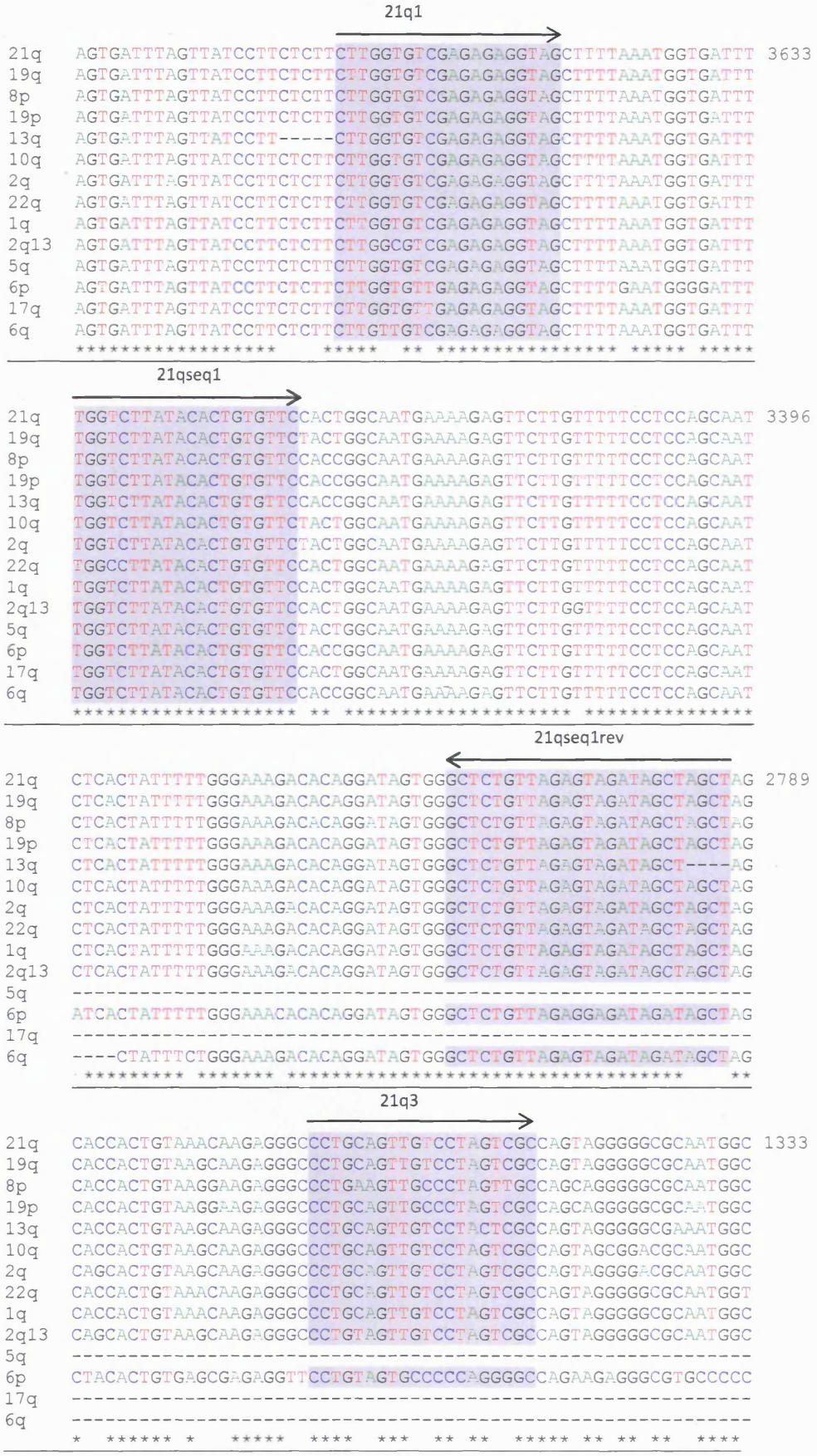
3.4.1 Development of STELA at additional chromosome ends

STELA was previously developed at XpYp and four autosomal telomeres 2p, 17p, 11q and 12p (Baird et al. 2003; Britt-Compton et al. 2006). The sub-telomeric sequence of these chromosome arms is sufficiently unique to enable telomere-specific STELA. Given the repetitive nature of sub-telomere sequence and the fact that similar sequences are shared amongst multiple chromosome ends it was difficult to obtain telomere-adjacent sequence that was sufficiently unique to allow chromosome specific amplification (Brown et al. 1990; Riethman et al. 2005). However, utilising the single-base variation between different ends, the sequence specific primers could be designed based on the putative sequences of 21q, 10q, 9p and 15q [Figure 3.1A and 3.1B; (Riethman et al. 2004)]. The 21q and 10q represented the telomeres of the 21q family, a sub-telomeric repeat family that share 90-98% homology. The telomere-adjacent DNA of 21q and 10q contains less than 600bp between the start of the telomere repeat array and the GC-rich dinucleotide repeat interspersed by a LINE (Blouin et al. 1995; Lynn et al. 2000). This satellite is refractory to PCR thus primer design was limited to the <600bp region [Appendix, Figure 3A-1].

The 16p family is represented by 9p and 15q, which share 97-99% sequence homology (Riethman et al. 2003; Riethman et al. 2004). The telomere adjacent DNA of 15q share 99.2% sequence homology with 1p, thus primers for 15q were designed utilising the single-base variation that distinguish 15q sequence from 1p and other members of the 16p family. Similarly, primers for 9p were designed utilising the single-base variation that distinguish 9p from other members of 16p family. However, the primer design at chromosome 9p was limited to the <300bp region between the GC-rich minisatellites (37bp-29bp-61bp; Appendix, Figure 3A-2) and the start of the telomere repeat array (Brown et al. 1990). The STELA was carried out as outlined in (Baird et al. 2003) using HEK293 DNA with modifications outlined in chapter 2. The specificity of the primers was determined by utilising clonal MRC5 in STELA experiments, it was not possible

Figure 3.1A. Sequence alignments for telomeres of the 16p family, 1p, 9p, 12p, 15q, 16p, XqYq, and interstitial 2q14. The regions of homology are indicated by *, gaps indicate mismatch. The hyphen (-) has been inserted to improve the alignment. The oligonucleotide primers used for telomere fusion assay and STELA are highlighted in grey and yellow respectively, with the arrows indicating the 5′-3′ orientation of the primers relative to the telomere. The telomere fusion oligo primers were designed on the putative sequence of 16p, thus the distances (nucleotide) from the repeat array of 16p are indicated on the right of each alignment. The 16p1 was used for telomere fusion PCR, while 16p1B or 16p2 was used for making the probe with 16pseq1rev and for nested-PCR during re-amplification of the putative fusion molecules. 15qA and 9p2 primer were used for 15q and 9p STELAs respectively.


16p1
16p1B
16p2
16pseq1rev
15qA
9p2
Start of telomere


21q1
21qseq1
21qseq1rev
21q3

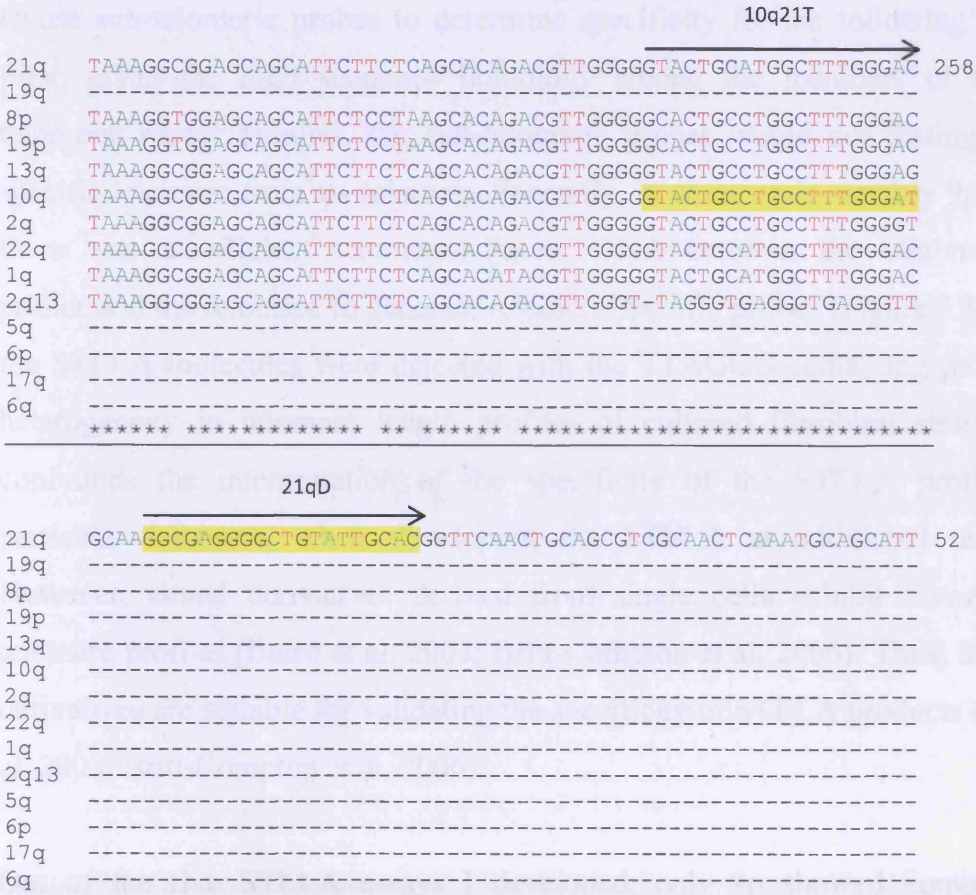


Figure 3.1B. Sequence Alignment from telomeres of the 21q family, 1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q, 22q and interstitial 2q13. The regions of homology are indicated by *, gaps indicate mismatch. The hyphen (-) has been inserted to improve the alignment. The oligonucleotide primers used for telomere fusion assay and STELA are highlighted in grey and yellow respectively, with the arrows indicating the 5′-3′ orientation of the primers relative to the telomere. The 21q1 was used for telomere fusion PCR, while 21qseq1 was used for making the probe with 21qseq1rev and for nested-PCR in reamplification of the putative fusion events involving members of the 21q family. 21q3 is upstream of the satellite DNA, which may be refractory to PCR. Downstream of the satellites lies the 10q21T and 21qD primer used for 10q and 21q STELAs respectively. The numbers indicated on the right side of each alignment are the distances (nucleotide) from the start of the telomere repeat array of 21q. The perfect alignment is shown by * and gaps indicate mismatch.

to use sub-telomeric probes to determine specificity for the following reasons: First, given the high sequence homology among the members of the sub-telomeric repeat families, the sub-telomeric probes would not distinguish the specific telomere from its relatives. Secondly, in some ends, notably 9p (16bp), there was insufficient telomere-adjacent DNA between the oligonucleotide primer and the telomere to generate telomere-specific probes (Figure 3.1A); thus the STELA molecules were detected with the TTAGGG-containing probe. The heterogeneity in telomere length profiles of cultured fibroblast strains often confounds the interpretation of the specificity of the STELA profile of a particular telomere, when developing the STELA at additional telomeres. However, clonal derivatives derived from single cells exhibit homogeneous telomere profiles (Baird et al. 2003; Britt-Compton et al. 2006). Thus, the clonal derivatives are suitable for validating the specificity of STELA products (Baird et al. 2003; Britt-Compton et al. 2006).

Out of the five STELA assays I developed, only 9p showed homogeneous distributions that are typical of those observed in clonal populations (Baird et al. 2003; Britt-Compton et al. 2006); this suggested that the assay was specific for the telomere being investigated. However, due to the sequence constrains in primer positioning at 9p (Figure 3.1A), STELA amplification at this telomere was of lower efficiency relative to that of telomeres of XpYp and 17p. Interestingly 9p could not be amplified in MCF7 (data not shown), an observation consistent with the presence of the polymorphism in the primer. The existence of null alleles has been reported with other successful STELAs; notably MRC5 contains one amplifiable allele at 17p and two at other ends, whilst the 11q telomere could not be amplified in IMR90 (Britt-Compton et al. 2006).

The STELA reactions for telomeres of 21q (Appendix, Figure 3A-3), 10q and 15q readily amplified telomere molecules that could be detected by telomere repeat containing probes (Figure 3.2). These distributions showed clear evidence of telomere erosion with ongoing cell division and, as observed previously at

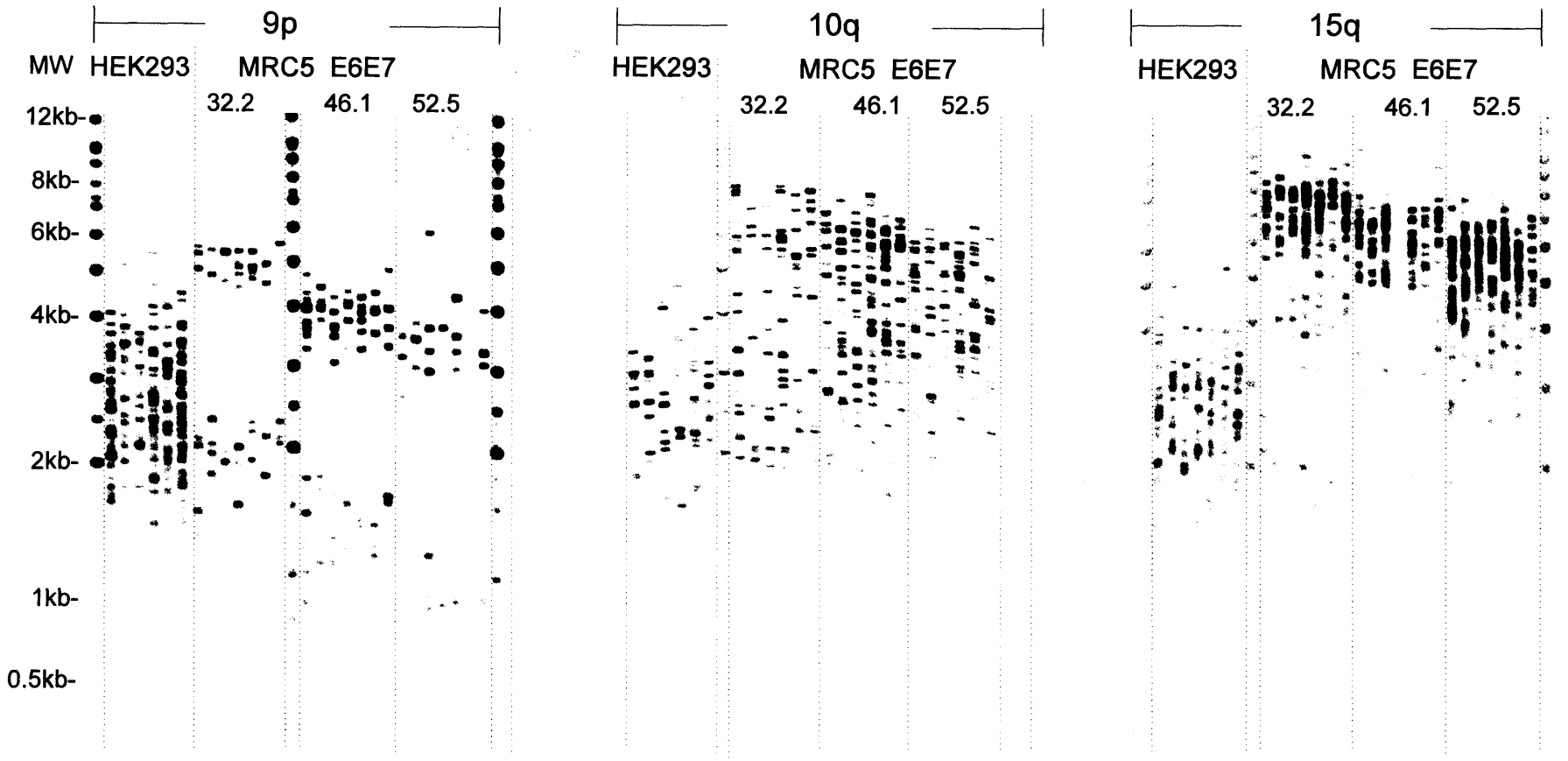


Figure 3.2. Single telomere length analysis (STELA) for the telomeres of 9p, 10q and 15q in HEK293 and MRC5 expressing E6E7 oncoproteins. Multiple (typically 6 per sample) long-range single-molecule PCR reactions were carried out with oligonuclotide primers targeting telomeres of 9p, 10q and 15q. The bands were detected by Southern hybridisation with TTAGGG-containing probe. The number of population doublings after which the MRC5 cells were harvested is indicated above MRC5 gels. Molecular weight marker (MW) is shown.

XpYp, the 10q assay had evidence of the complete loss of a telomere length distribution as the cells approach crisis (Figure 3.2). Moreover, the characteristic short telomere length profile of HEK293 cells that was apparent in all the assays has been identified at XpYp and 17p (Capper et al. 2007) as well as in the new 9p assay (Figure 3.2; Figure 3.3). However, the telomere length distributions in MRC5 clones were more heterogeneous than observed from DNA derived from the clonal cell populations. These distributions appear to be composed of three to four distributions. This was indicative of more than one telomere being amplified simultaneously. Thus whilst these assays do provide an indication of telomere length, at the chromosome ends of interest, the fact that they might be detecting more than one end lessened the utility of these assays. Thus, they were not used for subsequent analysis (Figure 3.2: Figure 3.3).

3.4.1.1 Telomere length in HEK293

Telomere length in HEK293 was estimated for XpYp, 17p and 9p telomeres. Previous analysis of XpYp has revealed inter-allelic telomere length variation, which was particularly apparent in analysis of clonal populations; this variation is established in the zygote and maintained throughout development (Baird et al. 2003). However, the telomere length profile of HEK293 did not suggest the existence of allelic-variation, thus it was essential to determine whether the population was homogeneous for any of the telomere-adjacent XpYp alleles. Direct sequence analysis of the XpYp telomere-adjacent DNA of HEK293 cells (Figure 3.3A) and allele-specific STELA analysis revealed two XpYp variant alleles (-427G/-415C or GC allele and -427A/-415T or AT allele). The two alleles exhibited identical telomere length distributions (t-test, P=0.3052; F-test, P=0.3850; Figure 3.3B and 3.3C) which are super-imposed on one another in a mixed HEK293 cell population. Interestingly, the AT-allele was under-represented with the population consisting of 75% GC and 25% AT allele not the expected 50:50 ratio (chi-square, P<0.000013; Figure 3.3B). To rule out the possibility of the lower amplification efficiency with AT allele-specific STELA, direct sequence analysis of the telomere-adjacent DNA of XpYp revealed the

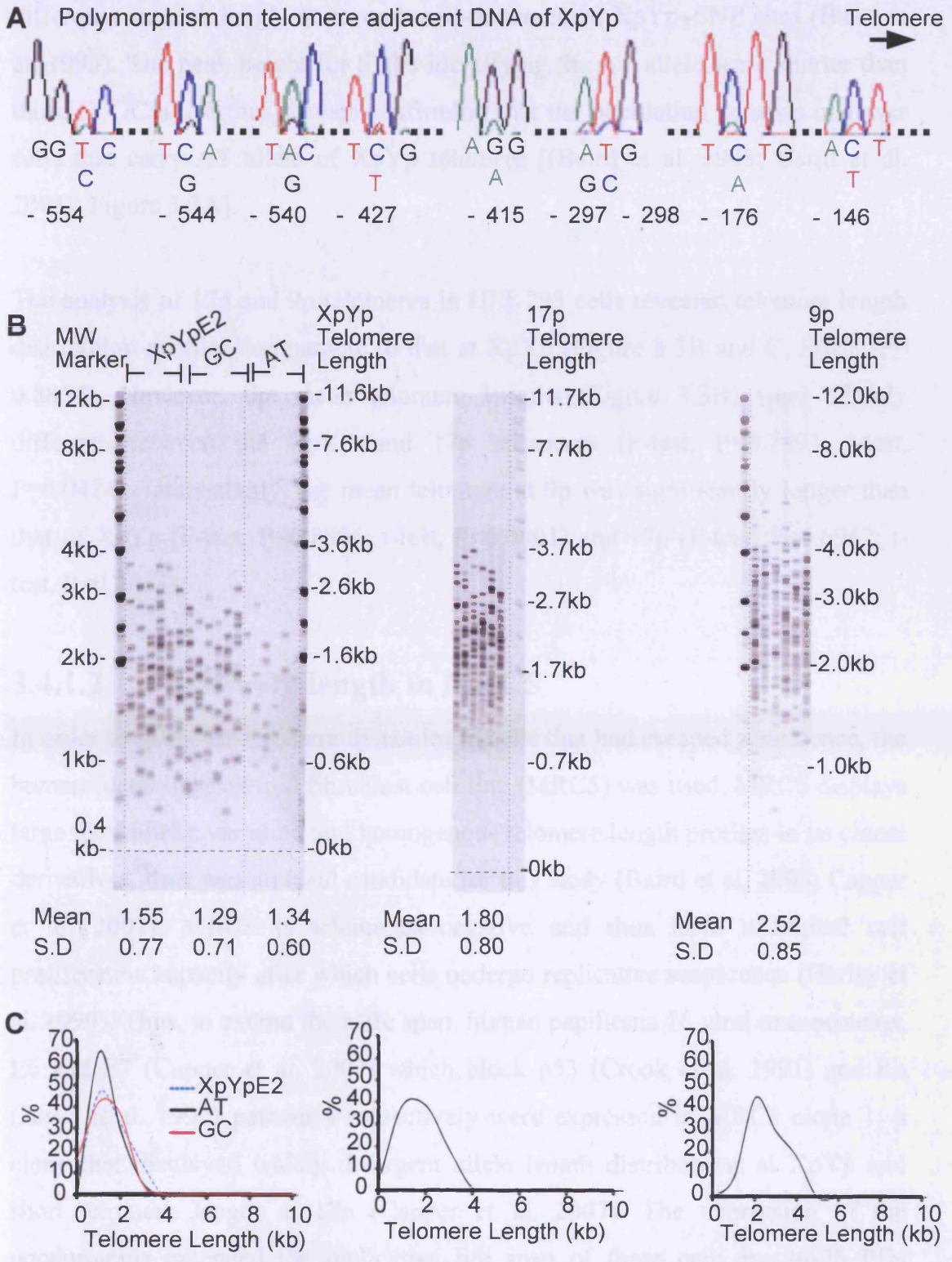


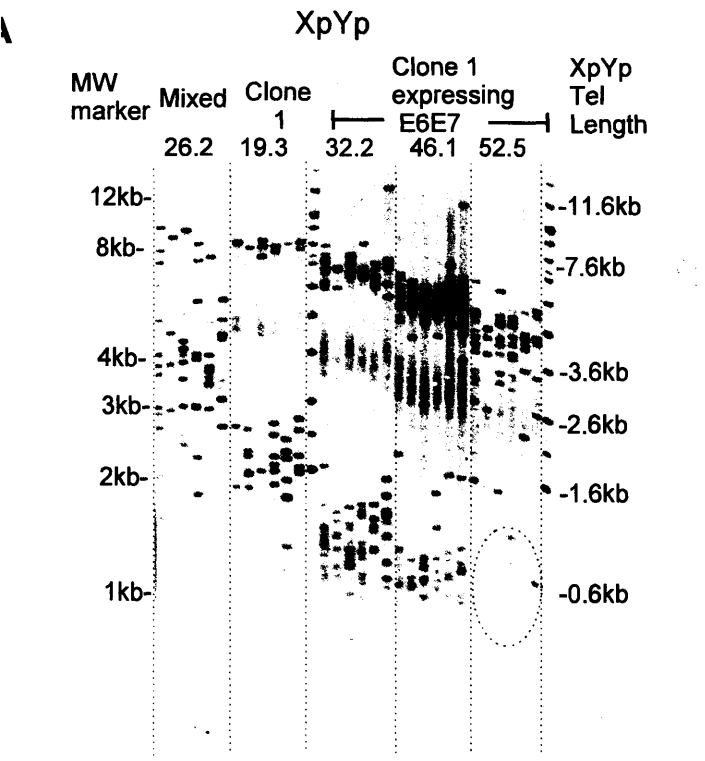
Figure 3.3. Telomere length analysis at XpYp, 17p and 9p telomeres in HEK293. A. The telomere adjacent polymorphism at the XpYp telomere. The allelic variation at XpYp was based on the polymorphism at -427 (G/A) and -415 (C/T). B. Gels illustrating the telomere distributions at the three telomeres. C. The modal distribution of the telomere length at the three telomeres; XpYp, 17p and 9p (left to right).

difference in peak height at several well-documented XpYp–SNP sites (Baird et al. 1995). The peak height for SNPs identifying the AT allele were shorter than those of GC allele thus further confirming that the population consists of fewer cells that carry AT allele of XpYp telomere [(Baird et al. 1995; Baird et al. 2003); Figure 3.3A].

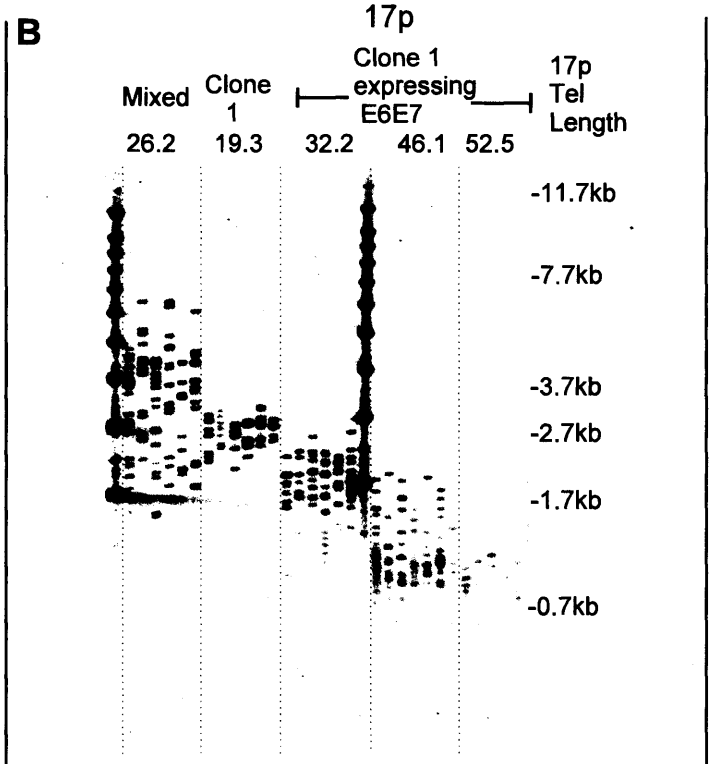
The analysis of 17p and 9p telomeres in HEK293 cells revealed telomere length distribution profiles comparable to that at XpYp (Figure 3.3B and C; F-test, P= 0.8857). However, the mean telomere lengths (Figure 3.3B) were slightly different between the XpYp and 17p telomeres (F-test, P=0.7893, t-test, P=0.0424). Interestingly, the mean telomere at 9p was significantly longer than that of XpYp (F-test, P=0.8806; t-test, P<0.0001) and 17p (F-test, P=0.6962; t-test, P=0.0013).

3.4.1.2 Telomere length in MRC5

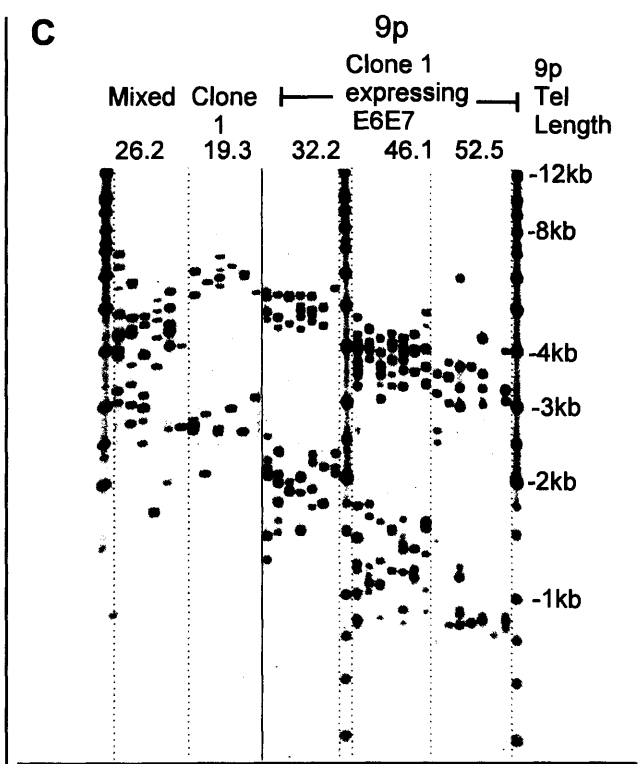
In order to study the telomere dynamics in cells that had escaped senescence, the human foetal lung diploid fibroblast cell line (MRC5) was used. MRC5 displays large inter-allelic variation and homogenous telomere length profiles in its clonal derivatives, thus was an ideal candidate for this study (Baird et al. 2003; Capper et al. 2007). MRC5 is telomerase-negative and thus have a limited cell proliferation capacity after which cells undergo replicative senescence (Harley et al. 1990). Thus, to extend their life span, human papilloma 16 viral oncoproteins, E6 and E7 (Capper et al. 2007) which block p53 (Crook et al. 1991) and Rb (Heck et al. 1992) pathways respectively were expressed in MRC5 clone 1; a clone that displayed widely divergent allele length distributions at XpYp and short telomere length at 17p (Capper et al. 2007). The expression of the oncoproteins extended the replicative life span of these cells by 26-28 PDs beyond senescence and cells continued dividing until crisis (Capper et al. 2007; Letsolo et al. 2009). To investigate telomere dynamics in these cells, STELA was carried out for telomeres of XpYp, 17p and 9p in differing cell populations of MRC5; proliferating mixed population (PD 26.2), young presenescent clone 1



XpYp	PD	Long allele Mean	Long allele SD	Short allele Mean	Short allele SD
Mixed population*	26.2	3.88	2.06		
Clone 1	19.3	7.45	0.23	1.73	0.35
Clone 1 expressing E6E7	32.2	6.60	1.12	0.94	0.29
	46.1	5.48	1.04	0.81	0.46
	52.5	4.64	0.57	d	d



17p	PD	Mean	SD
Mixed population*	26.2	3.24	1.13
Clone 1	19.3	2.50	0.23
Clone 1 expressing E6E7	32.2	1.71	0.38
	46.1	1.04	0.36
	52.5	0.77	0.20



9p	PD	Long allele Mean	Long allele SD	Short allele Mean	Short allele SD
Mixed population*	26.2	3.87	1.24		
Clone 1	19.3	5.89	0.49	2.66	0.30
Clone 1 expressing E6E7	32.2	4.95	0.42	1.88	0.43
	46.1	3.87	0.56	1.21	0.41
	52.5	3.32	0.34	1.02	0.42

Figure 3.4. STELA gels and the tables of descriptive data for telomeres of XpYp (A), 17p (B) and 9p (C) in human lung fibroblast cell line, MRC5. The gels illustrate the telomere length distributions while tables give the means and standard deviations (SD).* The means and standard deviation shown for mixed population are overall mean not allelic values. The mean telomere length for the shorter XpYp allele at crisis (PD52.5) was not determined as the allele had disappear (d) shown by eclipse. The numbers above each gel are population doublings (PD) after which cells were harvested.

(PD 19.3), post-senescent (PDs 32.2, 46.1) and from cells undergoing crisis (PD52.5). The proliferating MRC5 cells displayed heterogeneous length of the telomere at XpYp and 17p. Consistent with previous studies, the clonal populations exhibited homogeneous telomere length profiles characterised by bimodal distribution at XpYp telomere and a single distribution with narrow variance at 17p suggesting that it contains one amplifiable allele at 17p telomere [(Britt-Compton et al. 2006; Capper et al. 2007); Figure 3.4A; Figure 3.5]. Analysis of 9p revealed heterogeneous and homogeneous telomere length profiles in proliferating and clonal cells respectively (Figure 3.4C; Figure 3.5).

The apparent bimodal distribution in clones was similar to that observed in XpYp and was consistent with MRC5 containing two amplifiable alleles at 9p telomere. Like 17p and XpYp telomere, 9p telomere erodes as the cells continue proliferating beyond senescence to crisis. In addition, the telomere length variance increases i.e. distributions become wider as cells approach crisis. Interestingly, like XpYp, the shorter allele in 9p began to disappear as cells approach crisis (PD52.5; Figure 3.4A and C; Figure 3.5). We have previously demonstrated that at crisis, the shortest telomere consists of nine and three repeats in XpYp and 17p respectively (Capper et al. 2007). Similarly, the shortest telomere consists of approximately five repeats at 9p. However, due to close proximity of the 9p primer to the telomere, some shorter molecules might have not been detected.

The erosion rate due to end-replication losses, was estimated by determining the difference as a function of population doubling (PD) between modes of telomere length distribution plotted as histograms (Britt-Compton et al. 2006). The telomere erosion rates at 9p telomeres were comparable to those at XpYp and 17p. The erosion rates at the longer alleles of XpYp and 9p were 77bp/PD and 81bp/PD respectively, while the short 9p allele and that of the single 17p allele were both 48bp/PD. This observation is consistent with previous studies, in which the telomere erosion rates within individual clones were conserved among

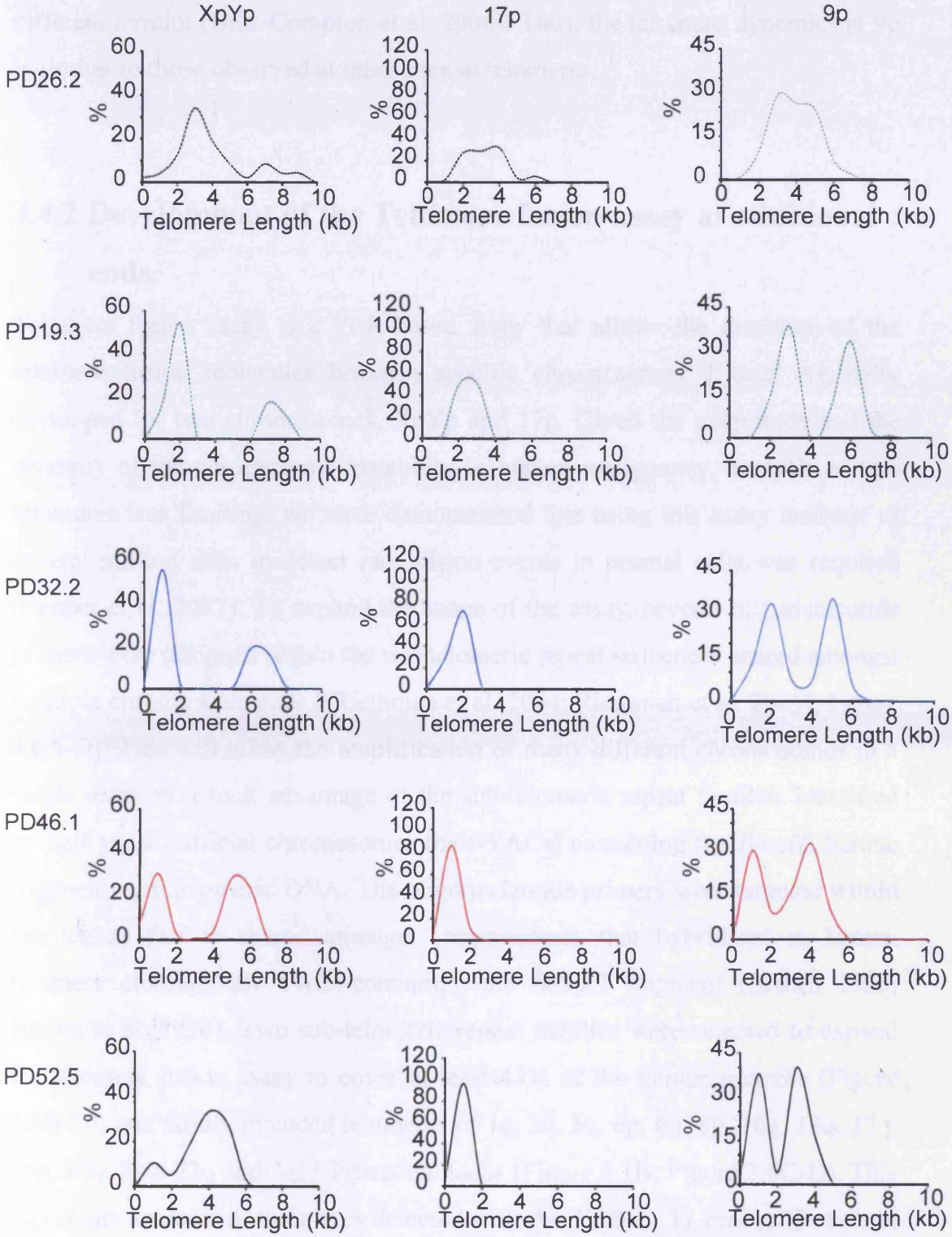


Figure 3.5. Modal distributions of telomere lengths at telomeres of XpYp, 17p and 9p in MRC5 cells. The mode shifts as cells continue dividing from young cells through senescence to crisis.

different termini (Britt-Compton et al. 2006). Thus, the telomere dynamics at 9p is similar to those observed at other human telomeres.

3.4.2 Development of the Telomere fusion assay at additional ends.

Telomere fusion assay is a PCR-based assay that allows the detection of the telomere fusion molecules between specific chromosomes. It was originally developed for two chromosomes, XpYp and 17p. Given the magnitude and the diversity of the chromosome instability in human malignancy, analysis of two telomeres was limiting: we have demonstrated that using this assay analysis of several million cells to detect rare fusion events in normal cells was required (Capper et al. 2007). To expand the scope of the assay, several oligonucleotide primers were designed within the sub-telomeric repeat sequences shared amongst multiple chromosome ends [(Riethman et al. 2004; Riethman et al. 2005); Figure 3.6A-D]. This will allow the amplification of many different chromosomes in a single reaction. I took advantage of the sub-telomeric repeat families identified by half yeast artificial chromosomes (half-YACs) containing the *BamH*I human fragment with telomeric DNA. The oligonucleotide primers were targeted within the region that is shared amongst chromosomes that hybridised to human telomere cloning half YACs-containing the *BamH*I fragment (Brown 1989; Brown et al. 1990). Two sub-telomeric repeat families were selected to expand the telomere fusion assay to cover at least 43% of the human genome (Figure 3.6B-D): one family included telomeres of 1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q, 22q and 2q13 interstitial locus (Figure 3.1B; Figure 3.6C-D). This represents a subset of telomeres detected with the TelBam 11 probes (Brown et al. 1990; Riethman et al. 2005). The second family represents telomeres detected by TelBam 3.4 probes (Brown et al. 1990; Riethman et al. 2005); these include 16p, XqYq, 9p, 1p, 12p, 15q and the interstitial 2q13-14 [Figure 3.1A; (Brown et al. 1990; Riethman et al. 2004)]. The primers for the Telbam 11 and Telbam 3.4 telomeres were designed within the putative telomere adjacent sequences of 21q

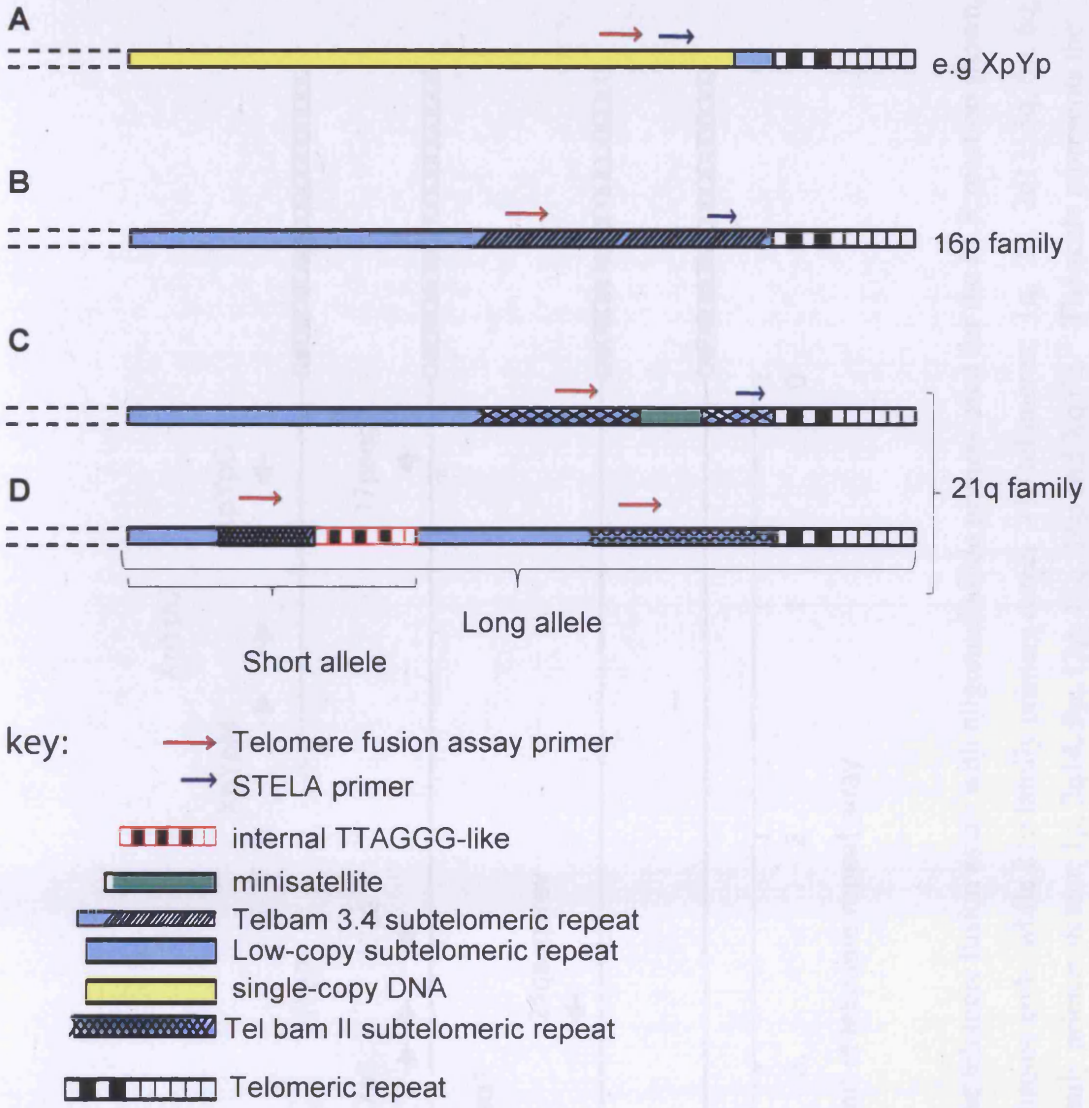


Figure 3.6. Diagrammatic representations of sequence organisation of subtelomere repeat families. A. The low-copy DNA is sufficiently unique for specific telomere analysis, telomeres that display this organisation include XpYp, 11q, 14q, 8q and 18q. 17p was included in this group because single nucleotide differences are sufficient for development of chromosome-specific analysis. B. The subtelomeric repeat family that hybridise to Telbam 3.4 probes, telomeres that display this organisation include 1p, 9p, 12p, 15q, 16p, XqYq and interstitial 2q14. C-D. Sequence organisation for telomeres that hybridise to Telbam 11. This comprises 10-20 telomeres which include 1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q, 22q and interstitial 2q13 etc. D. Illustrates the example of the telbam 11 family in which the longer and shorter alleles vary by thousands of kilobases. The interstitial telomere in the longer allele coincided with the telomere of the shorter allele, an example being 6p.

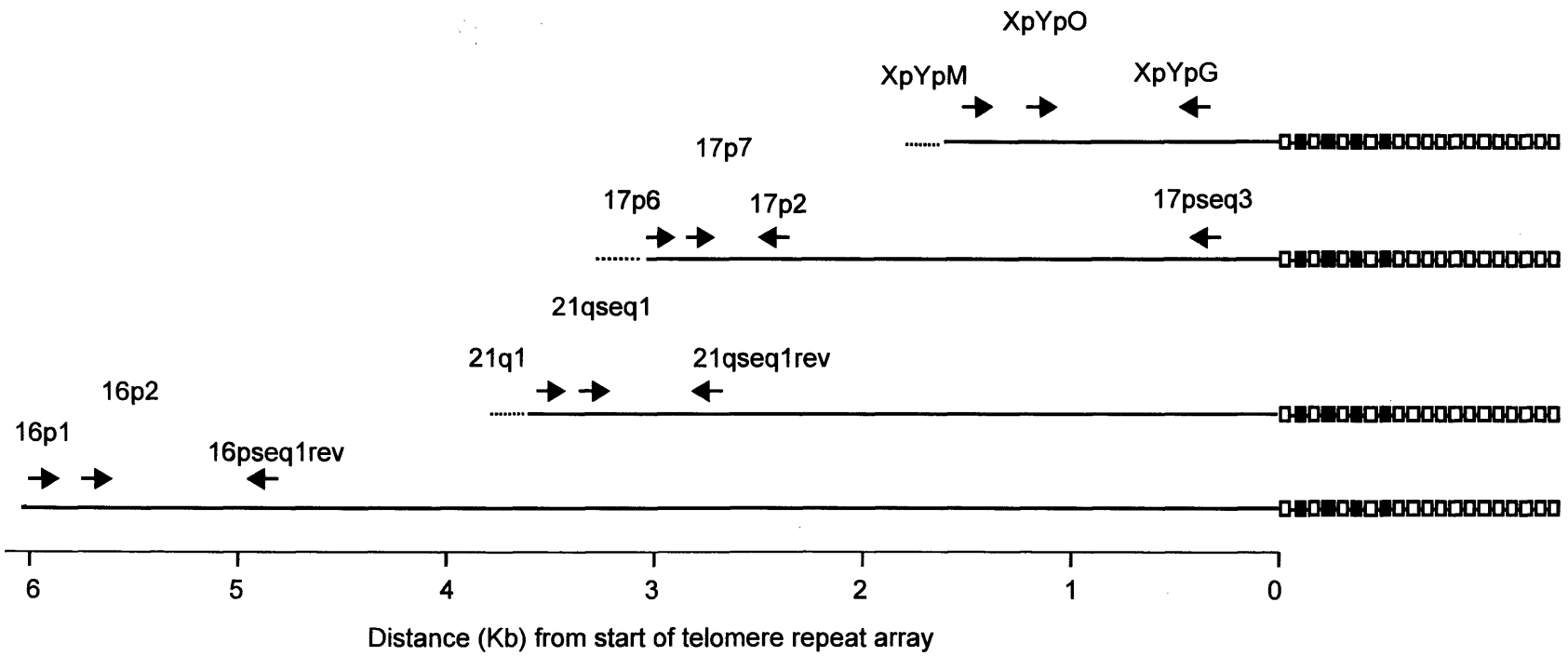


Figure 3.7. Schematic representation illustrating the telomere fusion assay with oligonucleotide primers used for the PCR reaction shown, XpYp and 17p primers are specific for those chromosome ends, while 21q family primers detect >10 telomeres; 1q, 2q, 2q13, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q, 22q ; and 16p family primers detect: 1p, 2q14, 9p, 12p, 15q, 16p and XqYq. The scale represents the relative distance of the oligos from the start of the telomere repeat array.

and 16p telomeres respectively (Figure 3.1A and B; Appendix Figures 3A-1 and Figure 3A-2). Thus the two families have been referred to as 21q and 16p families respectively (Letsolo et al. 2009). Originally the telomere fusion assay was limited to detecting deletions up to 3.0kb of the telomere adjacent DNA of 17p. While the improved assay, extended up to 3.5 kb and 6.0 kb proximal to the start of the telomere repeat array of the telomeres of 21q and 16p families respectively (Figure 3.7; Appendix, Figures 3A-1 and 3A-2).

The specificity of the primers was tested by PCR amplification of the telomere adjacent DNA and products validated by direct sequence analysis. Once appropriate primers were obtained, the telomere fusion assays were carried out as outlined in (Capper et al. 2007) with different two primer combinations (X:17, X:16, X:21, 17:21, 17:16, 16:21, 16:16, 17:17, 21:21 and X:X; Figure 3.8) in HEK293; a cell line in which we have demonstrated that it exhibited high frequency of fusion events between XpYp and17p (Capper et al. 2007). To ensure the specificity of the analysis, the fusion products for all primers were detected by sequential Southern hybridisations with the chromosome-specific telomere adjacent (XpYp, 17p) and family-specific (21q and 16p) probes (Figure 3.8). The products were detected with all probes indicating that the assay was capable of detecting specific telomeres being investigated. However, cross-hybridisation with 17p probe was expected as 21q family share some sequence homology with the 17p telomere-adjacent DNA (Figure 3.8, panel with ***). In addition to molecules detected by all probes corresponding to each combination, it was apparent that some molecules were detected with only one probe. For instance, in panel for X:17 of Figure 3.8, one molecule was detected with XpYp probe (indicated by an arrow) but not with 17p probe. Similar pattern is indicated in X:16 panel in which the arrowed molecule was not detected with XpYp probe but with 16p probe. This pattern of hybridisation is consistent with intra-allelic event such as sister chromatid exchange or the large deletion within the telomere adjacent DNA of either of the ends involved in the fusion events. This possibility

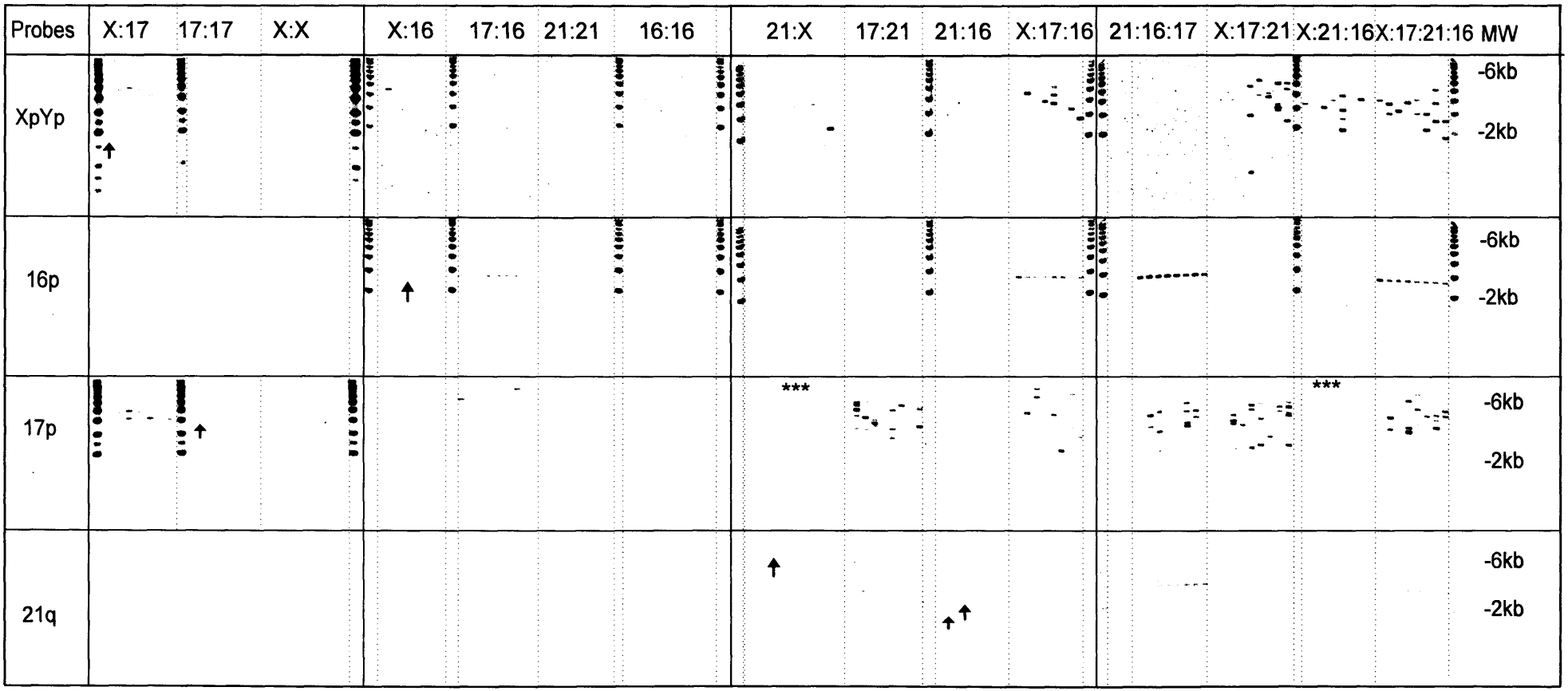


Figure 3.8. The telomere fusion gels illustrating all the possible primer combinations for telomere fusion assay. Multiple PCR reactions (8 per primer combination) were carried out with several primer combination using HEK293 DNA. The probes used for Southern hybridisations are shown. For the X:17, X:X and 17:17, the 16p and 21q group probes are not shown. The molecular weight (MW) marker is shown on the right for each panel. ***It was however not obvious if the bands detected with the 17p probe are due to failed stripping. Sister-chromatid like putative fusion molecules are shown with arrow-headed lines

is also confirmed in the assay carried out with a single primer (e.g. 17:17; Figure 3.8).

However, in assays involving detection of members of a family (e.g. arrowed molecules in X:21 or 16:21 assays), such hybridisation pattern could represent either intra-allelic events or fusion events between the members of the same family. To identity the chromosome ends involved in the fusion events, putative single fusion molecules were re-amplified by nested PCR and the chromosome ends verified by multiple direct sequence analysis of individual fusions followed by alignment (NCBI blast) against available near complete database human sequences. However since the sub-telomeric region of the telomeres within the family share high homology, it was difficult to determine the identity of the chromosomes involved in a fusion events between members of the same family (Letsolo et al. 2009).

In addition to specific fusion events, 21q and 16p probes produced several non-specific fragments that could not be isolated by nested PCR. These molecules are potentially PCR artefacts or cross-hybridisation of the 21q or 16p probe with interstitial loci that share homology with families. Furthermore, assays that involved 17p with either 16p and/or 21q i.e. 17:16, 17:21, generated a product at 2.5-3kb that was detected by 16p and 21q probes (Figure 3.8). Despite several attempts to characterise these molecules, it was not possible to isolate them thus their identities remained unknown. It is however possible that the molecule represented an amplification of the interstitial locus that contains a repetitive sequence such as a satellite that shares the sequence homology with the sub-telomere of the two families, which is characteristic of human genome. In support of this view, this pattern of hybridisation was observed in several other human samples analysed (data not shown, LR, TL, NH personal comm.).

The telomere fusion frequencies were estimated from the number of amplifiable input molecules based on the concentration of single diploid human DNA

molecule (6 picograms) relative to the amplified molecules, as measured by number of bands detected by individual probes. However, 21q and 16p probes produced non-specific bands and thus could not be used to provide reliable estimates of fusion frequencies; these probes are however useful for identifying the chromosome ends involved in each fusion event, which is informative for the subsequent analysis (Figure 3.8). Thus the frequencies of fusions were estimated from bands detected with either the XpYp or 17p probes (Figure 3.9 A and B). Combining XpYp, 17p and 21q primers, the frequency increased by 2.9x and 2.7x with XpYp and 17p probes respectively (Figure 3.9A and B). Similar increment was observed with X:17:16 and 17:16:21 assays (Figure 3.8). The diversity of the fusion events was confirmed by direct sequence analysis, which indicated that different additional ends were involved in fusion events. The combination of all the four primers; XpYp, 17p, 21q and 16p resulted in a further increase (4x) in number of detectable fusion events compared to XpYp and 17p alone [Figure 3.9; (Letsolo et al. 2009)].

Given the apparent success of the assay with HEK293, the improved assays were applied to the clonal MRC5, the telomerase-negative human fibroblast cell line expressing HPV 16 E6/E7 onco-proteins (Capper et al. 2007; Letsolo et al. 2009). We have demonstrated that these cells suffer extensive telomere erosion as the cells enter crisis in culture with evidence of complete telomere loss at XpYp, 17p and 9p [(Capper et al. 2007; Letsolo et al. 2009); Figure 3.4A-C]. Interestingly, as the cells continue to divide towards crisis the frequency of telomere fusion events increased. Similar to HEK293, the frequency of fusions increased accordingly with three and four primer assays relative to XpYp:17p alone. However, the frequency of fusions in MRC5 was so great that it seemed to interfere with the amplification creating smears on the gels (Figure 3.9A and 3.9B). The improved assay have also been applied to investigate telomere fusions in B-cells derived from chronic lymphocytic leukaemia patients supplied by Dr Chris Pepper, Cardiff university [Approved by South East Wales Research Ethics Committee (#02/4806)]. However, due to time limitation, this part of the project

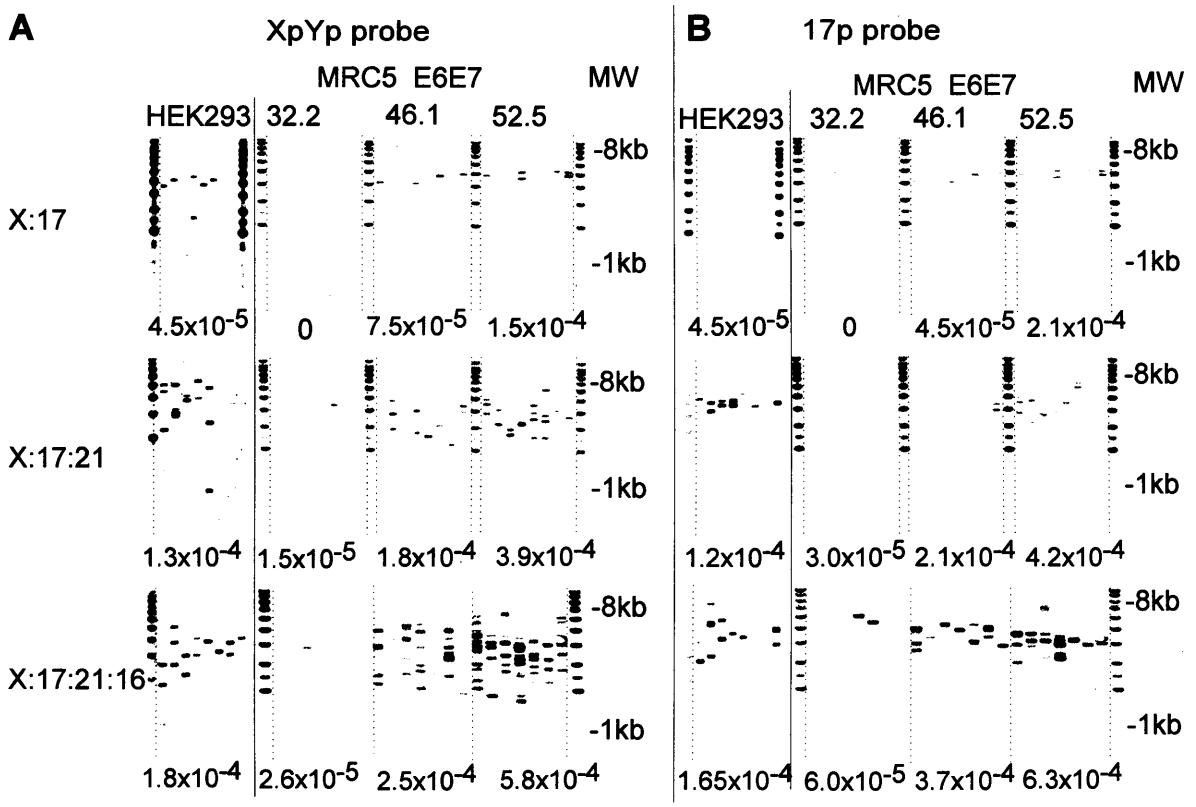


Figure 3.9. Telomere fusion assay in HEK293 and MRC5 cells. The telomere fusion gels indicating the improved assay from two primers (XpYp:17p) to 4 primers (XpYp:17p:21q:16p). The primer combinations used are indicated on the left while the probe used to detect the molecules are indicated above each gel. X for XpYp, 17 for 17p and 21 represents the 21q family of telomeres (1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q and 22q) and 2q13 intestitial locus. Whist 16 represents 16p family of telomeres (1p, 9p, 12p, 15q, 16p and XqYq) and 2q14 intestitial locus. The telomere fusion frequency as estimated from the number of amplifiable input molecules based on the concentration of single diploid human DNA molecule (6 picograms) relative to the amplified molecules, as measured by number of bands detected by either XpYp (A) or 17p (B) probes are indicated below each gel. The molecular weight markers (MW) are indicated on the right of each gel.

was handed-over to a colleague. Data derived during this part has contributed to our recent publication (Lin et al. 2010).

3.4.2.1 The upper limit for the detection of telomere fusion events.

Several studies have reported presence of sporadic telomere deletion events in normal human cells and tissues expressing telomerase (Baird et al. 2006; Britt-Compton et al. 2006). Such events could result in dysfunctional telomeres that are capable of fusion, which may contribute to genomic instability in human tissues. Indeed we have demonstrated that telomeres below the specific threshold of 12 repeats are capable of fusion (Capper et al. 2007). Given the low frequency ($8x10^{-5}$) of fusions in any cell, detection of telomere fusion events in normal cells and tissues would require large-scale analysis. In fact, in its original manifestation with XpYp and 17p, 10ng of DNA (1667 Molecules) per reaction, and several hundred PCR reactions were required to detect rare sporadic fusion events in normal cells (Capper et al. 2007). Thus there was a requirement to increase the efficiency of the fusion assay, by determining the maximum number of DNA (genome equivalents) molecules that could be analysed in a single fusion PCR without inhibiting the ability of the PCR to amplify what may be complex structures. We have detected higher frequency of telomere fusions between XpYp:17p in HEK293 than other telomerase-expressing cell lines such as W12 and Caski (Capper et al. 2007) but did not detect any fusions with MCF7 (Capper et al. 2007), despite both MCF7 and HEK293 cells expressing telomerase activity and maintaining their telomeres at a very short length (Capper et al. 2007). Thus, MCF7 and HEK293 were selected for an experiment aimed at determining the maximum amount of DNA that could be analysed in a single fusion PCR without inhibiting PCR. To achieve this, first I undertook an extensive analysis with increasing amount of diploid genome equivalents [1,667 (10ng) to 66, 667 (400ng)] per reaction (Figure 3.10A), to determine if XpYp:17p fusion events could be detected in MCF7. The telomere fusion events were not detected even with analysis of up to 2 533 346 diploid genome

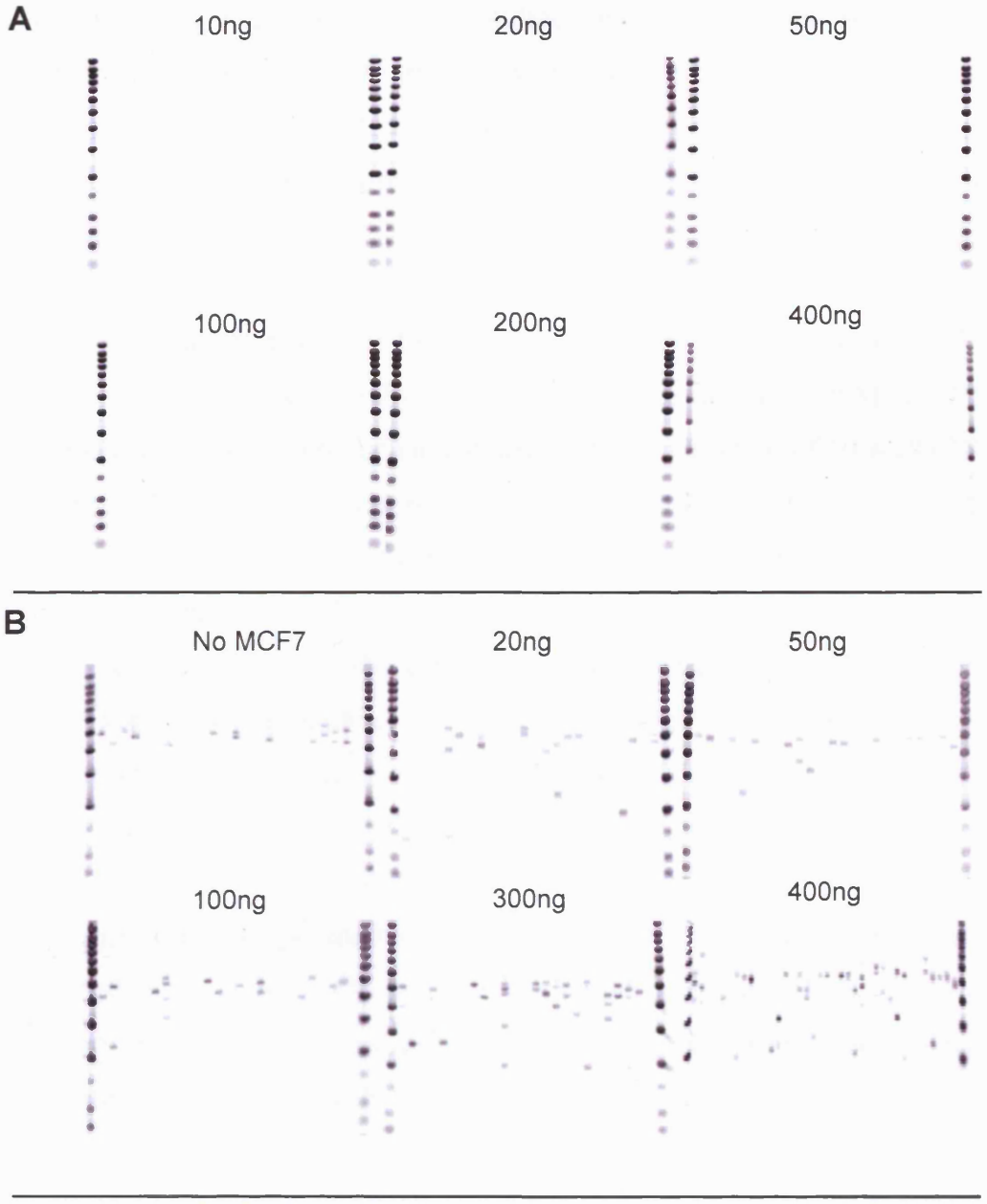


Figure 3.10. Illustrates the telomere fusion gels with increasing amounts of MCF7 either unspiked (A) or spiked (B) with equal amounts of HEK293 DNA. XpYp:17p telomere fusion assay was carried out with increasing amounts of MCF7 spiked with HEK293 (10 ng) DNA. C. The telomere variant map of the XpYp telomere in MCF7 and HEK293, V represents telomere variant repeat, and T represents TTAGGG repeat.

equivalents in a single experiment (400ng per reaction with 38 reactions). However, with this number of input DNA, the assay produced some background amplification products, which despite several attempts could not be isolated and were deemed to be artifactual products that are apparent with increased amounts of input DNA.

Given the absence of detectable fusion events after extensive analysis of MCF7, I carried out experiments whereby the increasing concentrations of MCF7 DNA was spiked with 10ng (1667 genome equivalents) per reaction of HEK293 DNA in a XpYp:17p telomere fusion reactions (Figure 3.10B). The frequency of the telomere fusions ($6.4x10^{-4}$) with this increasing number of genome equivalents remained comparable to that in the control HEK293 (10ng or 1667 diploid genome equivalents) up to the concentration of 310ng (300ng MCF7 + 10ng HEK293)/reaction. i.e. number of fusions observed in HEK293 (10ng) was similar to same amount when spiked into 300ng of MCF7 DNA. However, at the concentrations of DNA greater than 310ng up to 1.01 μg [168333 cells] per reaction, the fusion molecules could still be readily detected, however there was an increase in the background bands which made the estimation of the frequency difficult (data not shown). The putative fusion molecules from HEK293 were re-amplified and direct sequencing analysis revealed that indeed the molecules represented the fusion events between telomeres of XpYp and 17p.

The start of the human telomeres consists of the region often referred to as telomere variant repeat region (TVR) in which telomere variants are interspersed with TTAGGG repeats. The pattern of interspersion varies among different telomeres of the same individual as well as across the population (Baird et al. 1995; Coleman et al. 1999; Letsolo et al. 2009). Similarly, the analysis of the TVR regions of XpYp and 17p telomeres in HEK293 and MCF7 TRF2 knockdown cells (Capper et al. 2007) displayed distinct profiles [(Capper et al. 2007); Figure 3.11C]. Thus for the fusion events that showed a deletion on one side of the fusion point, the TVR pattern did confirm that the fusion events were

indeed from HEK293 but not MCF7; implicating that the fusion events could be detected even at higher input DNA concentration. However, too much DNA could result in the generation of artifactual bands. Therefore, I concluded that the highest concentration for detection of fusions was determined to be 310ng per PCR reaction [approximately 52000 diploid genome equivalents per reaction; (Figure 3.10B)].

3.4.2.2 Fusion frequency in MCF7 and normal MRC5

We have previously demonstrated that the cells that maintain telomere length distributions that extend to below a critical value of 12 TTAGGG repeats can be subjected to telomeric fusions (Capper et al. 2007). However, most normal human cells maintain their telomeres at longer length than this critical value. The analysis of normal cells has revealed that although telomeres are maintained at longer lengths in the normal cells, these profiles are superimposed by rare stochastic events which generate short telomeres that are capable of fusion (Baird et al. 2006; Britt-Compton et al. 2006). Such events have been detect in the young proliferating and senescent cells, as well as in cells expressing telomerase (Baird et al. 2006). The fusion of these truncated telomeres could be catastrophic as they may lead to large-scale genomic rearrangements through BFB cycles (Artandi et al. 2000). It is therefore essential to determine whether the rate of telomere fusion in normal human cells is similar to that in cancer cell lines using the improved telomere fusion assays. Two cell lines were selected, normal telomerase-negative human fibroblast cell line MRC5 and telomerase-positive breast cancer cell line MCF7. MRC5 displays two distinct allelic variation at XpYp, the two alleles differ in length as well as in the polymorphism of the telomere-adjacent DNA. This would allow the differentiation of the telomeres involving either of the XpYp alleles. We have detected an increased frequency of telomere fusion events in MRC5 expressing HPV16 E6E7 oncoproteins as cells continue proliferating to crisis. However, telomere fusion frequency was independent of the HPV status but appeared to be dependent on the telomere length distributions. Using the well-documented SNPs within the telomere-

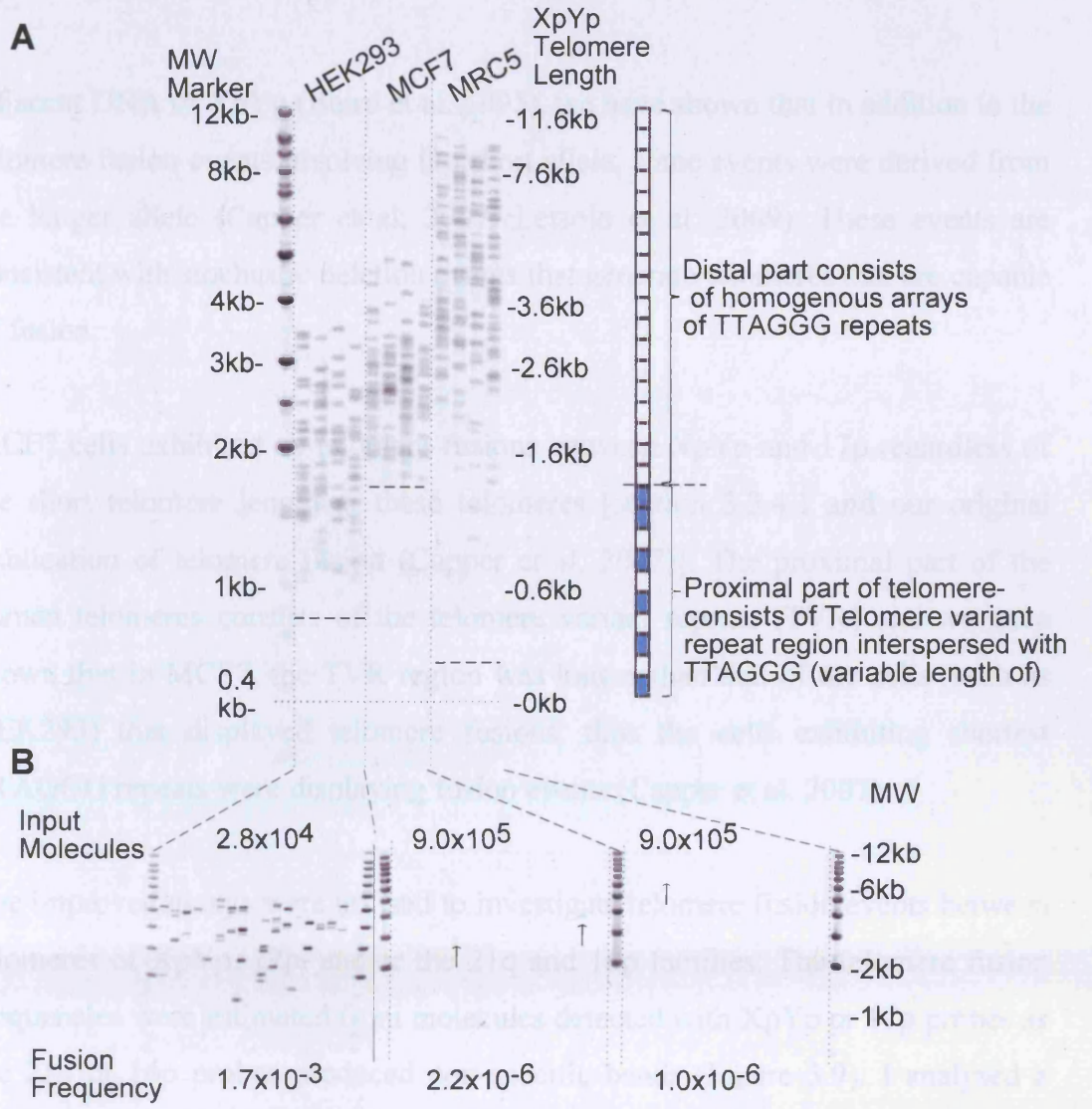


Figure 3.11. Telomere length distributions and telomere fusion frequencies in HEK293, MCF7 and MRC5. A) STELA gels illustrating the telomere length distribution at XpYp telomere detected by TTAGGG-containing probe. The distal end of the highly variable telomere variant region (TVR) for the longest allele is indicated by dashed line across each gel. The TVR for shorter alleles may be less extensive. The TVR and TTAGGG repeat regions are shown on the right. Blue boxes represent TVRs and blank boxes represent TTAGGG repeats. B) Telomere fusion gels between 21q, XpYp and 17p telomeres. The number of input molecules used in each experiment is shown above gels while telomere fusion frequencies based on XpYp probe are shown below the gels. The putative fusion molecules in MRC5 and MCF7 are very weak thus have been indicated with arrows. C) TVR map of one allele of XpYp telomere, V represents telomere variant repeats while T represents TTAGGG repeats.

adjacent DNA of XpYp (Baird et al. 1995), we have shown that in addition to the telomere fusion events involving the short allele, some events were derived from the longer allele (Capper et al. 2007; Letsolo et al. 2009). These events are consistent with stochastic deletion events that generate telomeres that are capable of fusion.

MCF7 cells exhibited no telomere fusions between XpYp and 17p regardless of the short telomere length at these telomeres [section 3.3.4.1 and our original publication of telomere fusion (Capper et al. 2007)]. The proximal part of the human telomeres consists of the telomere variant repeats (TVR) and we have shown that in MCF7, the TVR region was longer than that of the cells (such as HEK293) that displayed telomere fusions, thus the cells exhibiting shortest TTAGGG repeats were displaying fusion events (Capper et al. 2007).

The improved assays were utilised to investigate telomere fusion events between telomeres of XpYp, 17p, and/or the 21q and 16p families. The telomere fusion frequencies were estimated from molecules detected with XpYp or 17p probes as the 21q or 16p probes produced non-specific bands (Figure 3.9). I analysed a total of 4.83 x 10^7 of diploid genome equivalents in MRC5 (n=3.3x10^6) and MCF7 (n=4.5x10^7). Similar to the early study (Capper et al. 2007), no telomere fusion events between XpYp and 17p telomeres were detected in MCF7 (n=4.09x10^7) and MRC5 (n=3.5x10^5). In contrast, analysis with XpYp, 17p and 21q primers revealed the fusion events between XpYp or 17p and 21q family in both cell lines [Figures 3.11A and B). The average frequency of the fusion events in MCF7 (n=2.8x10^6) was undetectable and 3.7x10^{-6} for XpYp and 17p probe respectively (see Table 3A-1 in appendix). In MRC5 (n=1.7x10^6), the fusion frequencies were undetectable and 3.3x10^{-6} for XpYp and 17p probes respectively. In addition analysis with XpYp, 17p, 21q and 16p primers showed an increase in frequency for MRC5 (XpYp=3.3x10^{-6}; 17p=5.2x10^{-6}). To confirm that the molecules detected represented the telomere fusion events, the putative single telomere fusion molecules were isolated with nested PCR primers and the

identity of the chromosomes involved in a fusion event confirmed by direct sequence analysis. Despite several attempts, most of these molecules could not be isolated for characterisation, however one that was fully sequenced from MCF7 did represent the fusion event between 17p and 10q, with deletion on the telomere fusion adjacent DNA of both telomeres. Thus from these data, I concluded that the telomere fusion events could occur in normal cells although at a very low frequency despite maintaining their telomeres at a stable length. It will therefore be interesting to determine if these events occur in human tissues *in vivo*.

3.5 Discussion

In this chapter, I have described the development of STELA and fusion assays at additional telomeres, together with an analysis of base-line fusion frequencies in human cells. These will provide powerful tools for the analysis of telomere erosion, instability and fusion in human cells both *in vitro* and *in vivo*.

3.5.1 Telomere Length

STELA is a robust assay that allows the analysis of the telomere length of specific telomeres (Baird et al. 2003; Britt-Compton et al. 2006). However, the high sequence homology and variability of the sub-telomeric DNA pose a challenge during the development of the telomere specific primers. In addition, the variation among the sub-telomeric repeat families is not absolute thus targeting sequence differences between members of one family could still detect another telomere in a different family. For example, 6p telomere-adjacent DNA has the regions that hybridise weakly to the telbam3.4 probes and strongly to Telbam 11 probes (Brown et al. 1990). To compensate for these problems, "universal STELA" has been developed by others. It is based on similar principle as chromosome-specific STELA but estimates telomere length at almost all chromosomes (Bendix et al. 2010). However, unlike chromosome-specific STELA, the amplification efficiency is reduced for molecules greater than 7kb (Bendix et al. 2010). In addition telomere length estimated by this method

include variable length of sub-telomeric DNA (depending on the restriction site utilised), thus like TRF analysis the telomere length estimated by this "universal STELA" may be greater than the actual telomere length. Whilst "universal STELA" may be ideal for detection of very short telomeres (Bendix et al. 2010), it is not suitable for analysis of samples in which large variability in telomere length may be expected. Again since some chromosome such as 10q and 21q are not amplified by the universal method (Bendix et al. 2010), it was important to develop the chromosome-specific STELA at specific ends. It remains to be determined if the "universal STELA" can be improved further. Taking into consideration the limitations of the "universal STELA" and other methods as described in Chapter 1 of this thesis, chromosome-specific STELA which can detect broader range telomere length [0-25kb; (Baird et al. 2006)] was deemed to be the suitable method for this study.

Here, I have shown that the telomere length of specific autosomal telomeres can be analysed by STELA despite the high sequence homology among the multiple sub-telomeric families. Using single nucleotide variation, I have developed the STELA at 9p telomere. The telomere adjacent DNA of 9p shares 97-99% sequence homology with other telomeres that hybridise to the Telbam 3.4 probes (1p, 12p, 15q, XqYq, 16p and interstitial locus 2q14), which in the study have been named 16p family. The successful 9p assay will allow the investigation of whether the frequent LOH at 9p in premalignant conditions such as Barrett's oesophagus is related to telomere dynamics at this end (Galipeau et al. 1999). As shown by others, loss of telomere function can result in chromosomal rearrangements and LOH via BFB cycles (Gisselsson et al. 2001; Hemann et al. 2001). In addition, the chromosome instability in pre-malignant conditions such as ulcerative colitis and Barrett's oesophagus has been linked to loss of telomere function resulting from telomere erosion (O'Sullivan et al. 2002; Finley et al. 2006).

I have also attempted to develop the STELA for analysis of telomeres of 10q, 21q and 15q, however these assays failed to produce homogenous telomere distributions, characteristic of clonal populations in MRC5 clones, thus were considered not relevant for further analysis. Interestingly, the telomere length profile at these telomeres was similar to that of successful assays; XpYp, 17p and 9p in HEK293. In addition, in MRC5 cells undergoing crisis, these assays revealed that the telomere length were eroding as a function of cell division with evidence of complete loss of shorter molecules especially at 10q. The development of chromosome-specific STELA was more difficult at these ends because in addition to falling into different families, the sub-telomeric DNA of these ends is also shared with other telomeres that are not within the 21q and 16p family. For instance, 20q telomere shares sequence homology with some members of the 21q family. In addition, the design of STELA primers was restricted to within 300bp region from the start of the telomere repeat array, as upstream to these region (>300bp) lies the hyper-variable minisatellite in some members of the 16p family. While in 21q family, the region (>600bp) consists of the GC-rich dinucleotide repeat that is interspersed by the LINE and this could be refractory to PCR (Brown et al. 1990; Blouin et al. 1995). Similarly 21q and 10q could not be amplified by "universal STELA" (Bendix et al. 2010). Thus analysis of the telomeres of 21q and 10q may be carried out with other methods such as FISH.

I have shown that HEK293 exhibits a characteristic short telomere distribution at 9p telomere, similar to that reported at XpYp and 17p, indicating that the telomeres in this cell line are maintained within a similar range at which telomeres are capable of fusion despite expressing telomerase activity. Bryan and colleagues have shown that HEK293 cells exhibit variable telomerase activity with some cells showing no detectable telomerase activity. Thus, such cells exhibit short telomeres while cells with high telomerase activity tend to maintain longer telomeres (Bryan et al. 1998).

In MRC5, bimodal distribution at 9p was observed, suggesting that MRC5 contains two differently lengthed alleles at the 9p telomere, a type of distribution similar to that of the telomeres of XpYp, 12q, 2p and 11q (Britt-Compton et al. 2006). This bimodal distribution is consistent with allelic variation resulting from maternal and paternal telomeric contributions in the zygote that is maintained throughout life (Baird et al. 2003).

Previous studies revealed that as the cells divide towards crisis, the telomeres of XpYp, 17p and other autosomal telomeres were eroding with the evidence of complete loss of the telomeres (Britt-Compton et al. 2006). In this study, a similar pattern of erosion was observed at 9p telomere suggesting that these telomeres are subjected to similar telomere dynamics.

3.5.2 Telomere fusion assay

The human sub-telomeric regions are highly dynamic and variable regions that consist of duplications and sub-telomeric repeat sequences of varying size and composition (Brown et al. 1990; de Lange et al. 1990). I have taken advantage of this sub-telomeric variability and developed the telomere fusion assay at additional telomeres. The telomere fusion assay was originally developed for detection of telomere fusion events between XpYp and 17p (Capper et al. 2007). The analysis of two chromosome ends required analysis of several tens of thousands cells to detect such rare events in normal cells, thus was limiting. I have extended the telomere fusion assay to include at least 43% of the human genome. More telomeres with homology to the 21q and 16p families may become apparent as the sequence of all human telomeres is completed. These new assays will allow the detection of fusion events in samples derived from normal and diseased human tissues where the events could be very rare. Furthermore, the expanded assay will allow the analysis of broader range fusion structures and thus enhance further understanding of the mechanistic basis of short dysfunctional telomeres. In addition, using the improved assay, I have demonstrated that telomere fusion events can be detected between XpYp or 17p

and other telomeres in samples that we have detected no fusion events with the original assay (Capper et al. 2007). Thus the improved assay appears to be more robust in detecting what may be rare fusion events.

Although not always involved in human tumourigenesis, cryptic telomere deletions/amplifications are associated with idiopathic mental retardation and birth defects. Such terminal deletions or duplications may result in unbalanced translocations (Precht et al. 1998; Bonaglia et al. 2003; Feenstra et al. 2006; Bonaglia et al. 2008), one of the consequences of telomere dysfunction. For instance, terminal deletion of 2q and duplication of 15q resulting in partial monosomy 2q (2q37-2qter) and trisomy 15q (15q24-15qter) was observed in the foetus with facial dysmorphism, clenched hands and malpositioned toes (Chen et al. 2009). The development of the telomere fusion assay to cover a large majority of the human genome would facilitate the better understanding of the chromosome instability initiated by dysfunctional telomeres in a variety of disease settings.

Despite extensive analysis of telomere fusion events between telomeres of XpYp and 17p in MCF7 and MRC5, no telomere fusion events were detected. In contrast, the analysis in HEK293 has revealed high frequency of telomere fusion events. Unlike MCF7 and HEK293 cells which express telomerase activity (Kim et al. 1994), MRC5 cells exhibit no detectable telomerase activity (Harley et al. 1990). However, the young proliferating cells show longer telomere length profiles and thus telomere fusion events between these two telomeres could be so low that it was effectively difficult to detect. Indeed the shortest telomere in MRC5 at XpYp is several magnitudes longer than the threshold below which the telomeres are capable of fusion [(Capper et al. 2007); 12 TTAGGG repeats]. Additionally, we (Capper et al. 2007) and others (Ducray et al. 1999) have shown that MRC5 cells that have escaped senescence showed an increased frequency of end-to-end fusions once their telomeres have eroded to length at which they are capable of fusion. In contrast to the low frequency of fusions in normal

fibroblasts described in this study, higher frequency of fusions was described by karyotype analysis (Takubo et al. 2010). The difference between the data described in this study and data by Takubo et al may be due to differences in telomere length profiles of cells analysed. The data described in this thesis was derived from MRC5 cells displaying long telomere profiles while in TIG cells analysed by Takubo and colleagues, the shortest telomeres were 1.3 and 2.4kb (Takubo et al. 2010). It is possible that analysis of MRC5 displaying shorter telomere length may reveal comparable frequency of telomere fusions to those described by Takubo and colleagues.

However, lack of fusion events in MCF7 cannot be explained on the basis of telomere length and telomerase activity (Capper et al. 2007). We have demonstrated that one XpYp allele of MCF7 contains an extensive telomere variant repeat region (TVR), which is such that the pure TTAGGG repeats begin at 1.3kb while in HEK293 cells, the pure TTAGGG starts at about 400bp. By taking into account the TVR region, we have also shown that the cell line with the shortest telomere range also displayed fusion events (Capper et al. 2007). Apart from telomere length distribution, the status of p53, a tumour suppressor that protects the cells from physiology and environmental stress such as telomere dysfunction could account for the differences in telomere fusion frequency in the two cell lines. Although telomeres are short in MCF7, the presence of the wild-type p53 (Zhan et al. 1994) in these cells could be inhibiting the propagation of genomic instability by ensuring that cells with dysfunction telomeres exit the cell cycle. Evidently, disruption of the p53 function by expression of the HPV16's E6 oncoprotein, impaired the cells' response to γ-ray-induced p53 cell cycle arrest (Fan et al. 1995). Furthermore, induction of DNA damage at telomeres of MCF7 through use of T-oligos; molecules that mimic the 3′ overhang led to p53-dependent apoptosis and senescence (Yaar et al. 2007). Moreover, our recent work has demonstrated that disruption of the telomere structure through siRNA-mediated knockdown of TRF2 increased the frequency of telomere fusions between XpYp and 17p (Capper et al. 2007). Knockdown of TRF2 has been

shown to induce formation of telomere-induced foci, a marker of DNA damage resulting from telomere dysfunction and these are accompanied by p53-dependent apoptosis or senescence (van Steensel et al. 1998; Karlseder et al. 1999). Similarly, disruption of telomere structure through knockdown of POT1 induced p53-dependent apoptosis in MCF7 cells (Yang et al. 2005).

In contrast, in HEK293 the p53 pathway has been impaired during transformation with adenovirus 5 (Graham et al. 1977). Thus, the cells with dysfunctional telomeres can continue proliferating and accumulate chromosome aberrations. Evidently, the primary cells that are deficient in p53 and /or pRb continue dividing beyond senescence, with subsequent severe telomere erosion and genetic instability. *In vivo*, p53 deficiency has been reported to rescue the adverse effects of telomeres by cooperating with telomere dysfunction to accelerate carcinogenesis (Chin et al. 1999; Artandi et al. 2000). Furthermore, mice without telomerase activity experience extensive telomere shortening and accumulation of chromosome end-to end fusions. However, in the presence of functional p53, these mice ($mTR^{-/-}$) exhibit a low rate of telomere fusions compared to mice without functional p53 [$mTR^{-/-}$ $p53^{-/-}$; (Chin et al. 1999; Cosme-Blanco et al. 2007)]. In addition, it has been shown that the level of telomerase is variable at the cellular level in HEK293 cells, where subpopulations with undetectable to very low activity and subpopulations with high telomerase activity are detected. Furthermore, clonal populations with longer and shorter telomeres have also been detected in these cells (Bryan et al. 1998). Thus, p53 deficiency in those clones without telomerase activity could lead to telomere erosion and thus account for increased frequency of telomere fusions. To support this, recent work in our laboratory has isolated HEK293 subpopulations with longer telomeres that exhibited no telomere fusions and clones with shorter telomeres that exhibited fusion events (DMB personal comm.). Thus, the difference in telomere fusion frequencies between MRC5, MCF7 and HEK293 can be explained on basis of telomere length, telomerase activity as well as the intact p53 pathway.

3.6 Key findings

- Telomere fusion assay has been expanded to cover at least 43% of the human genome and this will allow the understanding on the mechanistic basis of the genomic instability generated by short telomeres.

- These will provide tools to study potentially rare fusion events in human tissues.

- The assay have also been extended to detect the events involving deletions of up to 6kb of the telomere adjacent DNA.

- Using the expanded assay, I have defined the fusion frequency in normal human cells *in vitro*.

- I have defined the upper limit at which the rare telomere fusion events can be detected, as 310ng per reaction.

- Despite extensive analysis of MCF7, the telomere fusion frequency remained very low.

- I have successfully developed STELA at an additional autosomal end, 9p. Using this assay, I have demonstrated that the telomere distribution at 9p is similar to that of other telomeres in HEK293 and MRC5.

4.1 Introduction

4.1.1 Telomere dysfunction

The primary function of the telomeres is to preserve genomic integrity and to prevent genomic instability arising from degradation, recombination or end-to-end fusions, thus telomeres distinguish the natural chromosome ends from the double-stranded breaks [DSBs; (Blackburn 1991; De Lange 2005)]. The loss of telomere function could result from gradual telomere erosion or stochastic telomere deletion events generating short dysfunctional telomeres (Capper et al. 2007). In addition, destabilisation of the telomere structure through experimental disruption of the telomere binding proteins such as TRF2 can lead to full length but dysfunctional telomeres (van Steensel et al. 1998).

Normal human somatic cells do not express telomerase and thus display a limited replicative capacity in culture (Hayflick and Moorhead 1961; Harley et al. 1990). According to the telomere hypothesis of ageing, this finite proliferative capacity is correlated with telomere length such that cells with longer telomeres would exhibit a longer replicative capacity (Allsopp et al. 1992). In addition, the replicative capacity of normal diploid cells can be extended by expression of hTERT the catalytic component of telomerase (Bodnar et al. 1998). In the presence of functional p53/Rb DNA damage response machinery, cells at the end of their replicative capacity enter replicative senescence; an irreversible cell cycle arrest which is believed to be a barrier to tumourigenesis (Karlseder et al. 2002; d'Adda di Fagagna et al. 2003; Choudhury et al. 2007). However, in the absence of a functional DNA damage response, cells with shortened/dysfunctional telomeres fail to undergo senescence and continue to proliferate until their telomeres become critically short (Chin et al. 1999). Critically short telomeres are capable of fusion and may form dicentric chromosomes (amongst others). During anaphase, dicentric chromosomes can form bridges, which in turn can break when the sister chromatids are pulled to opposed poles of the cell. If the breakage occurred at a different site from the original fusion point, this would

result in the loss or gain of genetic material in the daughter cells. The subsequent double-stranded breaks could drive successive cycles of fusion-bridge-breakage (Murnane and Sabatier 2004) and thus genomic instability. Hence, loss of telomere function can create chromosome instability such as non-reciprocal translocations (NRTs) and genetic amplifications/losses (Meltzer et al. 1993). Indeed, Artandi and colleagues have demonstrated that telomere dysfunction in ageing telomerase-negative mice results in NRTs, a typical feature of human carcinomas (Artandi et al. 2000). Anaphase bridges are surrogate markers of telomere instability and have been observed in early stages in human cancer cells and mouse cells. Furthermore, the frequency of anaphase bridges have been correlated with abnormal karyotypes in sarcomas, pancreatic and colon carcinomas (Gisselsson et al. 2001; Rudolph et al. 2001). Moreover, mice with knockouts in NHEJ proteins ligase IV and p53 exhibit lymphomas with chromosome rearrangements consistent with breakage-fusion-breakage cycles (Frank et al. 2000).

4.1.2 Intact shelterin complex inhibit telomere fusion

The loss of shelterin integrity or function induces inappropriate chromosome fusions through non-homologous end-joining and homologous recombination (van Steensel et al. 1998; Ferreira et al. 2004; Celli and de Lange 2005; Denchi and de Lange 2007; Verdun and Karlseder 2007).

Non-homologous end-joining is an error-prone repair mechanism in which broken ends are joined together after minimal processing. It depends upon the activities of ligase IV, XRCC4 and Ku/DNA PKcs (Kanaar et al. 1998; Smith and Jackson 1999; Karran 2000) and is the predominant repair mechanism that can operate at any phase of cell cycle. Another mechanism of DNA repair, homologous recombination; is mainly for repair of sister chromatids (Celli et al. 2006).

Several experiments aimed at investigating the consequence of loss of telomere function arising from loss of shelterin components, have revealed that telomere dysfunction induces DNA damage response and repair at telomeres (van Steensel et al. 1998; Hockemeyer et al. 2007). Thus, below I discussed briefly the role of specific shelterin components in inhibiting illegitimate repair and formation of end-to-end fusions.

4.1.2.1 TRF2 and Telomere fusions

TRF2, the shelterin component that plays a crucial role in telomere capping represses illegitimate NHEJ and HR at telomeres thus functional TRF2 is essential at all stages of the cell cycle. During G_1 phase, TRF2 is a main repressor of NHEJ thus allowing DNA replication to proceed while, post-replication at G_2, TRF2 cooperates with POT1 to repress NHEJ (Celli and de Lange 2005; Hockemeyer et al. 2006; Konishi and de Lange 2008). Evidently, TRF2 deficiency results in fusions at G_1 phase (Konishi and de Lange 2008). The role of TRF2 in inhibiting inappropriate chromosome fusions is further enhanced by studies, which show that disruption of the TRF2 sub-complex i.e. TRF2 and its interacting partners, induces chromosome fusion (van Steensel et al. 1998; Bae and Baumann 2007). For instance, human RAP1; a TRF2-interacting factor protects telomeres from illegitimate non-homologous end-joining through interaction with TRF2 (Bae and Baumann 2007; Sarthy et al. 2009). In addition, knockout of another TRF2-interacting factor, PARP-1 in mice results in elevated frequency of telomere fusions, abundant chromosomal breaks and aneuploid cells compared to the wild type mice (d'Adda di Fagagna et al. 1999). Furthermore, loss of Ku, a TRF2-interacting factor in mouse embryonic fibroblasts results in increased telomere fusions with long tracts of telomere repeats, consistent with telomere uncapping without telomere shortening (Samper et al. 2000).

Moreover, TRF2 cooperates with Ku to repress HR at the telomeres. Evidently, mouse cells lacking Ku70 [$Ku70^{(-/-)}$] have normal telomeres and do not activate DNA damage response. However, in the absence of functional TRF2, these

$Ku70^{(-/-)}$ mouse cells exhibit increased frequency of telomere sister chromatid exchanges, a form of homologous recombination, predominant in cells maintaining their telomeres through telomerase-independent mechanism(s) called alternative lengthening of telomeres [ALT; (Celli et al. 2006)]. Furthermore, mutant TRF2 lacking the DNA-binding domain (ΔB) represses NHEJ but results in rapid erosion of telomeres as well as formation of t-loop sized telomere circles implicating the processing of the Holliday junction at the t-loop. Indeed such deletions were dependent upon the action of XRCC3, a nuclease involved in processing of Holliday junctions (Wang et al. 2004), an intermediate stage in homologous recombination.

4.1.2.2 POT1 and telomere fusions

The single-stranded binding protein, POT1 is essential for maintaining telomere integrity and genome stability by repressing NHEJ and HR at telomeres. In support of this, loss of POT1 function results in loss of overhang, TTAGGG-containing fusions, anaphase bridges, ring and dicentric chromosomes (Hockemeyer et al. 2005; He et al. 2006; Hockemeyer et al. 2007); suggesting that loss of POT1 function may result in recognition of telomeres as sites of DNA damage.

In addition, deficiency of Pot1a in mouse cells induced telomere elongation, increased frequency of telomere sister chromatid exchanges and telomere circles (t-circles); characteristic features of ALT, a homologous recombination-based telomere maintenance mechanism (Cesare and Griffith 2004; Wu et al. 2006). Similarly, a mutant mouse pot1b results in increased frequency of both leading and lagging strand telomeric sister chromatid exchanges (T_SCE) in MEFs (He et al. 2006). Likewise, loss of pot1 in *S.pombe* results in telomeric loss and cell death, with survivors having circularised chromosomes, a potential processing of t-loops (Baumann and Cech 2001). Together these findings suggest that POT1 is essential for repressing inappropriate repair at telomeres.

4.1.3 This study

The end-capping function of telomeres is mediated by TRF2 and associated proteins (van Steensel et al. 1998). Inhibition of TRF2 in human and mouse telomeres through expression of dominant mutant TRF2 induces Ligase IV-mediated chromosomal fusions (van Steensel et al. 1998; Smogorzewska et al. 2002; Celli and de Lange 2005). These fusions are characterised by long tracts of TTAGGG repeats, and are a result of covalent linkage of the G-strand to the C-strand (Smogorzewska et al. 2002).

In contrast to TRF2-deficient cells, the telomere fusion events have been observed in mammals in the absence of NHEJ components, implicating the existence of an alternative mechanism that is employed during repair of short dysfunctional telomeres (Maser et al. 2007). Evidently, in telomerase-deficient mutants of yeast and plants, dysfunctional telomeres result in fusion events independent of ku70, DNA PKcs and Ligase IV (Baumann and Cech 2000; Heacock et al. 2004; Maser et al. 2007). Thus to investigate the mechanism that underlies the fusion of short telomeres, a PCR-based telomere fusion assay was developed for analysis of telomere fusion events between the telomeres of XpYp and 17p (Capper et al. 2007). The advantage of the assay is the ability to isolate individual fusion events and examine their molecular structure using direct sequence analysis. Using this assay, we have demonstrated that the fusion of short telomeres is accompanied by large sub-telomeric deletions, short patches of micro-homology and minimal telomere repeats at the fusion point (Capper et al. 2007). The assay has since been extended to include at least 43% of the human genome [chapter 3: (Letsolo et al. 2009)]. Thus, the aim of the work described here was to isolate and characterise the telomere fusion events generated by short telomeres and investigate whether the chromosome instability we have observed between XpYp and 17p was typical of dysfunctional telomeres in the human genome. The study will detect broader range fusion events and possibly assist in the understanding of the mechanism that underlies the repair of short dysfunctional telomeres. The data can also be used to compare fusion profiles

generated from human tissues, specific disease situations and importantly experimental systems testing the mechanistic basis of fusion.

4.1.4 Sample Preparation and Statistics

The HEK293 and MRC5 cells have been discussed in chapter 3. The statistical analysis was carried out as described in chapter 3.

4.2 Results

4.2.1 Telomere fusion analysis

The improved telomere fusion assay was used to isolate and characterise fusion events in HEK293, a telomerase-positive epithelial cell line that maintains short telomere length distributions (Figure 3.3B-C) and has been shown to exhibit increased frequency of fusions between XpYp and 17p (Capper et al. 2007). Telomere fusion events were also characterised from MRC5, telomerase-negative human fibroblast cell line expressing HPV16 E6E7 onco-proteins. These cells suffer extensive erosion of telomeres as the cells enter crisis in culture (Capper et al. 2007), with evidence of complete telomere loss at XpYp, 17p and 9p (Figure 3.4A-C; Figure 3.5).

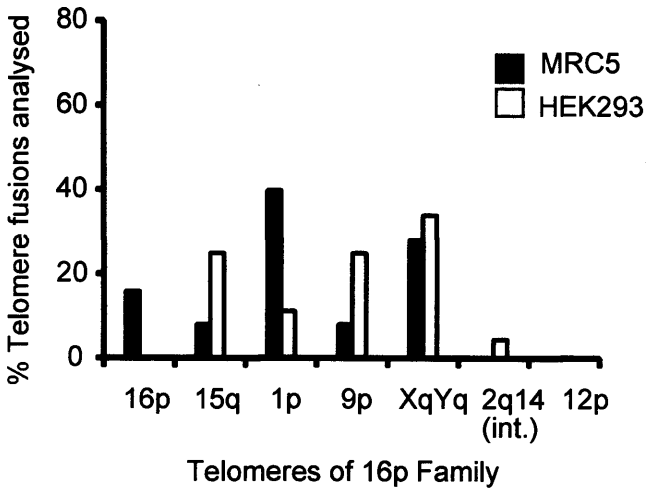
The DNA was analysed with multiple PCR reactions, typically 7-18 each containing 1667 diploid genome equivalent. The amplified products were detected by sequential Southern hybridisation with telomere-adjacent probes. After which the putative fusion molecules were isolated by PCR with nested primers prior to direct sequence analysis. The direct sequence analysis of the putative telomere fusion events verifies the chromosome ends involved in the fusion events and allows the examination of the molecular structure of fusion events as well as the investigation of the underlying mechanistic basis of fusion. However, fusion events between members of the 16p or 21q telomere families only could result in the formation of palindromic sequences making the sequence

analysis technologically challenging. Therefore, for the initial analysis, fusion events were investigated between 21q family of telomeres (1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q and 22q and interstitial 2q13), XpYp and/or 17p and then XpYp, 17p and/or 16p family (1p, 9p, 12p, 15q, 16p, XqYq and interstitial 2q14) and between telomeres of the 21q and 16p families. I have shown that using the modified assays, the number of detectable molecules were increased, indicating that the assays were capable of detecting fusion events between additional telomeres (Letsolo et al. 2009).

Initially, 298 fusion events between XpYp or 17p and 21q and 16p families were sequenced; 125 from HEK293 [n=84 and n=41 for 21q and 16p families respectively], 173 in MRC5 (n=102 and n=71 for 21q and 16p families respectively). An additional 10 events involving fusion between the telomeres of 16p and 21q family were sequenced in HEK293, making a total of 308 sequenced fusion events. I have detected fusion events between XpYp or 17p with telomeres of 21q and 16p families, and fusion events between members of 21q and 16p families indicating that the assay was capable of detecting fusion events between multiple chromosome ends being targeted.

The telomerase-positive cell line HEK293, cells display uniformly short telomere length distributions at all the telomeres analysed with STELA (Figure 3.2; 3.3B-C). This appears to be reflected in the fusion analysis where it was apparent that several members of 21q and 16p families were involved in fusion events [Figure 4.1A and B]. In contrast, fusion events in MRC5 were limited to a subset of chromosome ends. Notably, within the 16p family [Figure 4.1A] several members were detected in telomere fusion events while only 21q and 10q, representing the 21q family were detected. This suggests that in the telomerase-negative MRC5 E6E7 cells, a subset of telomeres have eroded to within the telomere length range at which fusion occurs (Figure 4.1B).

A



B

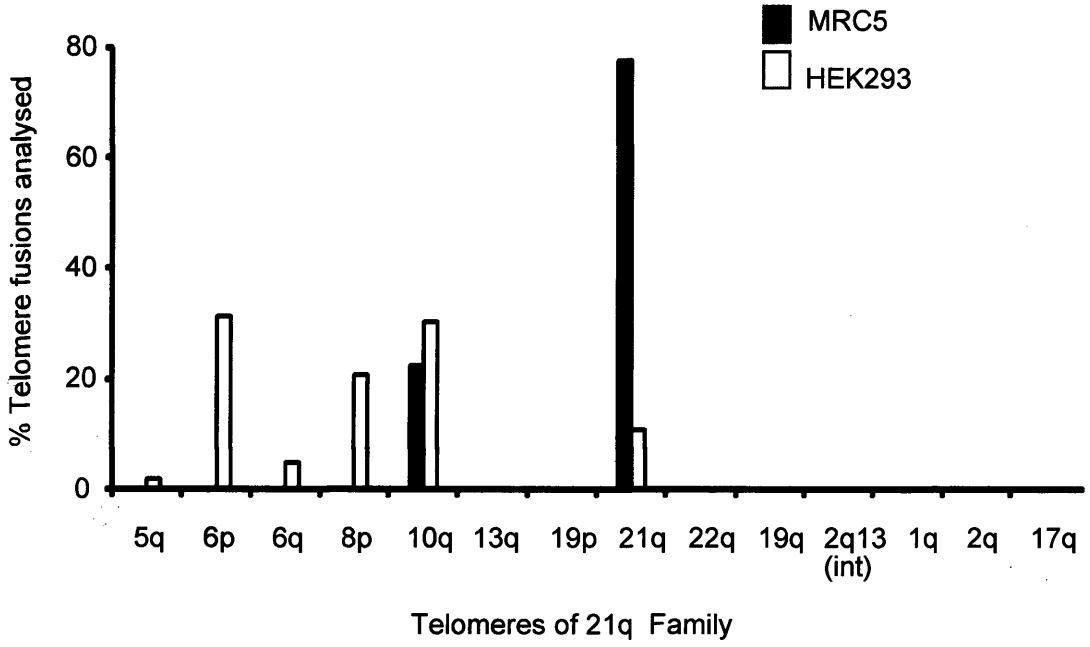


Figure 4.1. Histograms illustrating the different ends that were detected in fusion events for 16p (A) and 21q (B) families in HEK293 (blank boxes) and MRC5 cells expressing HPV16 E6 E7 onco-proteins (black boxes)

4.2.2 Internal structure of telomere fusion events

4.2.2.1 Clonal events

The fusion events are amplified from single molecules, thus presence of same fusion in separate reactions from the same DNA sample would suggest that fusion events have been derived from separate cells and hence is a clonal event. In HEK293, all the 84 events derived from 21q family were unique while 15% (6/41) events derived from 16p family were sequenced more than once [Table 4.1]. In contrast, 25% of fusion events in MRC5 [(26/102 for 21q family; 17/71 for 16p family] were sequenced more than once and the remaining 75% were unique. Interestingly, in MRC5, each event sequenced more than once was derived from cells within the same sample i.e. an event that is detected more than once within the same sample of MRC5 expressing E6E7 (PD46.1). In addition, it was apparent that some events were occurring in several cells, such that they were sequenced 10x. Such events were occurring in cells undergoing crisis [PD 52.5; Table 4.1], implicating that once formed, fusion events can be stable throughout cell cycle. The specific clonal fusion events could dominate the culture and result in clonal expansion. Clonal expansion has been implicated as one of the early lesions that may predict progression to malignancy (Maley et al. 2006). These data is consistent with the observation that non-reciprocal translocations in carcinomas and early stage neoplasia are clonal (Artandi et al. 2000).

4.2.2.2 Subtelomeric deletions

A striking feature of all the 308 fusion events sequenced was that fusion was accompanied by the deletion of either one or both telomeres. The deletion events included the proximal telomere variant repeat region, extending into the telomere-adjacent DNA close to the limit of the assay (6.1kb). In this section, I discuss the nature of the deletion events involving 21q and 16p families, XpYp and 17p.

Table 4.1. Details of the fusion events that were detected more than once in HEK293 and MRC5

HEK293								
Chromosomes involved	Number of TTAGGG	Deletion size 21q/16p	Deletion size XpYp/17p	Microhomology Nucleotides (number)	Insertion	Population doubling	Microhomology nucleotide (sequence)	Number of events
9p;17p	0	883	0	0	0	n/a	none	2
15q;17p	0	1925	2244	0	4	n/a	none	2

MRC5								
Chromosomes involved	Number of TTAGGG	Deletion size 21q/16p	Deletion size XpYp/17p	Microhomology Nucleotides (number)	Insertion	Population doubling	Microhomology nucleotide (sequence)	Number of events
15q;XpYp	0	4107	950	3	0	46.1	CAG	2
16p;17p	0	2285	1737	4	0	46.1	ACAA	3
16p;17p	0	1839	248	2	9	52.5	TC	2
16p;17p	0	3642	248	0	0	52.5	none	2
1p;XpYp	0	1199	1464	3	0	46.1	AAA	2
21q;17p	3.5	1486	0	0	0	46.1	none	2
21q;XpYp	0	2078	500	4	0	46.1	CTGG	4
21q;XpYp	0	1320	986	4	0	52.5	GGGC	10
21q;XpYp	0	1607	301	0	0	52.5	none	3
21q;XpYp	0	1299	1205	3	0	52.5	AGC	3
21q;XpYp	2.5	1237	0	0	no	46.1	none	2
XqYq;XpYp	0	2187	314	2	0	52.5	AG	3
XqYq;XpYp	14	5601	0	4	0	52.5	CCCT	3

4.2.2.2.1 21q and 16p Families

The 21q family consists of 1q, 2q, 2q13 (interstitial), 5q, 6q, 6p, 8p, 10q, 13q, 17q 19p, 19q, 21q, 22q and other ends that may be apparent with further sequencing of human genome. In HEK293, several of these ends were detected in telomere fusion events (Figure 4.1B). The mean sub-telomeric deletion was 1.19kb ±0.53 (SD), with the largest and the minimum deletions being 2.79kb and 0.24kb respectively (Table 4.2). In MRC5 on the other hand only two telomeres, 21q and 10q within this family were detected during direct sequencing analysis.

Whilst the deletion profiles in MRC5 cells appeared to be similar to that observed in HEK293, the means and variances of the two profile were different ($P<0.0001$; Figure 4.2A; Figure 4.3, panel A) with the mean deletion size (±SD) being 1.50kb±0.35 and 1.19kb ±0.53 in MRC5 and HEK293 respectively (Table 4.2). The combined mean deletion (±SD) for telomeres of 21q family for both MRC5 and HEK293 was 1.34kb ±0.44.

Notably, in MRC5 cells, the frequency of deletion events was less in the region around 1kb from the telomere (Figure 4.2A). This may be due to the presence of a satellite repeat interspersed with a LINE in that region in some members of the family, namely, 22q, 21q, 1q, 2q and 10q (Blouin et al. 1995). This was more apparent in MRC5 where only 21q and 10q were detected in fusion events (Figure 4.2A). Thus to examine if the satellite was refractory to PCR, the assay was carried out with the oligonucleotide [subtel2], downstream of the satellite. Subtel 2 is an oligonucleotide that is shared amongst the members of 16p and 21q families [appendix figures 3A-1 and 3A-2]. Despite several attempts, the amplification across the satellite DNA failed (data not shown). These data suggest that the satellite sequence located on 22q, 21q, 1q, 2q and 10q could be refractory to PCR. However, using the subtel2 assay, the fusion frequency was 1.8x lower than that of the 21q1 [appendix Figure 4A-1A and 4A-1B, panels 1 and 2]. It was however not known whether the frequency was reflective of the ends that have eroded beyond the subtel2 primer site or the low amplification

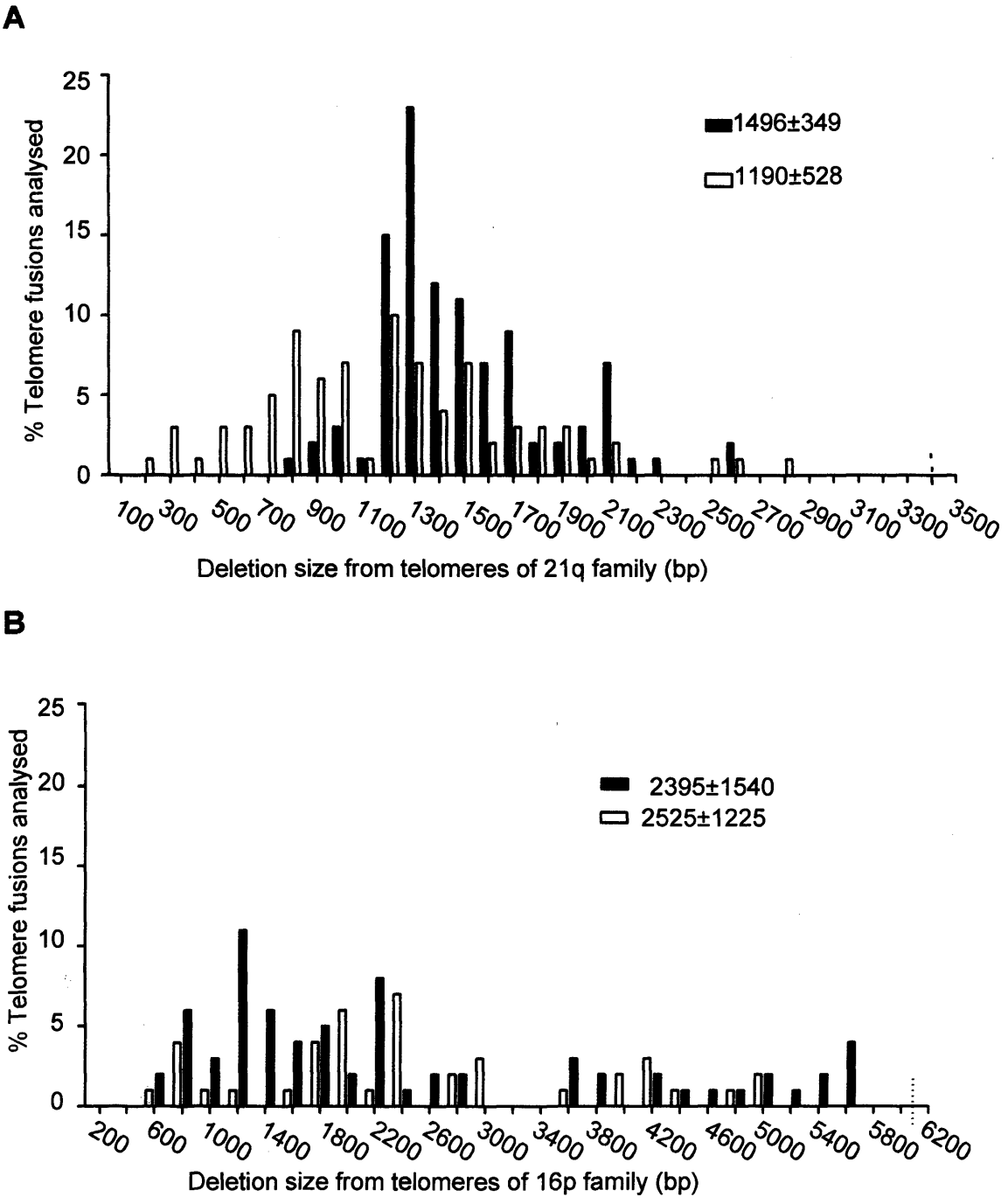


Figure 4.2. Histograms illustrating the deletion size from the start of telomere repeat array of telomeres of 21q (A) and 16p (B) families, in telomerase -positive HEK293 (open bars) and telomerase -negative MRC5 expressing E6E7 (solid bars). The limit of the assay is indicated with the dotted line. Means±SD are shown for each data set.

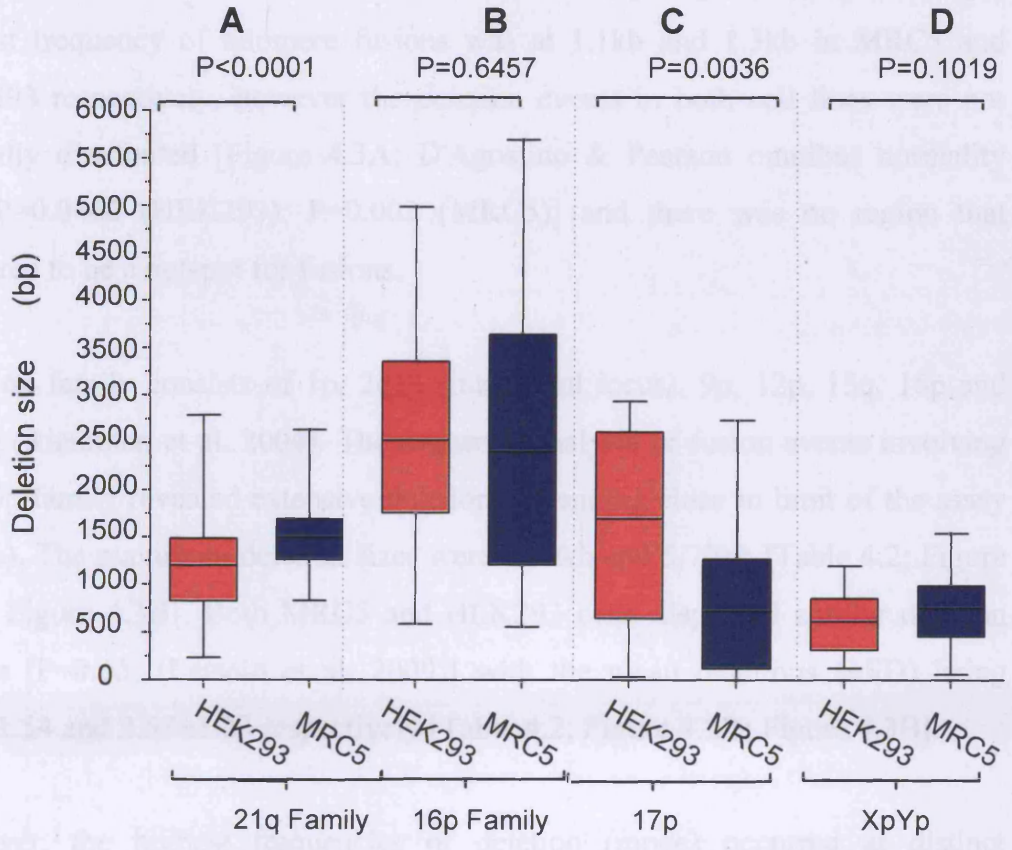


Figure 4.3. Box whisker plot summarising the size of subtelomeric deletions from analysis of telomere fusion events derived from HEK 293 (red boxes) and MRC 5 expressing E6E7 oncoproteins from all PD points sampled (blue boxes). The line within the box represents the mean deletion size, the lower (bottom of box), upper (top of box) quartiles, minimum (bottom whisker) and maximum (upper whisker) deletions size are shown. A-D. illustrate deletion sizes from the telomeres of 21q family, 16p family, 17p and XpYp respectively. The P-values for pair-wise comparison of the data are shown for each data set

efficiency of the primer. Within the 21q family, the deletion events extended close to the limit of the assay, of 3.4kb from the start of the telomere repeat array. However, the distribution was random with no clear correlation between the distance from the start of the repeat array and the frequency of fusion events. The highest frequency of telomere fusions was at 1.1kb and 1.3kb in MRC5 and HEK293 respectively, however the deletion events in both cell lines were not normally distributed [Figure 4.3A; D'Agostino & Pearson omnibus normality test, P=0.0460 (HEK293); P=0.002 (MRC5)] and there was no region that appeared to be a hotspot for fusions.

The 16p family consists of 1p, 2q14 (interstitial locus), 9p, 12p, 15q, 16p and XqYq (Riethman et al. 2004). The sequence analysis of fusion events involving the 16p family revealed extensive deletions extending close to limit of the assay (6.1kb). The maximum deletion sizes were 4.99kb and 5.70kb [Table 4.2; Figure 4.2B; Figure 4.3B]. Both MRC5 and HEK293 cells displayed similar deletion profile [P=0.65; (Letsolo et al. 2009)] with the mean deletions (±SD) being 2.40±1.54 and 2.53±1.23 respectively [Table 4.2; Figure 4.2B; Figure 4.3B].

However, the highest frequencies of deletion (mode) occurred at distinct distances from start of telomere repeat array [MRC5, 2.2kb; HEK293, 0.88kb] with the data derived from HEK293 being normally distributed [P=0.2561] whilst that from MRC5 was not [P=0.0072]. Interestingly, the frequency of the fusion events distal to the mode appeared to remain constant per unit length [Table 4.2; Figure 4.2B]. Thus, the data derived from fusion events involving telomeres of 16p show that the subtelomeric deletion can be very extensive and may extend beyond the limit of the assay.

Within the telomere-adjacent DNA of telomeres of 16p family lies the 29bp repeat array, which terminates 515bp from the start of the telomere array (Brown et al. 1990; de Lange et al. 1990). The 29bp repeat is a minisatellite that has 85% GC content. Downstream of the minisatellite lies another 37bp repeat array

Table 4.2 Descriptive statistics summary of deletion size from telomere fusion data derived from HEK293 and MRC5.

	16p group		21q group		XpYp		17p	
	MRC5	HEK293	MRC5	HEK293	MRC5	HEK293	MRC5	HEK293
Mean	2395	2525	1496	1190	772	609	715	1680
Standard Error	183	191	34	58	52	78	166	287
Median	1839	2328	1386	1180	756	540	289	1879
Mode	2187	883	1320	1180	986	853	248	2244
Standard Deviation	1540	1225	347	528	390	341	864	1034
Minimum	556	586	826	235	62	126	2	33
Maximum	5696	4986	2633	2790	1543	1198	2737	2938
Count	71	41	102	84	56	19	27	13
Confidence Level (95.0%)	364	387	68	114	104	164	342	625

(Brown et al. 1990; de Lange et al. 1990). These satellites could be refractory to PCR. Consistent with this view, the minimal deletion size observed in fusion events involving the telomeres of the 16p family was 586bp and 556bp in HEK293 and MRC5 respectively [Figure 4.2B; Figure 4.3B]. The presence of the minisatellite in this region could also account for failure to detect the fusion events containing telomeric repeats from the 16p family.

To explore if the minisatellite was refractory to PCR, the fusion assay was carried out with oligonucleotide primers [subtel2; subtel1; appendix Figure 3A-2] that were located downstream of both repeat arrays. Subtel 2 detects fusion events involving telomeres of 21q and 16p families while subtel 1 detects fusion events that involve the telomeres of the 16p family only [appendix Figure 4A-1, panels 3 and 4]. The putative fusion molecules detected with subtel1 could not be isolated and characterised as semi-nested PCR amplification failed.

We have previously illustrated that the analysis of fusion events between 17p and XpYp telomeres in MCF7 TRF2-knockdown cells resulted in fusion events that contained extensive TTAGGG repeats on both sides of the fusion junction (Capper et al. 2007). Thus, analysis of these cells was undertaken with the expectation of detecting fusion events that contain TTAGGG repeats from members of the 21q or 16p families. Despite multiple attempts, fusion events involving these ends were not detected in these cells. However, it was not known if the fusion events generated between these ends were too large to be resolved by gel electrophoresis or could not be amplified with the assays. Thus it remains to be determined if the assays are capable of detecting fusion events that contain telomere repeats from the telomeres of 16p family. As mentioned previously, the amplification of the fusion events containing telomere repeats from the telomeres of 16p family could be inhibited by the presence of the minisatellite mentioned above.

4.2.2.2.2 XpYp and 17p telomere

The deletion events extending into the sub-telomeric DNA of XpYp and 17p have been described previously for fusion events that involve XpYp with 17p (Capper et al. 2007). The analysis of fusion events involving telomeres of XpYp and 17p has revealed that the fusion events were accompanied by large deletions extending to the limit of the assay [3kb (at 17p) and 1.7kb (at XpYp); (Capper et al. 2007)]. In this study, sub-telomeric deletions for fusion events involving 17p or XpYp with telomeres of the 21q and 16p families are described. A total of 191 [HEK293, n=73; MRC5, n=118]; and 107 [HEK293, n=52; MRC5, n=60] fusion events involving XpYp and 17p respectively were sequenced. Notably, only 39% [MRC5, n=56/118; HEK293, n=19/73] of the telomere fusion events that involved XpYp showed a deletion extending into the telomere-adjacent DNA [Table 4.2].

Both MRC5 and HEK293 cells displayed similar deletion profiles at the XpYp telomere [P=0.1019] with a combined mean deletion (±SD) of 0.69±0.37kb [Table 4.2]. The highest frequency of deletion (mode) in both cell lines [0.986kb (MRC5); 0.853kb (HEK293)] was occurring around same distance, with data being normally distributed [Figure 4.3D; Figure 4.4A; HEK293, P=0.1970; MRC5, P=0.2570]. Interestingly 91% (MRC5) to 100% (HEK293) were clustered within 1200bp of the telomere repeat array [Figure 4.3D; Figure 4.4A] with distributions beyond 1200bp appearing to be randomly distributed extending to the maximum deletion of 1.198kb and 1.543kb in HEK293 and MRC5 respectively, very close to the limit of the assay, 1627bp.

Similarly, the deletion profile at 17p was extending close to the limit of the assay 3.1kb [HEK293, 2.9kb; MRC5, 2.7kb]. However, only 36% of fusion events [MRC5, n=27/60; HEK293, n=13/52] involved a deletion extending into the 17p telomere-adjacent DNA [Figure 4.3C; Figure 4.4B]. In 17p, the deletions were randomly distributed with the minimal and maximum deletion being 33bp/2938bp and 2bp/2737bp; and the highest frequency (mode) of deletion

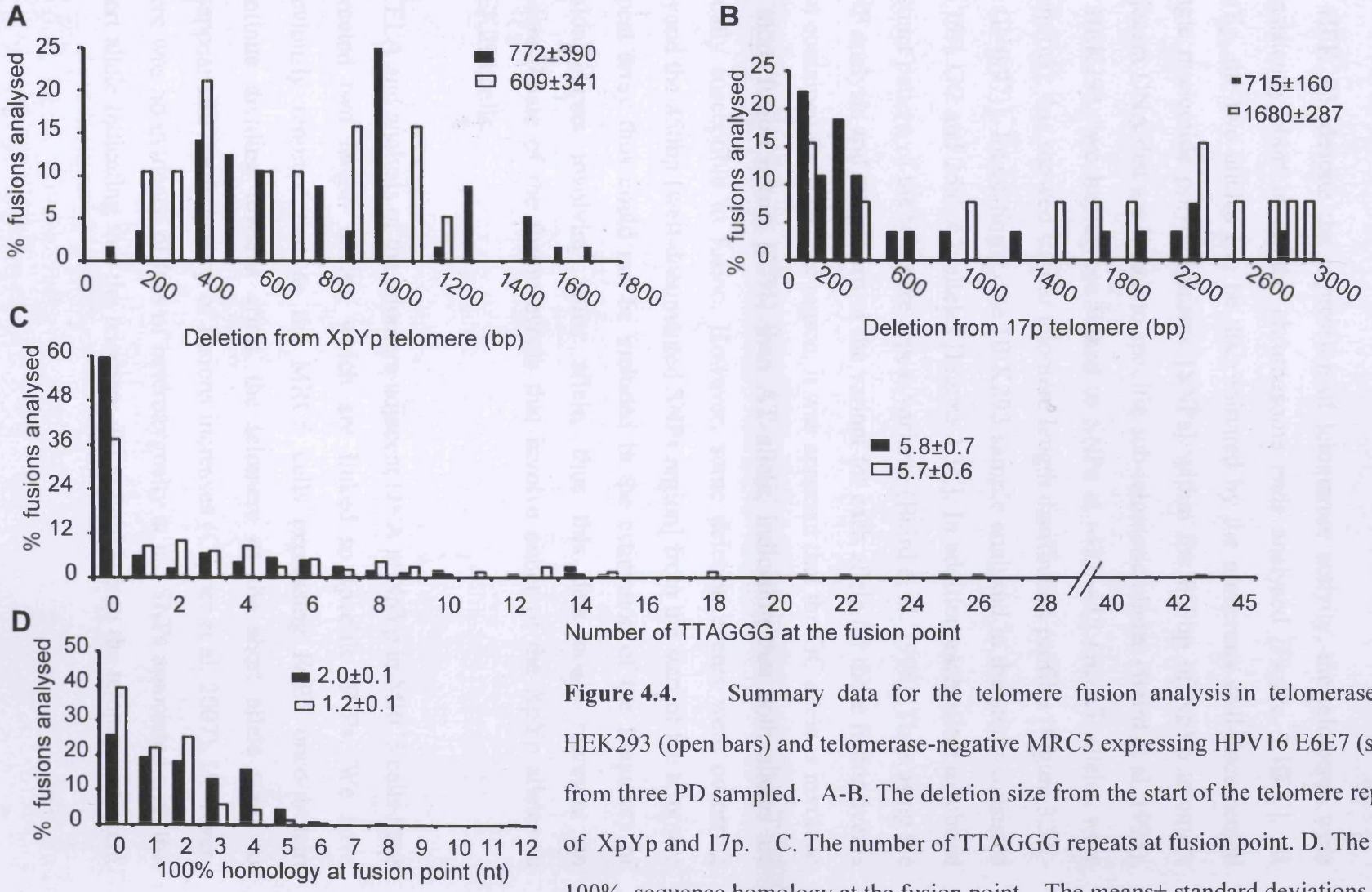


Figure 4.4. Summary data for the telomere fusion analysis in telomerase-positive HEK293 (open bars) and telomerase-negative MRC5 expressing HPV16 E6E7 (solid bars) from three PD sampled. A-B. The deletion size from the start of the telomere repeat array of XpYp and 17p. C. The number of TTAGGG repeats at fusion point. D. The size of the 100% sequence homology at the fusion point. The means± standard deviations (SD) are shown for each data set.

being at 2.244kb and 0.248kb in HEK293 and MRC5 respectively [Table 4.2; Figure 4.3C; Figure 4.4B].

In HEK293, despite the expression of telomerase activity, the telomeres were consistently short at all the chromosome ends analysed [Figure 3.3B-C]. At XpYp, the two alleles can be discriminated by the numerous well-documented single nucleotide polymorphisms [SNPs] within the 850bp of XpYp telomere adjacent DNA that are linked to specific sub-telomeric alleles (Baird et al. 1995). In HEK293, two haplotypes linked to SNPs at -427/-415 GC/AT alleles were identified; this showed similar telomere length distribution profiles [Figure 3.3A-C; (P=0.97)]. Interestingly, the HEK293 sample analysed in the study consisted of 75% GC and 25% AT alleles [Figure 3.3C]. In addition each allele exhibited distinct pattern of the telomere repeat variants (Baird et al. 1995). Thus using the SNP analysis and the pattern of the variant for each allele for those fusion events that contained the proximal region, it was apparent that the GC allele is involved in more fusion events (67%) than AT allele, indicating that both alleles are equally susceptible to fusion. However, some deletion events were occurring beyond the 850bp [well-documented SNPs region] from the start of the telomere repeat array thus could not be included in the estimation of the frequency of fusion events involving either allele, thus this data could represent an underestimate of the fusion events that involve either of the XpYp alleles in HEK293 cells.

STELA and analysis of the telomere-adjacent DNA at XpYp in MRC5 cells have revealed two length alleles, which are linked to specific SNPs. We have previously reported that as the MRC5 cells expressing E6E7 onco-protein continue dividing towards crisis, the telomere of the short allele starts to disappear and the frequency of fusions increases (Capper et al. 2007). However, there was no evidence of loss of heterozygosity at the SNPs associated with the short allele indicating that the telomere did not erode into the telomere-adjacent

DNA but was involved in fusion events with other telomeres (Capper et al. 2007).

Consistent with this, two alleles were involved in fusions with 17p (Capper et al. 2007). The current data has further revealed that indeed the short and the longer length alleles were involved in fusion events with the members of the 21q and 16p families. However, due to frequent deletions some of which were extending beyond the well-documented 850bp telomere adjacent DNA of XpYp, the ratio of events that involve either allele was not determined. The observation that both long and short XpYp alleles are involved in fusion events is consistent with large-deletion events at the longer allele resulting in short dysfunctional telomeres that are capable of fusion (Capper et al. 2007) coupled with telomere erosion at the short allele to range at which telomeres may be subjected to fusion.

4.2.2.3 TTAGGG and Micro-homology

In addition to large deletion events, the fusion events were characterised by limited TTAGGG repeats at the fusion point, with 38% to 60% of fusions in HEK293 and MRC5 respectively, containing no TTAGGG repeats at the fusion junctions [Figure 4.4C; Table 4.3; Figure 4.5A]. Of those that did contain TTAGGG repeats, the highest was 26.3 and 43 repeats in HEK293 and MRC5 respectively [Figure 4.4C; Table 4.3; Appendix Figure 4A-2A]. However, the mean (±SE) TTAGGG repeats was similar in both cell lines (5.7±0.6; 5.8±0.7), with the minimum being 0.3 and 0.2 respectively. In HEK293 cells, 80-90% of the fusion events that had TTAGGG repeats were occurring at the proximal part of the telomere that contains telomere variant repeats [TVRs; Figures 4.5C and 4.5G]. This proximal 1-3kb of human telomeres is highly variable and consists of canonical telomere repeat sequences, TTAGGG, interspersed with telomere variant repeats (Allshire et al. 1989; Baird et al. 1995). The telomere variant repeats are not bound by the telomere binding proteins TRF1 and TRF2 (Bianchi et al. 1999; Baumann and Cech 2001). This coupled with extreme variability of TVR, indicates that the TVR region is non-functional. This data is consistent

with this view, as it appears that telomeres that have eroded to within the TVR region are capable of fusion.

The fusion events that contained the longest TTAGGG tracts in MRC5 were observed in cells that have continued to proliferate for 6-8PDs beyond senescence. i.e. The two fusion events that contained long tracts of telomere repeats (21 and 43) were detected at PD32.2 where the telomere repeat arrays were longer, whilst fusion events derived from cells undergoing crisis (PD52) were characterised by large deletions on both sides of the fusion junction.

Out of the 308 fusion events sequenced, only one event revealed telomere repeats on each side of the fusion point, in this case 6.5 repeats from 17p and 3.5 repeats from 10q [Figure 4.5B]. This indicates that the assays are not refractory to inverted repeats on either side of the fusion point, consistent with our previous study (Capper et al. 2007).

Table 4.3. Descriptive statistical data of number of TTAGGG repeats and microhomology sequences at fusion point for fusion events derived from HEK293 and MRC5. S.E is standard error; S.D is standard deviation of the mean.

	Number of TTAGGG at fusion point		Micro-homology at fusion point			
					data excluding zero	
	HEK293	MRC5	HEK293	MRC5	HEK293	MRC5
Mean	5.7	5.8	1.2	2.0	2.0	2.7
SE	0.6	0.7	0.1	0.1	0.1	0.1
Median	3.8	4.4	1.0	2.0	2.0	2.0
Mode	1.0	1.0	0.0	0.0	2.0	1.0
SD	5.5	6.1	1.2	1.8	1.0	1.6
Minimum	0.3	0.2	0.0	0.0	1.0	1.0
Maximum	26.3	43.0	5.0	12.0	5.0	12.0

In addition to the characteristic large sub-telomeric deletions and lack of TTAGGG repeats at fusion junctions, the majority of fusion events [60-74% in HEK293 and MRC5 respectively] exhibited short patches of nucleotide homology at the fusion point between the participating telomeres [1-12nt, mean 1.7nt; Table 4.3; Figure 4.4D; Figure 4.5A-D and 4.5F-H]. These micro-homologies at fusion point showed a significant bias towards a G:C content of 39% A:T, 61% G:C (chi square, P= 0.002) in comparison to the A:T/G:C content of the telomere-adjacent sequences being investigated which consist of 48% A:T and 52% G:C.

4.2.2.4 Insertions and complex events

In addition to the fusion events between the chromosome ends described above, it was apparent that complex events could accompany the fusion of short dysfunctional telomeres. A subset of the fusion events (34/298) comprising 11% were characterised by an insertion of genomic sequences at the fusion junction between the chromosomes being studied. All of these insertions except six were made up of the simple insertions of 1-34 nucleotides [Figure 4.5E]; these insertions were too small for their origin to be identified. Four insertions that were large enough to be identified were mapped to interstitial sites in the human genome, near documented fragile sites. One was derived from 7p22.1, one from 8q24.3 [Figure 4.5H] and the other two from Xp22.1 [appendix Figure 4A-2F; (Chen et al. 1998; Debacker and Kooy 2007)]. Two additional fusion events contained an insertion between the partner chromosomes. These were distinct however because they involved fusion of one telomere to telomere-adjacent DNA of same telomere in reverse orientation. In MRC5, where two XpYp alleles can be distinguished by the SNPs within the telomere adjacent DNA, it was clear that these insertions were derived from the same allele as the one fused to it, and were therefore intra-allelic [Figure 4.5G].

In addition, fusion events were detected between XpYp and/ or 17p and the interstitial loci adjacent to the ancestral telomere-telomere fusion locus, 2q14; a

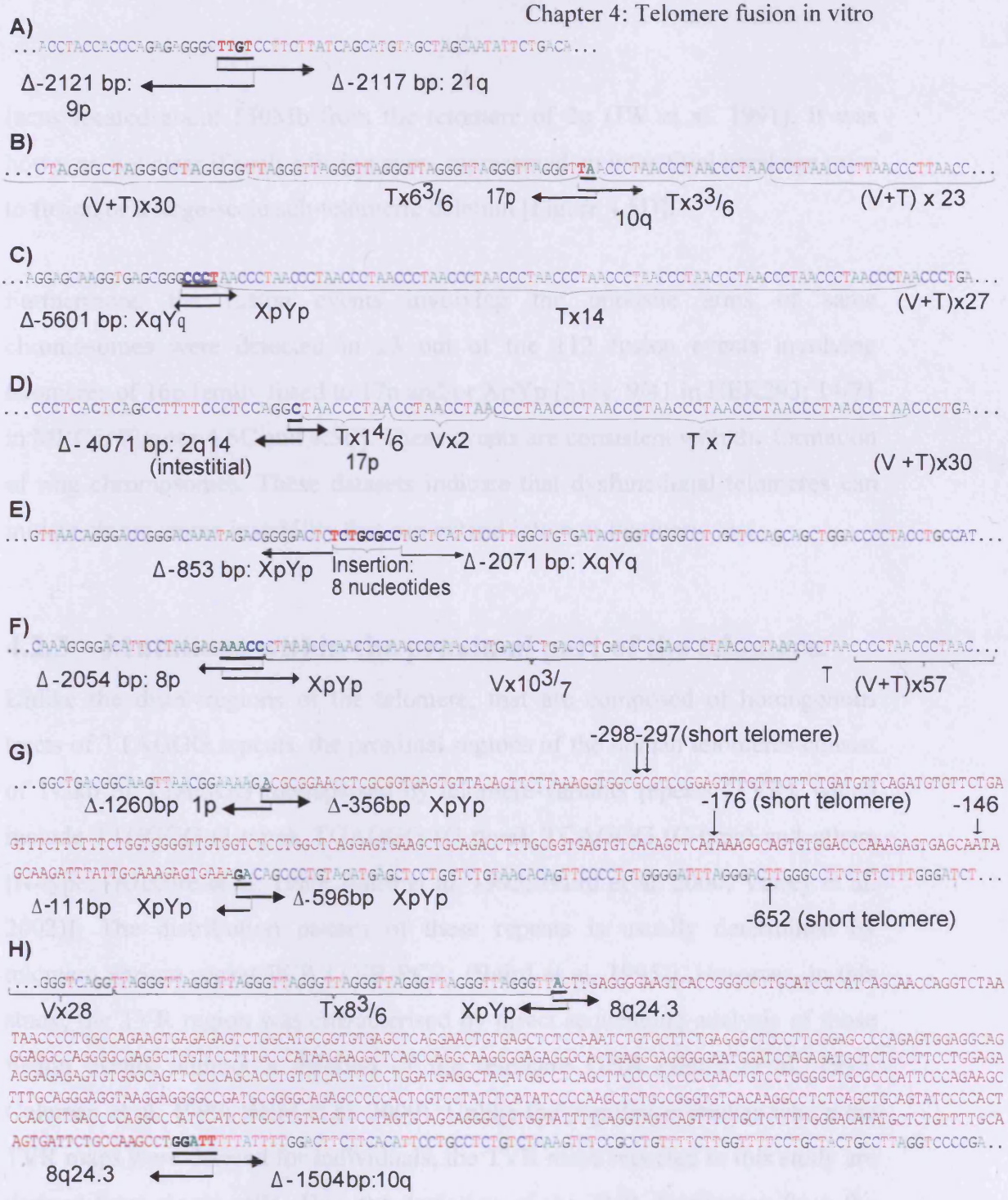


Figure 4.5. Examples of DNA sequences for the telomere fusion events. A) 16p:21q group fusion. B) 17p:10q showing a fusion with TTAGGG on either side of the fusion point.C) XpYp:XqYq showing large deletion event in XqYq telomere-adjacent DNA. D) fusion of 17p to the intestitial locus 2q14. E) XpYp:XqYq with a simple insertion. F) XpYp:8p showing fusion at the proximal part of the telomere G) 1p:XpYp, an example of an event with insertion from same telomere (XpYp) in reverse orientation, SNPs identifying the short allele are indicated with arrows.H) XpYp:10q, an event with large insertion from non-telomeric locus 8q24.3. large insertions are shown in brown. The TTAGGG (T) and variants (V) are indicated for each sequence. Microhomology nucleotides are bolded and underlined

locus located about 130Mb from the telomere of 2q (JW et al. 1991). It was however, not clear if such a fusion event represented an interstitial breakage prior to fusion or a large-scale sub-telomeric deletion [Figure 4.5D].

Furthermore, the fusion events involving the opposite arms of same chromosomes were detected in 23 out of the 112 fusion events involving telomeres of 16p family fused to 17p and/or XpYp [21%; 9/41 in HEK293; 14/71 in MRC5; Figures 4.5C and 4.5E]. These events are consistent with the formation of ring chromosomes. These datasets indicate that dysfunctional telomeres can initiate chromosome instability that can extend into non-telomeric loci.

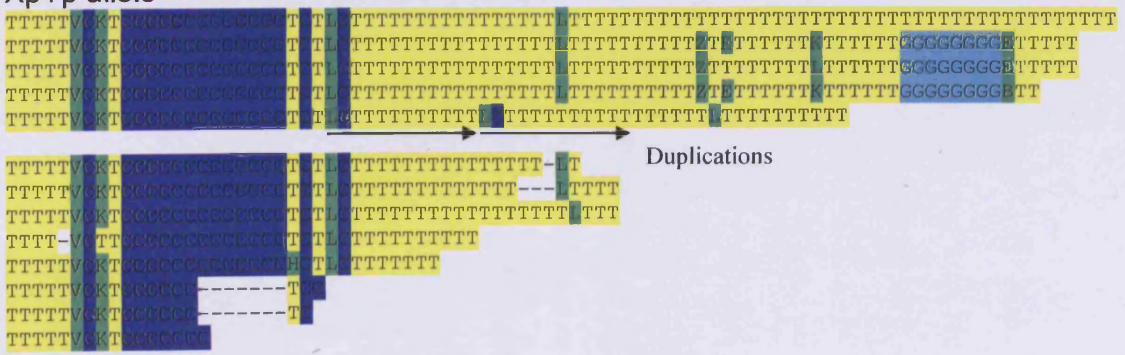
4.2.3 Mutations within the proximal part of the telomere.

Unlike the distal regions of the telomere, that are composed of homogenous tracts of TTAGGG repeats, the proximal regions of the human telomeres consist of 1-3kb of TTAGGG interspersed by telomere-variants repeats (TVR), which include TTGGGG (J-type), TGAGGG (G-type), TCAGGG (C-type) and others [N-type; (Allshire et al. 1989; Baird et al. 1995; Baird et al. 2000; Varley et al. 2002)]. The distribution pattern of these repeats is usually determined by telomere variant repeat PCR [TVR-PCR; (Baird et al. 1995)]. However, in this study, the TVR region was characterised by direct sequencing analysis of those fusion events without a deletion of the telomere DNA (Baird et al. 1995; Coleman et al. 1999; Baird et al. 2000). Unlike the population studies where the TVR maps were derived for individuals, the TVR maps reported in this study are derived from single cells. Thus the deviation of the TVR distribution from the parental as determined by aligning all the TVR maps, would imply a mutation within a specific cell. In addition, the presence of the same pattern of deviation from the putative parental TVR pattern in more than one event derived from same cell strain would imply that such mutation was stably maintained through cell division. Detailed analysis of XpYp telomere and other autosomal telomeres 16p, 16q and 12q in different populations have revealed extensive allelic

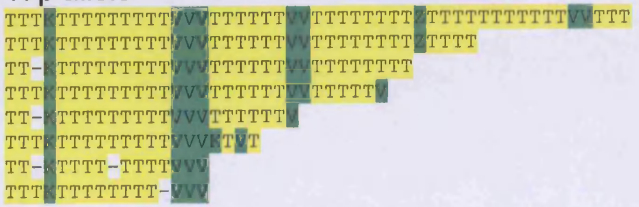
Figure 4.6. See next page for legend

MRC5

XpYp allele



17p allele

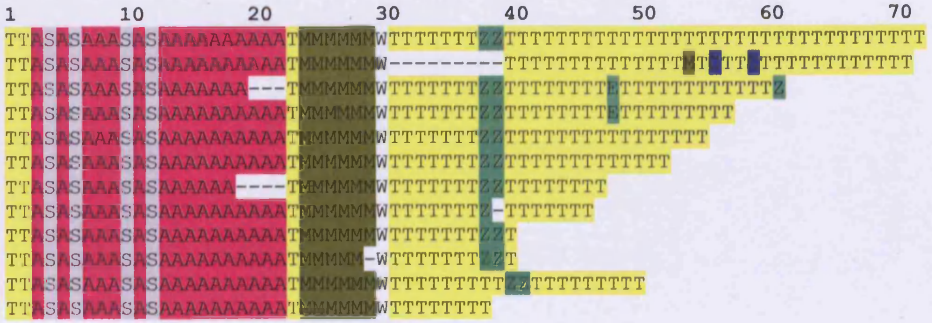


KEY	
TTAAGG	D
TTAGAGGG/TTAAGGG	B
TTTAGGG	V
TGAGGG	G
TTAGGG	T
TTGGGG	J
TTCGGG	A
TTCGGGG	S
CTAGGG	M
CTAGGGG	W
TCAGGG	C
TAGGG	E
TAGGGG	H
TTAGAG/TTAGCG	I
TTAGGGG/TCAGGGG	K
TCGGG/TTCGG/TTGGG	Y
TTAGG	Z
TTTGGG	L
TTTTAGGG	P

HEK293

17p

Allele 1



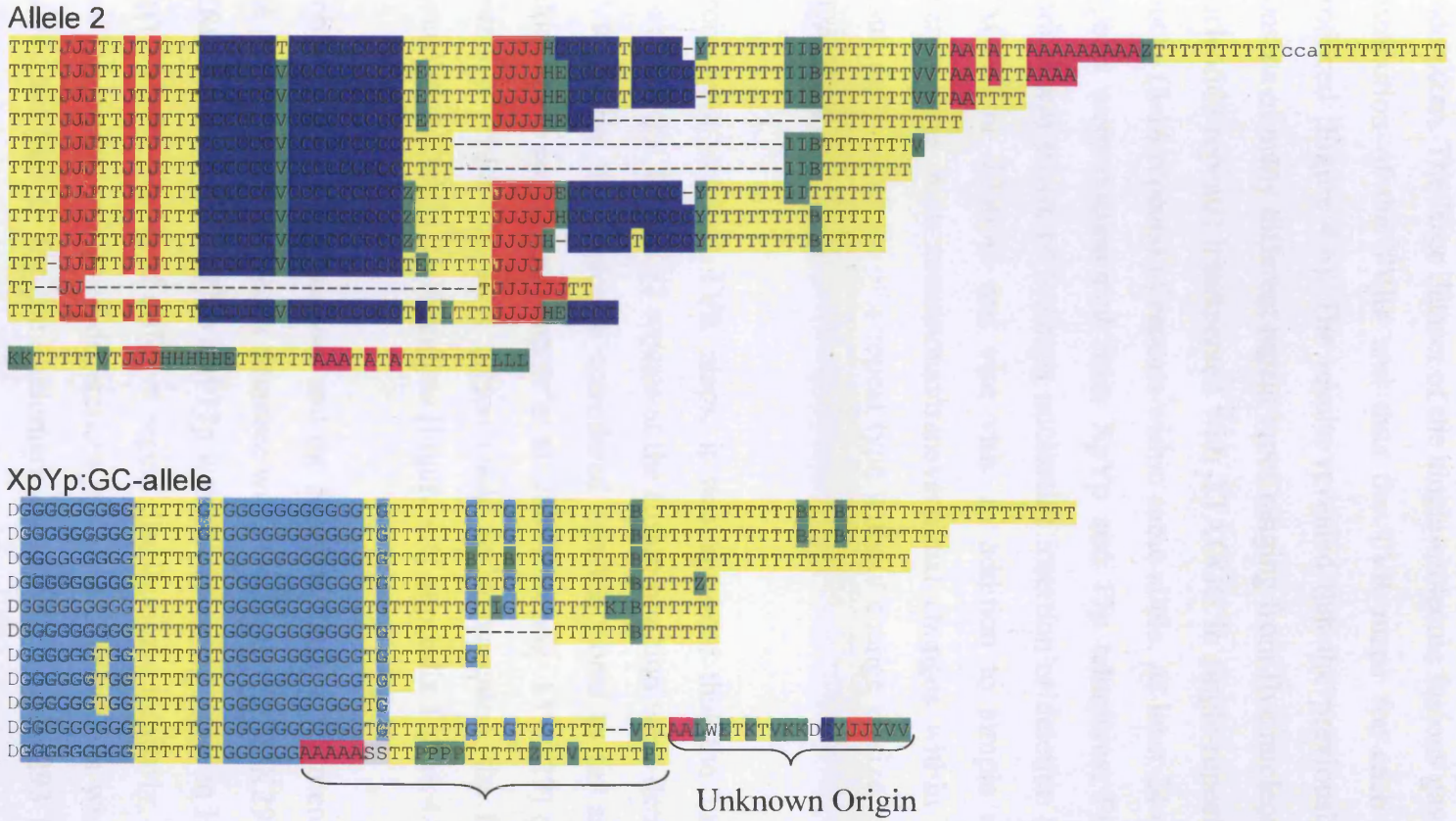


Figure 4.6. Telomere variant repeat structure derived from telomeres of XpYp and 17p in HEK293 and MRC5 E6E7 cells. The key consists of letter used to denote the different repeats types, the gaps (-) were introduced to improve the alignment and thus represent putative expansions and contractions in repeats blocks. The duplication is shown by arrows.

variation and hypervariability at the proximal end of the repeat array (Baird et al. 1995; Coleman et al. 1999; Baird et al. 2000). Thus, to investigate whether such variability was reflective of the mutational spectrum at a single-cell level, a detailed analysis of the TVR region of the XpYp and 17p telomeres was undertaken. The large dataset of the single-molecule fusions gave comprehensive distribution of the TVRs and thus the TVR maps for each cell strain were produced [Figure 4.6]. The results revealed that the previously named N-type consists of many different repeat types ranging from five nucleotide repeat to >9-nucleotide repeats; interspersed with TTAGGG in single-repeats and/or varying blocks (3-16 repeats) of repeats within same allele. At least 24 different types of repeats were documented from XpYp and 17p telomeres. Furthermore, each variant was subjected to single nucleotide insertion or deletion e.g. TTAGGG to TAGGG or TTAGG and vice visa. In addition to simple deletion/insertion events, there were transitional/transversional changes within the repeat array resulting in a change of a repeat type without change in size. e.g. TTAGGG to TCAGGG.

From these detailed TVR maps, it was apparent that the fusion events that contained the TTAGGG repeats at the fusion junction were derived from within TVR region; this region is considered non-functional as far as the capping of telomeres is concerned (Capper et al. 2007). In only 14% (29) of the 201 fusion events in which the TVR region could be documented, the fusion point was located distal to the TVR regions [Figure 4.6; appendix Figure 4A-3].

Combining the TVR analysis and the SNPs within the telomere-adjacent DNA, the allelic variation at each telomere was determined in HEK293 and MRC5. In MRC5, one allele at XpYp and 17p was characterised while in HEK293, the two XpYp alleles displayed different repeat distributions. Similarly, at 17p telomere, two alleles that exhibited differences in repeat distributions were characterised. Allelic analysis of the same telomere e.g. XpYp in HEK293 has revealed the mutational changes in the pattern of variants distribution. There was evidence of

contractions/expansions of the repeat blocks resulting from deletions/insertions of single or blocks of repeats. In addition, duplications of up to 12 repeats were also observed. Within a specific allele, the variation in the distribution profile could be attributed to simple events such as single nucleotide additions or deletions, converting one repeat type to another. Furthermore, the size of the repeat block was either contracting or expanding. Some of these events were detected more than once, indicating that the event was derived from more than one cell. This data suggested that once the event occurred, it can be stably maintained through cell division and thus result in clonal expansion. This is consistent with the clonal fusion events described in the study. However, the mutation events within the TVR region are unlikely to arise as a consequence of the fusion events as they were detected in independent and unrelated fusion events.

In addition to simple events, complex events involving insertion of the block of repeats with no obvious origin were characterised immediately adjacent to the fusion point in two fusion events [Figure 4.6; appendix Figure 4A-3]. It was however not clear whether they represented a mutational event resulting from the processing of sequences at the fusion junction or the insertion from another telomere.

4.3 Discussion

4.3.1 Telomere fusion analysis

Human cancers arise through accumulation of genetic alterations. One of the mechanisms that may confer tumourigenic potential is the state of the telomere, thus telomere-induced chromosome instability is a subject of extensive research in cancer studies. Telomere dysfunction through loss of the telomere cap or telomere attrition has been shown to result in telomere fusions (van Steensel et al. 1998). The fused chromosomes can break and fuse again fuelling large-scale chromosome instability such as amplifications, deletions and complex

rearrangements, which are some of the early lesions that drive tumourigenesis in epithelial cancers (Artandi et al. 2000; Rudolph et al. 2001). Previous studies have shown that telomeres are subjected to processes that generate short telomeres (Murnane et al. 1994; Baird et al. 2006; Capper et al. 2007). Thus to determine the fate of the critically short telomeres, the PCR-based technique that detect fusion events between XpYp and 17p telomeres was developed. That study revealed that fusion of dysfunctional telomeres was accompanied by large sub-telomeric deletions, lack of TTAGGG and short patches of nucleotide homologies at the fusion point (Capper et al. 2007). The assay has since been extended to include at least 43% of the human genome (Letsolo et al. 2009). Here, I have described a comprehensive analysis of the telomere fusion events between telomeres of XpYp, 17p, 16p family [1p, 9p, 12p, 15q, 16p, XqYq and interstitial 2q14] and 21q family [1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q, 22q and interstitial 2q13] in HEK293 and MRC5 cells that have escaped senescence. In telomerase-positive cell line, HEK293, all telomeres analysed displayed short length profile with increased frequency of telomere fusions. It appears that telomerase in this cell line is not sufficient to counteract the effect of telomere erosion. Indeed it has been shown that some of the clones within HEK293 lack detectable telomerase activity and thus maintain short telomeres (Bryan et al. 1998). Interestingly recent work in our lab has isolated clones that maintained longer telomeres and did not display any telomere fusions (DMB personal comm.).

In the absence of telomerase gradual telomere loss in human fibroblasts as a function of replicative age could contribute to cell senescence (Harley et al. 1990; Levy et al. 1992). However, inactivation of the p53/Rb pathways by oncogenic transformation extends the replicative capacity of fibroblast for several PDs. This extended life span results in further telomere erosion until cells reach crisis, the state characterised by extensive chromosome instability and cell death. However, cells that survive are generally aneuploid, maintain stable telomere lengths and express telomerase activity or a recombination-based

mechanism (Wright and Shay 1992; Ducray et al. 1999). To investigate the chromosome instability in cells that have bypassed senescence and continued proliferating to crisis, we have extended the replicative life span of the human lung fibroblast cell line, MRC5 through forced expression of HPV16 E6E7 oncoproteins (Capper et al. 2007). The analysis of these cells has revealed that as the cells continue dividing towards crisis, the telomere erosion was inversely proportional to frequency of fusion events. The longer alleles were becoming shorter while eroded shorter length alleles were actual disappearing. The short telomeres that are disappearing tend to fuse with other telomeres. Because the longer alleles are subjected to random deletions, they could become uncapped and are involved in telomere fusions. This was apparent in analysis of MRC5 cells, which have two different lengthed XpYp alleles that could be distinguished by SNPs within the telomere-adjacent DNA. It was apparent that both alleles were involved in fusion events; consistent with the data that in addition to end-replication losses resulting in gradual telomere erosion as a function of time, telomeres were also subjected to random deletion events, both generating short, dysfunctional telomeres capable of fusion (Capper et al. 2007).

Unlike in HEK293, where several ends were involved in fusion events, in MRC5, two ends, 10q and 21q representing the members of 21q family were detected in fusion events with XpYp or 17p. Absence of other ends in fusion events could suggest that in MRC5, only a subset of chromosome ends have eroded to within the length range at which fusion is detected. In support of this, STELA at the telomeres of 2p, 11q and 12q have revealed that MRC5 exhibited longer telomeres at these ends compared with XpYp and 17p, with none of the telomeres exhibiting length within the range that yields telomere fusions (Britt-Compton et al. 2006; Capper et al. 2007). In addition, telomere fusion analysis did not reveal any fusion events involving XpYp, 17p and these ends [(Capper et al. 2007); DMB personal comm.]. These data is consistent with the view that telomeres need to erode to critical threshold prior to being subjected to fusion. An alternative explanation could be the existence of large allelic variation at sub-

telomeres, extending beyond the scope of our assays, i.e. if the cells are carrying longer sub-telomeric alleles, the positions of the oligonucleotide primers used in these assays could be located several kilobases away from the start of the telomere repeat array and thus the size of fusion molecules could be beyond the amplifiable range of PCR products. Large allelic variation at sub-telomeres has been described at 16p telomere in which its several alleles vary by as much as 260kb (Wilkie et al. 1991), thus involvement of such large alleles in fusion events would be beyond the scope of these assays.

The minimum deletion size in telomeres of 16p and 21q families was restricted to the regions proximal to the GC-rich satellite DNA. This could be refractory to PCR, several attempts were employed to examine these possibilities and it was found that at the telomeres of 21q family, notably 21q and 10q, the telomere fusion events that contain telomeric repeats could be detected. However, the frequency of such events was very low regardless of the oligonucleotide primers used for detecting the telomeres of both families simultaneously. Therefore, I concluded that the extended assays [3.5kb at 21q family and 6.1kb at 16p] could be limited to detecting the fusion events with deletions upstream of the satellite DNAs.

4.3.2 Mechanistic basis of telomere fusions

The comprehensive analysis of the telomere fusion events between multiple chromosomes described here indicated that fusion of short dysfunctional telomeres is accompanied by deletion of telomeric and sub-telomeric DNA; this is consistent with our previous study of fusion events between XpYp and 17p (Capper et al. 2007; Letsolo et al. 2009). The large deletions of up to 5.6kb are of interest especially in telomeres of the 16p family, in which the deletions occur within the Wiskott-Aldrich syndrome protein and SCAR homologue (WASH) gene family (Linardopoulou et al. 2007; Riethman 2008b; Riethman 2008a). Although their function is not yet known, the data indicates that the deletion

within the sub-telomeric region can be very extensive extending to coding DNA within the region. This could create significant mutational burden that may result in misregulation of sub-telomeric genes thus leading to diseases. Indeed several terminal deletions have been associated with many human diseases, for instance, terminal deletions of 16p result in alpha-thalassemia (Hatton et al. 1990; Wilkie et al. 1990; Flint et al. 1994); terminal 10q deletion syndrome is associated with typical craniofacial appearance and varying degrees of psychomotor retardation found in patients with 10q-syndrome (Petersen et al. 1998).

Whilst the mechanism that underlies this type of event is not yet known, the characteristic profile consisting of large deletions at telomeric loci is consistent with other observations: for example, the repair of I-sceI-induced double strand breaks near telomeres generated large deletions (Zschenker et al. 2009). In addition, in cdc13 mutants of *Saccharomyces cerevisiae*, the processing of single-stranded DNA can result in telomere and sub-telomeric deletions extending up to 8kb from the start of the telomere repeat array (Maringele and Lydall 2002).

In addition to deletions, the fusion events lacked telomeric TTAGGG repeats on either or both side of the fusion point. Consistent with data described here, chromosome fusions that lack telomere repeats at fusion junction have been described in normal (Takubo et al. 2010) and transformed (Zou et al. 2009) human fibroblasts. Furthermore, more than 70% of fusions contained patches of homology nucleotides at the fusion point, of these 7% contained five nucleotides or more at the fusion point. Such fusion events are distinct from those generated through LIG4-mediated non-homologous end joining (TRF2-dependent); a mechanism involved in repair of long unprotected telomeres (van Steensel et al. 1998). The fusion events generated when the function of TRF2 is inhibited are characterised by long tracts of the TTAGGG repeats on either side of the fusion point (van Steensel et al. 1998). The mechanism that underlies fusion events described here is consistent with some form of an error-prone processing that is

facilitated by GC-rich micro-homologies. Alternative NHEJ pathways have been described in mammalian cells and tumours (Wang et al. 2003; Wang et al. 2006) as well as in class switch recombination in lymphocytes during synthesis of the immunoglobins in the absence of Ku70 and ligase 4 (Boboila et al. 2010). Such mechanisms are independent of DNA-PKcs and Ku, and may rely on the GC-rich micro-homologies to stabilise the complex prior to ligation of the ends together (Wang et al. 2003; Wang et al. 2006). The well-characterised alternative mechanism to NHEJ, which operates as a backup to NHEJ is micro-homology-mediated end-joining (MMEJ). Whether MMEJ represents one mechanism or several alternative mechanisms to NHEJ is controversial, such that the clear distinction need be made when discussing MMEJ and alternative NHEJ mechanisms. It is clear however that not all alternative end-joining events are MMEJ and similarly, some alternative end-joining events are distinct from MMEJ (Mcvey 2008).

MMEJ is an error-prone, ku-independent repair than involves use of micro-homologies, which has been described in yeast and *Xenopus* (Ma et al. 2003). It is characterised by the use of 5-25bp micro-homology sequences during the repair of double-stranded breaks. In addition, such repair is accompanied by deletion of the DNA flanking the break (Yu and Gabriel 2003). A subset of the fusion events described here is consistent with micro-homology mediated end joining that involves use of 5-25 nucleotides (micro-homology sequences). More fusion events (93%) contained less than five nucleotide micro-homologies. Although the fusion events have two subsets (i.e. micro-homologies greater than or less than 5 nucleotides) of micro-homologies, the mechanism is consistent with some form of error-prone DNA repair mechanism which as yet is unknown whether is distinct from MMEJ or represents an alternative or subtype of MMEJ. In support of this, DSBs induced by the yeast site-specific homing endonuclease I-*Sce*I in KU70$^{-/-}$ mammalian cells are repaired via MMEJ. However, some breaks are repaired using micro-homology-independent pathways (Bennardo et al. 2008). Like the repair of I-SceI-induced DSBs, which consists primarily of

large deletions, the mutational profile of the fusions described here is similar to that involving an alternative mechanism to NHEJ (Richardson and Jasin 2000; Zschenker et al. 2009).

In mammals, MMEJ has been associated with large deletions, complex rearrangements, translocations and cancer (Weinstock et al. 2007). For instance mice lacking functional p53 and NHEJ components, ku or lig4 develop pro b-cell lymphomas with multiple translocations characterised by presence of 2-8 nucleotide micro-homologies (Difilippantonio et al. 2000; Ferguson et al. 2000). The chromosome fusion events that are consistent with MMEJ have been observed in $tert^{-/-}/Ku^{+/+}$ mutants of Arabidopsis (Heacock et al. 2004; Heacock et al. 2007), *Drosophila* and yeast (Ma et al. 2003). In addition, telomere fusions that are independent of the NHEJ component, DNA PKcs has been described in mice without telomerase (Maser et al. 2007). This pathway is error-prone resulting in large deletions and is characterised by patches of micro-homologies at the fusion point with a bias towards G:C content (Audebert et al. 2008).

4.3.3 Telomere fusion has potential to form ring chromosomes

The fusion events involving the XpYp and XqYq were frequently detected, these events have a potential to form ring chromosomes. Formation of ring chromosomes is often accompanied by loss of chromosome ends, enabling the arms to fuse together (Miller and Therman 2001). However, ring formation can also occur with only one end being lost as in a case where the broken chromosome fused with the opposite telomere region (Arnedo et al. 2005). The ring chromosomes can also form without loss of genetic information as in case of telomere-telomere fusion. Although, they do not result in loss of genetic information, ring chromosomes have been observed in many human diseases. For instance, ring chromosome 20 is associated with epileptic seizures, behaviour disorders and mental retardation (Schinzel and Niedrist 2001), ring chromosome 14 (Schmidt et al. 1981) is associated with mental retardation, while ring X

chromosome is associated with turner syndrome (Dennis et al. 1993; Miller and Therman 2001). It has been postulated that while they do not result in loss of genetic material, the ring syndromes are a result of the instability of the ring during mitosis. Indeed, it has been shown that the ring chromosomes can form anaphase-bridges, which could induce continuous rearrangement through breakage-fusion-bridge cycles (Gisselsson et al. 2001; Miller and Therman 2001). Ring chromosomes are frequently detected in tumour cells and sarcomas, however the frequency of ring chromosomes is lower (<10%) for carcinomas (Gisselsson 2002). One of the mechanisms that generate ring chromosomes is loss of telomere function resulting in fusion of the two arms of the same chromosomes. Such ring chromosomes are unstable during mitosis (Miller and Therman 2001; Sumner 2003). The chromosomal breakage-fusion-bridge events have been implicated as mechanisms that increase the genetic heterogeneity of tumours (Gisselsson et al. 2000).

4.3.4 Telomere dysfunction can induce complex rearrangements

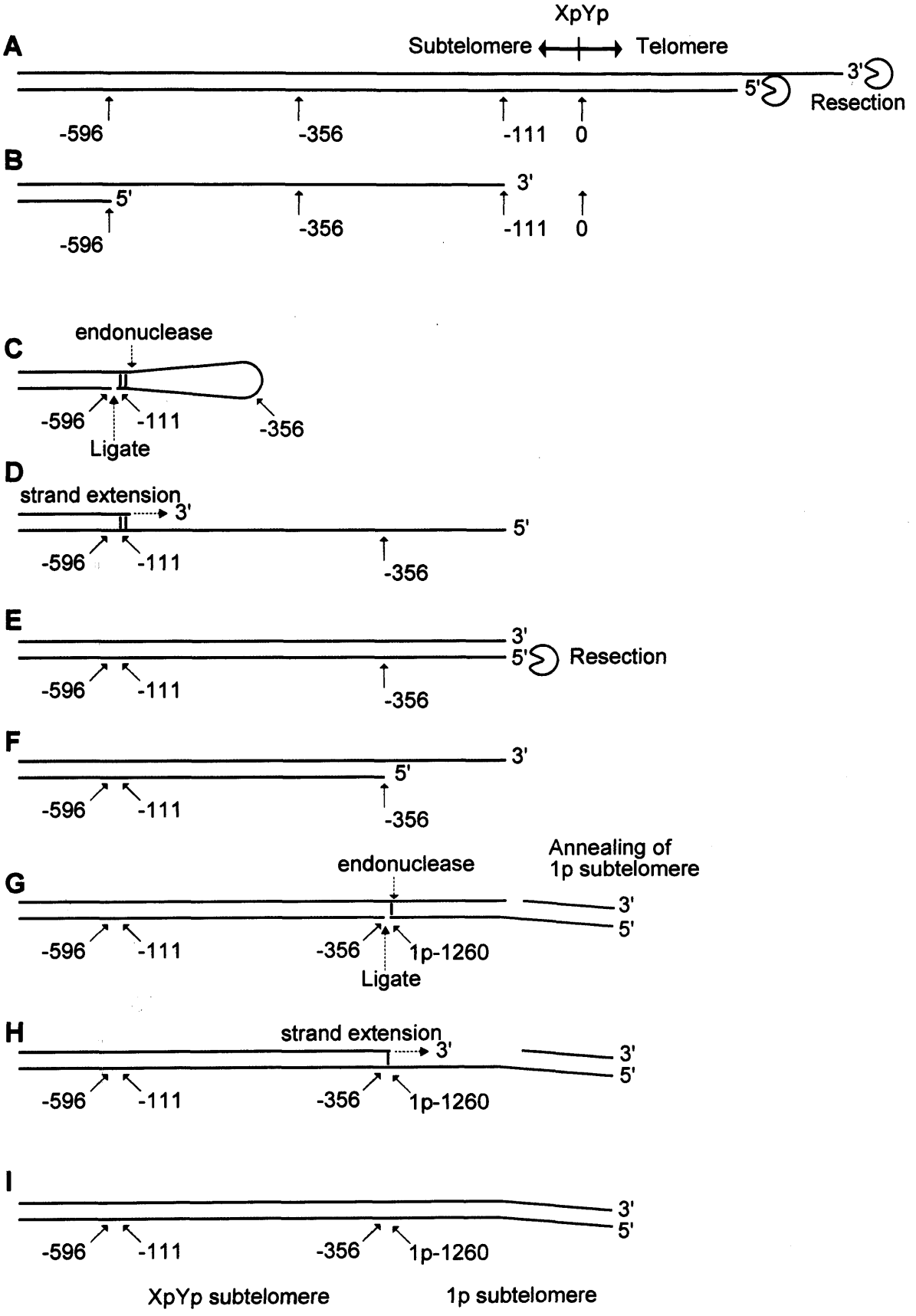
This study has also detected the fusion events that contained an insertion of the telomere-adjacent DNA derived from one of the ends involved in a fusion event. Using the informative SNPs in the telomere-adjacent of XpYp, it was apparent that the inserted DNA was derived from the same allele as the one involved in a fusion event in reverse orientation; these are consistent with intra-allelic events. The mechanism by which the segment of the same chromosome fuse in reverse orientation is not known but could include a sister chromatid exchange resulting in inverted repeats at the fusion point; which have been shown to be unstable thus creating a fragile site that could break in successive cell divisions. Once broken an uncapped chromosome can then fuse to another telomere resulting in the type of fusion event described (Lo et al. 2002). It was however, not possible to verify the intermediate type of fusion using our assays, as the assays can only detect sister chromatid-type fusions when at least one of the telomere repeats has been deleted creating an imperfect repeat (Capper et al. 2007). In our recent

publication, we have proposed an alternative mechanism, in which the telomeres are processed by an exonuclease that creates single-stranded overhang. Once established the overhang may loop back and invade the sub-telomeric DNA. Then the looped DNA can be processed by endonucleases, which generate the 5′ overhang. The resulting overhang can then fuse to another telomere or a double strand break [Figure 4.7; (Letsolo et al. 2009)].

4.3.5 Complex events involving documented fragile sites.

The fusion events that involved non-telomeric loci (2q14, 7p22.1, Xp22.1 and 8q24.3) were identified near the known fragile sites; 2q13 (FRA2B), 7p22 (FRA7B), Xp22.3 (FRAXB) and 8q24.3 [FRA8D; (Buttel et al. 2004)]. Fragile sites are generally stable in normal cells; however, in tumours they can be involved in chromosomal aberrations such as deletions and translocations. For instance, terminal deletion involving 7p22 is reported in 22% of endomentrial-derived tumours (Gogusev et al. 1999). Similarly, an increase in copy number at 8q24 (c-myc gene; an oncogene mapped within 8q24) is frequent in human tumours. It has been shown that telomerase activity is correlated with gains of this locus, which support the fact that c-myc induces telomerase in human cells. Indeed Wang and colleagues have shown that c-myc induces telomerase activity in normal human mammary epithelial cells and human diploid fibroblasts, by increasing the expression of hTERT, a catalytic subunit of telomerase (Wang et al. 1998). Further support comes from the report that the promoters of hTERT have c-myc binding sites (Wu et al. 1999). The involvement of the locus in fusion events in cells undergoing crisis could suggest that the cells are activating a telomere maintenance mechanism and thus will become immortal. Indeed studies have shown that in mammals, telomere dysfunction can activate senescence, however cells that bypass senescence through oncogenic transformation continue dividing until they reach crisis, the mortality stage II that is characterised by large-scale chromosome instability and death (Wright and Shay 1992). However, cells that survive, activate a telomere maintenance

Figure 4.7. Illustrating a model, for the sequence of events, that may account for intra-allelic event in which Insertion of same allelic DNA in inserted in reverse orientation. (A–B) Unprotected, short telomeres are susceptible to nycleolytic attack that generates a 3′-overhang within the telomere-adjacent DNA. (C) The 3′-end of the overhang (−111) folds back upon itself and anneals adjacent to the −596 position, this is ligated and endonucleolytic cleavage creates a 5′-overhang structure which can be rendered double-stranded by strand extension (D). Further resection (E) to the −356 position creates a substrate (F) for annealing and ligation of the 1p sub-telomere at position −1260 (G). Endonucleolytic cleavage removes the unpaired 3′-overhang from XpYp (G), strand extension and ligation complete the fusion (H–I). [reproduced from Letsolo et al 2009 with permission from producer, DMB]


XpYp
A
Subtelomere
Telomere
3'
5'
Resection
-596
-356
-111
0
B
3'
5'
-596
-356
-111
0
C
endonuclease
-596
-111
-356
Ligate
D
strand extension
3'
5'
-596
-111
-356
E
3'
5'
Resection
-596
-111
-356
F
3'
5'
-596
-111
-356
Annealing of
1p subtelomere
endonuclease
G
3'
5'
-596
-111
-356
1p-1260
Ligate
H
strand extension
3'
3'
5'
-596
-111
-356
1p-1260
I
3'
5'
-596
-111
-356
1p-1260
XpYp subtelomere
1p subtelomere

mechanism notably telomerase, 90% of the time and continue dividing indefinitely. Thus, the cells containing these fusion events near the myc site could activate c-myc, which in turn would activate its target genes including telomerase and thus bypass crisis and continue proliferating. However, it is not obvious if this event represents the terminal deletion at 8q, which result in telomere instability thus fuelling large-scale genome instability.

Several translocations near 8q24.3 locus and terminal amplifications involving the locus 8q24.3 have been reported in breast cancer (James et al. 1997) and other malignancies. For example, the locus 8q24.21 which is adjacent to the fusion detected here is associated with B-CLL risk plausibly through differential expression of c-**myc**. If similar type of fusions occurs *in vivo,* this could lead to larger scale rearrangements and hence genomic instability that could drive tumour progression in susceptible individuals (Crowther-Swanepoel et al.). While being of clinical significance in several other lymphomas, the locus 8q24 poses a cancer risk in several solid cancers including hereditary and non-hereditary prostate, colorectal, endometrial (Setiawan et al. 2007) and breast cancers (Schumacher et al. 2007; Fletcher et al. 2008).

Thus, whatever the mechanism that underlies these events, it is apparent that short dysfunctional telomere can initiate large-scale rearrangements that may extend to non-telomeric loci.

4.3.6 Mutational spectrum with the TVR region

The proximal part of the human telomere consists of complex patterns of TTAGGG repeats interspersed with telomere variant repeats (TVR) such as TGAGGG, TCAGGG, TTGGGG (Allshire et al. 1989; Brown et al. 1990; Baird et al. 1995; Coleman et al. 1999; Varley et al. 2002).

The large dataset derived from direct sequence analysis of telomere fusion events at the single cell level enabled the alignment of TVR-maps for each allele within the same cell strain. The data has also revealed inter-allelic variation at the same telomere within the same cell strain. This is consistent with previous studies in which several alleles at the XpYp, 16p and 12q have been described (Baird et al. 1995; Coleman et al. 1999; Baird et al. 2000). In HEK293, two XpYp alleles were associated with a specific haplotype, consistent with previous studies in human populations (Baird et al. 1995).

The analysis of the TVR distribution from separate cells within same cell strain has revealed intra-allelic variation arising from duplications, expansion or contraction of repeat blocks as well as single nucleotide deletions and insertions resulting in expansions and contractions of individual repeats. Thus, similar intra-allelic processes are operating at these sequences. In addition, transversions and transitions, which change one repeat type to another, were observed. These events are consistent with intra-allelic processes such as errors arising during replication such as slippage (Weber and Wong 1993; Richards and Sutherland 1994), as well as the intra-molecular recombination such as unequal sister chromatid exchange [review in (Britt-Compton and Baird 2006)]. Similar events which included variant repeat-type switching and intra-allelic events involving deletions and insertions have been described in ALT+ cells as well as in human populations (Baird et al. 1995; Coleman et al. 1999; Baird et al. 2000; Varley et al. 2002). In addition, the mutational processes that underlie the variability of the TVR region are similar to those described at other tandem repeat loci (Mahtani and Willard 1993; Weber and Wong 1993).

In addition to simple events, two fusion events in which the blocks of repeats with no obvious origin were inserted adjacent to the fusion junction were observed. This could represent the insertion of additional DNA from another telomere. The observed levels of variability within the telomere variant region may be a consequence of telomere fusion process. However, it is highly likely

that the repeat changes occurred prior to fusion events as the mutations were observed in more than one independent fusion events. In addition, such events were seen in more than one event from same sample as well as from different PD samples in MRC5. However, it was noted that in MRC5, a cell line grown under controlled conditions, the variability in the variants at early PD32.2 was rare while in fusion events occurring later on as in PD52, the events were occurring and becoming stable overtime. The mutations within the TVR at regions adjacent to the fusion point could be a result of processing during fusion event. Whilst, those occurring distal to the fusion point could have arisen prior to fusion event and thus, were stably maintained through cell division.

Recent work has shown that one of the N-type repeats CTAGGG are highly unstable in male germ-line (Lim et al. 2009; Mendez-Bermudez et al. 2009). By aligning the different TVR maps within the same cell strain, no particular repeat type seemed to be replaced or added more often than others. Thus, I concluded that in this study no telomere variant repeat was more unstable.

4.4 Key findings

- Using the extended and improved assay, I have isolated and characterised the telomere fusion event in telomerase-positive HEK293 and in telomerase-negative human fibroblast (MRC5) that have bypassed senescence.
- I have shown that fusion of short telomeres in human cells are characterised by large deletion extending close to the limit of the assay (6kb), short patches of microhomologies, insertions and complex rearrangements regardless of the telomerase status of the cells (Letsolo et al. 2009).
- This dataset is consistent with the notion that in addition to repair through chromosome healing, telomere loss can be restored through mechanism that form chromosome fusions resulting in translocations, dicentric chromosomes and ring chromosomes. Although the mechanism of chromosome fusions generated by dysfunction telomeres in human cells is not yet known, it is consistent with some form of error-prone ku-independent mechanism, which creates large deletions and utilises micro-homologies.
- Analysis of the proximal part of the telomere has revealed that the mutational processes that underlie variant repeats are intra-allelic. The single-cell data described here is consistent with previous studies in human populations.
- The data will be used to compare fusion profiles generated from human tissues, in normal and specific disease situation and importantly in experimental systems testing the mechanistic basis of fusion.

5 Chapter 5: Telomere dynamics in normal human skin

5.0 Summary

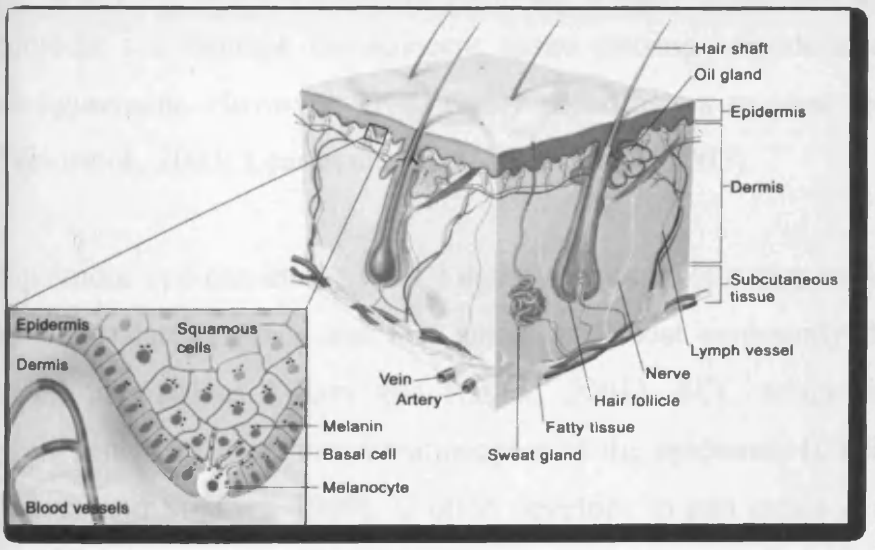
Normal somatic tissues exhibit low-level or undetectable telomerase activity. Consequently, their telomeres erode as a function of age due to "end-replication" problem (Harley et al., 1990). Telomere erosion can trigger replicative senescence, an irreversible growth arrest that cells have to bypass to progress to malignancy, thus senescence represents a potential tumour suppressive mechanism (Allsopp *et al.*, 1995; Allsopp *et al.*, 1992; Campisi *et al.*, 2001).

In vitro studies have demonstrated that telomeres are subjected to processes which generate short telomeres that are capable of fusion (Baird *et al.*, 2003; Britt-Compton *et al.*, 2006; Capper *et al.*, 2007). The objective of this study was to translate this knowledge of telomere dynamics *in vitro* to human tissues *in vivo*. To accomplish this, the high-resolution single-molecule approaches; STELA and the improved telomere fusion assays were utilised to investigate telomere length erosion, instability and fusion in normal human tissues. The samples derived from human skin of nineteen (19) individuals which included melanocytic naevi and the adjacent tissue, were subjected to telomere length and fusion analysis. The study revealed that the mean telomere length at XpYp and 17p was significantly shorter in melanocytic naevi than in adjacent tissue across the sample collection ($P<0.05$). In addition, the mean telomere length at 17p in naevi and associated adjacent tissue was shorter than that of XpYp ($P<0.05$).

Analysis of the fusion events between the telomeres of XpYp, 17p and 21q family revealed large sub-telomeric deletions, short patches of micro-homologies at the fusion point and complex events involving loci near fragile-sites known to confer increased susceptibility to skin cancer. Consistent with *in vitro* data, normal skin tissues are subjected to telomere erosion, instability and fusion.

5.1 Introduction

The human skin is the largest organ in the body that forms a barrier between internal and external environment. It is divided into two layers, the dermis and the epidermis (Figure 5.1). The dermis consists of the extracellular matrix components, the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic and blood vessels. The dermis consist of various types of cells such as endothelial cells, inflammatory cells and fibroblasts, which constitutes the larger source of dermal DNA (Sugimoto *et al.*, 2006). Whilst the epidermis is a self-renewing tissue that proliferates from the basal layer at the turnover of up to 45 days throughout lifetime (Hoath and Leahy, 2003). It consists largely of basal and squamous keratinocytes (95%), interspersed with melanocytes (melanin-producing cells), Merkels cells (sensory receptors) and Langerhans cells [dentritic cells; Figure 5.1; (Gilchrest et al., 1999; Hoath and Leahy, 2003)]. The discussion on this chapter is limited to the brief description of keratinocytes, melanocytes and related skin cancers.



(Modified from: www.ccsb.org/Cancer/Summary/CDR0000062802)

Figure 5.1. The anatomical cross-section of human skin illustrating different layers of the skin; epidermis, dermis with associated compartments and subcutaneous fat tissues (top right). Magnifying through section illustrates different cells that reside in the epidermis as well as part of the dermis (bottom left).

5.2 Skin cancer

Skin cancer is one of the most common forms of cancer in western countries (Brewster et al., 2007; Hussain et al.; Jung et al., 2010). This study is limited to brief discussion on basal and squamous cell carcinoma [also known as non-melanoma or Keratinocyte carcinoma] and melanoma.

5.2.1 Non-Melanoma/ keratinocyte carcinoma

Basal cell carcinoma (BCC) is the most common but least deadly form of skin cancer especially in men than women (Armstrong and Kricker, 2001; Brewster et al., 2007). It arises from the basal keratinocytes, cells lining the deepest layer of the epidermis (Blanpain *et al.*, 2007; Janes *et al.*, 2002). Most if not all BCC occur on sun-exposed areas such as face, neck, scalp, shoulders and back. Thus, major risk factor for BCC is chronic UV-exposure such as short intense episodes of sunlight (Armstrong and Kricker, 2001; English *et al.*, 1997). Other risk factors are outlined in Table 5.1A. The clinical features include nodules, plaques, ulcers etc (Rubin *et al.*, 2005). Although easily treated at early stages, advanced tumours can damage the adjacent tissue causing considerable destruction and disfigurement. However, BCC rarely metastasises to vital organs (Albert and Weinstock, 2003; Lear et al., 1998; Rubin et al., 2005).

Squamous cell carcinoma (SCC) is the second most common type of skin cancer in Caucasians, Asians and Hispanics, and most commonly diagnosed type in black populations (Alam and Ratner, 2001). SCC arises from uncontrolled proliferation of squamous keratinocytes of the epidermis (Gilchrest et al., 1999; Nemes and Steinert, 1999). It often develops in sun exposed areas (Armstrong and Kricker, 2001; Hussain et al., 2010), thus major risk factors include cumulative chronic exposure to UV radiation from the sunlight and artificial tanning lamps (English et al., 1998; Karagas et al., 2002). Like BCC, it is curable at early stages but can spread to other tissues if not treated (Alam and Ratner, 2001; Gloster and Neal, 2006). Although common, keratinocyte carcinomas account for fewer fatalities compared to melanoma.

Table 5.1A. Risk factors associated with Non-melanoma skin cancers

Cancer-type	Risk factors	References
Basal and Squamous cell carcinoma	• Fairer skin, • Blond or red hair • Blue, green or grey eyes	(Armstrong and Kricker, 2001; English et al., 1998).
	• Immuno-suppression and chronic infections as in HIV or HPV infection, after chemotherapy and anti-rejection drugs-treatment.	(Albert and Weinstock, 2003; Moloney et al., 2006)
	• Genetic disorders o xeroderma pigmentosum o nevoid basal cell carcinoma syndrome	(Albert and Weinstock, 2003; Giannotti et al., 2003)
	• Risk is positively associated with age and being male, however women are also affected	(Albert and Weinstock, 2003; Rubin et al., 2005)

Table 5.1B. Risk factors associated with Melanoma

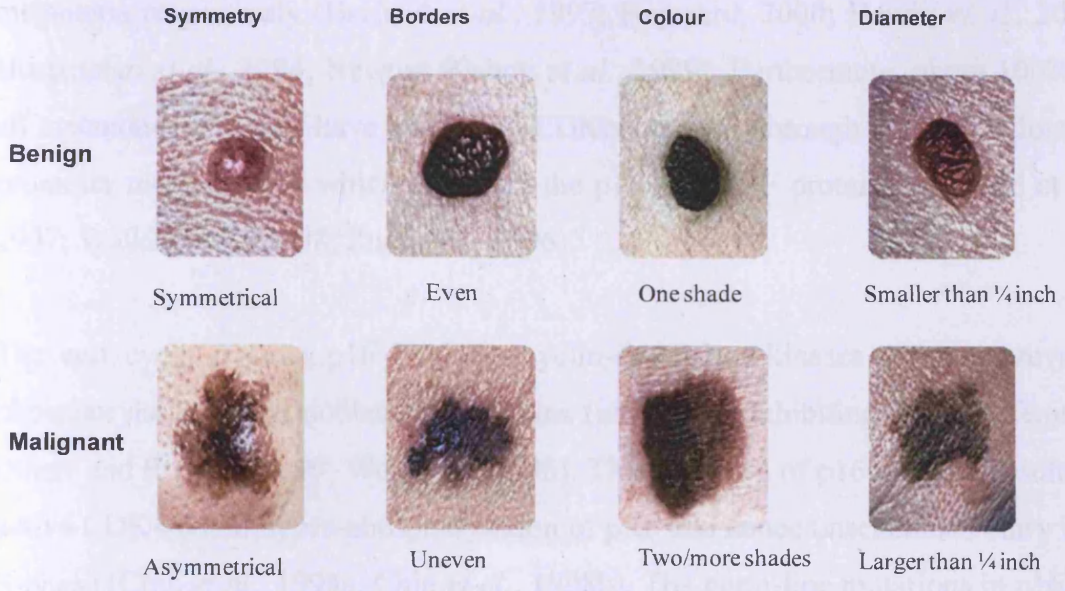
Cancer-type	Risk factors	References
Melanoma	• Chronic exposure to UVA and UVB rays from the sun and/or UV tanning booths. • Fairer skin, green, blue or grey eyes, and red or blonde hair	(Armstrong and Kricker, 2001; Gilchrest *et al.*, 1999; Han *et al.*, 2009; Tucker and Goldstein, 2003) (Thompson *et al.*, 2005).
	• Compromised immune system as in: o lymphoma, HIV infection o After chemotherapy or radiation-treatment and in patients taking anti-rejection drug after organ transplant.	(Armstrong and Kricker, 2001; Chin et al., 1998a; Thompson et al., 2005)
	• Family and personal risks • A personal history of any of the skin cancers such as keratinocyte carcinomas. • Personal history of melanoma is associated with increased risk of recurrence. • First-degree (50% increased risk) and Second-degree relatives of melanoma patients • Familial atypical multiple mole melanoma syndrome (FAMMM)-[also known as dysplastic naevus syndrome (DNS)], a condition in which atypical melanocytic naevi are detected in a melanoma-prone individual, are at greater risk of developing melanoma	(Li *et al.*, 2006) (Chaudru et al., 2004)

5.2.2 Melanoma

Cutaneous melanomas are the rare but most aggressive and fatal forms of skin cancer (Armstrong and Kricker, 2001; Jemal et al., 2003). They are curable when detected early, however, untreated, they can metastasise to other parts of the body and cause serious illness and death (Chin *et al.*, 1998a). Melanomas are malignant tumours that originate in the melanocytes; the cells that produce a pigment (melanin) that gives the skin, hair and eyes their colour (Gilchrest *et al.*, 1999). They are usually black or brown and rarely skin-coloured, pink, red, purple, blue or white (Chin *et al.*, 1998a; Gloster and Neal, 2006). Every individual is at risk of melanoma however certain factors such as melanocytic naevi, genetics and others (Table 5.1B), increases the risk of developing melanoma. The incidences of melanomas are on the rise in western countries, with an approximate increase rate of 5% reported in the past four-to-five decades (Armstrong and Kricker, 2001; Thompson et al., 2005). Therefore, there is considerable interest in understanding the genetic and environmental mechanisms that drive disease risk, initiation and progression.

5.2.3 Melanocytic naevi

Melanocytic naevi (also known as moles) are benign tumours, which arise from uncontrolled proliferation of melanocytes (Robinson *et al.*, 1998; Skender-Kalnenas *et al.*, 1995). They consist of functionally viable melanocytes (Bandyopadhyay *et al.*, 2001; Bennett, 2003; Bennett and Medrano, 2002; Mooi and Peeper, 2006) which have undergone senescence following oncogenic mutations (Davies *et al.*, 2002; Ichii-Nakato *et al.*, 2006; Michaloglou *et al.*, 2005; Pollock *et al.*, 2003). Although they predispose to melanoma in susceptible individuals, melanocytic naevi can remain dormant for many years (Bennett and Medrano, 2002; Hayward, 2000). Common naevi are small brown blemishes, out-growths or beauty marks that appear at early childhood or adolescent (acquired) or are present at birth (Congenital). They may also be caused by chronic UV exposure especially before the age of 20 (Takata and Saida, 2006).



Adapted from: www.skincancer.org/melanoma

Figure 5.2. Benign and Malignant melanocytic naevi. Changes in size, symmetry, appearance and colour of moles can signify that moles could be cancerous.

The definition of the dysplastic naevi (also known as atypical mole) is still controversial but they represent naevi that have a distinct appearance from the common mole. The risk of developing melanoma is directly proportional to the number of atypical (dysplastic) moles (>100) i.e. the more moles an individual possesses, the higher the risk of developing melanoma (Skender-Kalnenas et al., 1995). In addition, the changes in atypical mole such as symmetry, colour, borders and size can suggest the development of cancer (Figure 5.2). In support of this, primary melanomas are associated with pre-existing dysplastic naevi (Kraemer et al., 1983).

5.2.3.1 Genetics

The melanoma tumour suppressor gene, CDKN2A is mapped to 9p21. It encodes for the two cell cycle inhibitors, $p16^{INK4a}$, (referred to hereafter as p16) and $p14^{ARF}$ (referred to hereafter as ARF) which are transcribed from separate promoters and read into different reading frames (Bennett, 2003; Sharpless, 2005). Germ-line and somatic CDKN2A mutations or deletions have been linked to familial and sporadic

melanoma respectively (Harland *et al.*, 1997; Hayward, 2000; Hewitt *et al.*, 2002; Hussussian *et al.*, 1994; Newton Bishop *et al.*, 1999). Furthermore, about 100% of all melanoma cell lines have an altered CDKN2A gene, through either deletions or promoter methylations, which all affect the p16 and ARF proteins (Harland et al., 1997; Walker et al., 1998; Zuo et al., 1996).

The cell cycle protein, p16 binds to cyclin-dependent kinases 4/6 and prevents phosphorylation of retinoblastoma proteins (pRb); thus inhibiting cell proliferation (Sherr and Roberts, 1999; Weinberg, 1995). Thus, the loss of p16 function results in active CDK4/6 and hyper-phosphorylation of pRb and hence unscheduled entry into S-phase (Chin *et al.*, 1998a; Chin *et al.*, 1998b). The germ-line mutations in p16 are associated with multiple large or dysplastic naevi and melanoma (Hayward, 2000; Thompson *et al.*, 2005).

ARF on the other hand, is a potent tumour suppressor that induces G_1 and G_2 cell cycle arrest through its interaction with MDM-2, a protein that degrades p53 to inhibit cell cycle arrest. Thus, ARF binds to MDM-2 and blocks p53 degradation thereby stabilising p53, and hence allows cell cycle arrest (Kamijo *et al.*, 1997; Zhang *et al.*, 1998). In addition, ARF can block uncontrolled cell proliferation independent of p53 pathway (Ha *et al.*, 2007).

Another melanoma susceptibility gene is the gene for CDK4 located on 12q13. The germ-line mutations in CDK4 gene have also been reported in melanoma-prone families (Soufir *et al.*, 1998; Zuo *et al.*, 1996).

An additional tumour suppressor gene, phosphatase PTEN, a protein-lipid that inhibits AKT activation by Phosphatidylinositol 3′ kinase (PI3K) is mapped to 10q. The loss of heterozygosity at the locus 10q23 and somatic mutations at PTEN have been reported in melanomas (Isshiki *et al.*, 1993; Wu *et al.*, 2003).

Finally, NRAS is a proto-oncogene often mutated in melanomas (Polsky and Cordon-Cardo, 2003) through mutations that result in p21RAS onco-protein. This hybrid protein remains bound to GTP and active. This activates downstream RAF, a protein that has three isoforms; A-, B- and C-RAF. Interestingly B-RAF mutations are common in melanocytic naevi, regardless of whether acquired, congenital or dysplastic and melanomas (Pollock *et al.*, 2003). Importantly $BRAF^{V600E}$, a mutant which induces senescence, is expressed in 69-88% of melanocytic naevi, both acquired and congenital regardless of their location, i.e. regardless of whether they were located on sun-exposed or non-exposed areas (Davies et al., 2002; Ichii-Nakato et al., 2006; Michaloglou et al., 2005; Pollock et al., 2003).

5.2.3.2 Cellular senescence in melanocytic naevi (Moles)

Cellular senescence is the loss of ability to proliferate after a finite number of cell divisions (Hayflick and Moorhead, 1961). It is considered a crucial barrier that cells have to bypass to allow continued cell division required for progression to malignancy, thus senescence represents a potential tumour suppressive mechanism (Campisi, 2001; Sharpless and DePinho, 2004; Wright and Shay, 2001). Two main pathways effecting normal cellular senescence in culture have been reported, the well-known pathway is that first described in fibroblasts. In culture, human fibroblasts cease to divide after 40-70 population doublings and undergo pRb and p53 dependent growth arrest (M1). M1 can be bypassed through oncogenic transformation of cells with viral oncoproteins such as HPV16 E6 E7 or SV40 large T antigen (Crook *et al.*, 1991; Ducray *et al.*, 1999; Shay *et al.*, 1993). Significantly, expression of the catalytic subunit of telomerase (hTERT), an enzyme that maintains telomere length can also circumvent M1 (Counter *et al.*, 1992). Thus in most normal somatic cells that lack hTERT, telomere erosion triggers p53/pRb dependent cell cycle exit called replicative senescence (Allsopp et al., 1992; Harley et al., 1990).

An alternative route that occurs earlier than M1 has been described in keratinocytes and epithelial cells. This cell cycle exit referred to as M_0 appear to be pRb-

dependent but p53-independent (Kiyono *et al.*, 1998; Ramirez *et al.*, 2001). In support of this, immortalisation of these cells requires both HPV16 E6E7 and hTERT, implicating that culture stress may play an important role in cell cycle arrest of these cells (Kiyono *et al.*, 1998). Indeed, improvement of culture conditions, such as growing the cells with fibroblast "feeder" cells, extended the life span of these cells until they undergo the p53 and pRb senescence and are immortalised through forced expression of hTERT alone (Ramirez *et al.*, 2001).

Similarly, culture conditions play an even more significant role in the timing of cell cycle arrest in melanocytes, which vary from 8-90pd in neonatal cells to 2.4-40pd in adult melanocytes (Bennett, 2003; Bennett and Medrano, 2002). Several studies have indicated that senescence in melanocyte cultures is accompanied by upregulation of p16, dephosphorylation of pRb and down regulation of CDK2 and CDK4 among others (Bandyopadhyay and Medrano, 2000; Bandyopadhyay *et al.*, 2001) .i.e. unlike senescence in fibroblast cultures, p21/p53 pathway appears to play little or no role in melanocyte senescence (Ha et al., 2007; Serrano et al., 1996; Sviderskaya et al., 2002) . This view is backed by studies in human melanocytes carrying wild type ARF but non-functional p16, which exhibited elevated levels of p21 and p53 proteins which are often not upregulated in normal melanocytes (Bandyopadhyay et al., 2001; Sviderskaya et al., 2003), indicating that senescence of normal melanocytes is similar to the M_0 cell cycle arrest (Kiyono *et al.*, 1998; Ramirez *et al.*, 2001).

It is possible that normal melanocytes senesce through the mechanism that act against the $BRAF^{V600E}$ mediated oncogenesis. The expression of such mutated gene has been reported to induce cell cycle arrest in human melanocytes, with cells expressing senescence-associated beta–galactosidase, a senescent marker, accompanied by p16 expression. However, 25-30% of such senescent cells did not express any p16, implicating that other factors may be driving senescence in these melanocytes (Bennett, 2003; Davies *et al.*, 2002; Michaloglou *et al.*, 2005).

Another genetic change that drives senescence is telomere dysfunction (Campisi et al., 2001; Herbig et al., 2004; Karlseder et al., 2002). However, the role of telomere biology in melanocytic naevi senescence is controversial. As discussed above, it is believed that senescence of normal melanocytes in culture is dependent on p16 [M_0; (Bennett, 2003)]. However, improvement of culture conditions can extend the life span of melanocytes to another growth arrest (Bandyopadhyay et al., 2001) that is dependent upon p53 and pRb, which is also triggered by telomere dysfunction (Herbig et al., 2004), suggesting that telomere biology may play a role in senescence of melanocytes. In support of this, some studies have demonstrated that indeed progressive telomere attrition triggers human melanocytic senescence. In addition, ectopic expression of hTERT did extend the life span of melanocytes (Bandyopadhyay *et al.*, 2001; Bennett, 2003).

5.3 Telomere biology in Skin

Human skin exhibits low-level telomerase activity (Bacchetti, 1996). When the skin was dissected high levels of telomerase activity were detected in the basal layer of the epidermis while the dermis was telomerase-negative (Harle-Bachor and Boukamp, 1996; Yasumoto *et al.*, 1996). In addition, immortalised skin keratinocytes are telomerase-positive regardless of their immortalisation method (Harle-Bachor and Boukamp, 1996). However, the normal skin fibroblasts do not express any detectable telomerase activity (Kim *et al.*, 1994).

Telomerase activity varies among different skin conditions and cancer types. Previous studies have demonstrated that melanoma cell lines are telomerase-positive (Kim *et al.*, 1994). In addition, low or undetectable telomerase activity has been reported in benign melanocytic proliferations, while high telomerase activity was detected in cells at S-M phases within the dysplastic naevi (Rudolph *et al.*, 2000). Furthermore, all skin cancer samples are telomerase-positive regardless of the skin cancer type. However, BCC exhibits a high relative telomerase activity compared to SCC and melanoma. Moreover, telomerase activity is increased in aggressive melanoma being highest in tumours with deepest invasion. i.e.

Telomerase activity in melanomas increases with disease progression to metastatic melanoma (Rudolph *et al.*, 2000; Taylor *et al.*, 1996). In addition to skin cancer, telomerase activity has also been reported in some non-malignant skin conditions such as UV-damaged skin, hyper-proliferative skin diseases; psoriasis and poison ivy dermatitis, peritumoural samples and actinic keratoses (Sugimoto et al., 2006; Taylor et al., 1996). It was however, not clear in these studies whether telomerase activity was exhibited by skin cells or infiltrating lymphocytes, which are known to be telomerase-positive (Hiyama et al., 1995; Igarashi and Sakaguchi, 1997).

Telomere erosion plays contradicting role in different skin cancers. i.e. The shorter telomere lengths are associated with decreased risk of melanoma (Han *et al.*, 2009) and an increased risk of basal cell carcinoma while there is no association between the telomere length and squamous cell carcinoma risk. Furthermore, there is no association between the number of moles and telomere length on the skin cancer risk while short telomeres in peripheral blood are associated with decreased mole counts and size (Bataille *et al.*, 2007).

5.4 This study

In normal cells, telomeres erode as a function of age due to "end-replication" problem (Harley *et al.*, 1990; Watson, 1972). Progressive telomere erosion has been implicated as mitotic "clock" that limits cell proliferation in the absence of telomerase activity (Allsopp *et al.*, 1995; Allsopp *et al.*, 1992; Harley *et al.*, 1990). Eroded telomeres can initiate chromosome instability resulting in large-scale rearrangements similar to those observed in epithelial cancers (Artandi *et al.*, 2000; Murnane, 2006; Murnane and Sabatier, 2004; Rudolph *et al.*, 2001).

Several studies have demonstrated that telomeres are subjected to stochastic deletion events resulting in shorter telomeres than those in senescence cells *in vitro* (Baird et al., 2003; Britt-Compton et al., 2006); these short telomeres are dysfunctional and are capable of fusion (Capper *et al.*, 2007; Letsolo *et al.*, 2009). We have further demonstrated that telomere length and frequency of telomere

fusion correlated with severity of the disease in human blood malignancy, chronic lymphocytic leukaemia (Lin et al., 2010). Thus, this work was undertaken to investigate if such events were occurring in normal and senescent human tissues *in vivo*.

Telomere length instability and fusion were investigated in melanocytic naevi and adjacent tissue. Melanocytic naevi provide an important model for cell senescence as cells undergo oncogene-induced senescence and remain dormant for long periods even though they pose an increased risk to melanoma development in susceptible individuals (Robinson *et al.*, 1998). This study will enable the investigation of whether telomere dynamics is implicated in the senescence of melanocytes. In addition, it will indicate whether telomere dynamics plays a significant role in progression of benign naevi to malignancy. Furthermore, defining the fusion frequency in normal tissues will serve as a reference when determining if frequency of these events is elevated in pre-malignant conditions such as Barrett's oesophagus (chapter 6).

5.5 Sample Collection, Preparation and Statistical analysis

The naevi samples were obtained from 19 females born between 1955 and 1974 who volunteered for adult twin study registry at St Thomas hospital, London. The ethical approval for use of these samples was granted by Guy's and St Thomas NHS Trust ethics committee (Bataille et al., 2007). Using a clean scalpel, the naevi samples were macro-dissected and separated from adjacent tissue (DMB personal comm.). Thus the DNA extracted from either the naevi or adjacent tissues may contain the traces of DNA from other cells of the skin such as keratinocytes of the epidermis and any cells of the dermis.

DNA was then extracted by standard Phenol/Chloroform methods (Sambrook *et al.*, 1989). Samples used for STELA and telomere fusion assays were diluted as described in (Baird *et al.*, 2003; Letsolo *et al.*, 2009).

The D'Agostino & Pearson omnibus normality test was used to assess if the data was normally distributed. For pair-wise comparison, Mann-Whitney test (for medians) and unpaired t-test (for means) were used with F-test (for variance) to analyse not-normally-distributed and normal distributed data respectively. The two-tailed P-value, $P<0.05$ was considered statistically significant while $P<0.001$ was considered extremely significant.

5.6 Results

Here, I describe work undertaken to investigate telomere length instability and fusion in normal skin samples consisting of melanocytic naevi and the adjacent tissue. STELA was used to determine the telomere length at telomeres of XpYp and 17p. The telomere fusion events were investigated between telomeres of XpYp, 17p and 21q family: 1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q, 22q and interstitial 2q13.

Data was derived from the analysis of the nineteen [19] individuals, consisting of 17 melanocytic naevi and 19 naevi-adjacent tissue samples.

5.6.1 Telomere length distributions in Melanocytic naevi

The analysis of the seventeen [17] melanocytic naevi revealed considerable telomere length heterogeneity within each sample at both XpYp and 17p, with variation of up to 12.3kb [17p; 20562M] and 12.4kb [XpYp; 32811AM; Figure 5.3; Table 5.2]. This is consistent with the variation that has been observed in senescent populations (Baird *et al.*, 2003; Britt-Compton *et al.*, 2006). Similarly, large inter-individual variation in mean telomere lengths was observed with maximum variation of 3.9kb at XpYp and 2.18 kb at 17p telomeres [combined mean telomere lengths (±SD), XpYp=4.85±0.90kb; 17p=4.3±0.7kb].

The telomere of 17p has been implicated as one of the shortest in human genome (Martens *et al.*, 1998). Using STELA, 17p telomere was typically the shortest in some senescent clones (Britt-Compton *et al.*, 2006). Consistent with this, 17p

Table 5.2. Summary of the telomere length data from melanocytic naevi at XpYp telomere. Range is the difference between the shortest and the longest telomere. The shortest telomeres (blue bolded font) and the biggest difference between the telomeres (red bolded) across the sample collection are indicated. Standard deviation measures how widely the values are dispersed from the mean telomere length.

Sample id	XpYp mean telomere length (kb)	Standard Deviation	Shortest telomere (kb)	Longest telomere (kb)	Range	17p mean telomere length (kb)	Standard Deviation	Shortest telomere (kb)	Longest telomere (kb)	Range
98592M	5.42	2.09	1.90	13.32	11.42	4.57	1.58	2.45	9.19	6.73
95981M	4.22	2.17	1.39	9.26	7.87	5.45	2.16	1.28	11.72	10.45
32811AM	4.13	2.75	0.21	12.60	12.39	3.27	2.31	0.14	8.96	8.83
32811BM	5.10	2.70	0.44	11.67	11.23	3.37	2.27	0.14	9.74	9.60
20561M	5.45	3.11	1.92	10.97	9.06	4.10	2.26	1.09	7.69	6.60
20562M	5.06	2.90	0.68	12.00	11.32	5.20	2.78	1.23	13.48	12.25
52984M	5.84	2.61	2.15	11.65	9.50	4.92	2.55	1.36	11.46	10.10
93942M	3.57	1.93	1.43	10.71	9.29	4.37	1.83	0.41	8.73	8.32
2492M	4.35	2.58	0.73	11.67	10.94	3.96	1.81	0.97	7.83	6.86
2493M	4.79	2.64	0.55	10.52	9.97	4.42	1.88	1.05	9.30	8.26
94041M	3.15	2.03	0.32	10.04	9.72	4.09	1.85	1.59	9.40	7.81
97051M	5.00	2.63	1.68	11.99	10.31	5.05	2.45	1.38	11.72	10.35
18261M	5.13	2.77	1.36	11.63	10.27	4.34	1.38	2.10	7.78	5.68
93983M	5.12	2.37	2.17	9.37	7.19	3.33	1.05	1.48	5.71	4.23
06651M	4.14	2.09	1.35	9.45	8.10	4.15	2.23	1.41	8.96	7.55
06652M	4.94	2.22	1.20	11.66	10.46	3.45	1.80	0.97	8.69	7.72
98591M	7.05	3.03	2.13	14.04	11.91	4.76	1.60	1.52	8.51	6.99

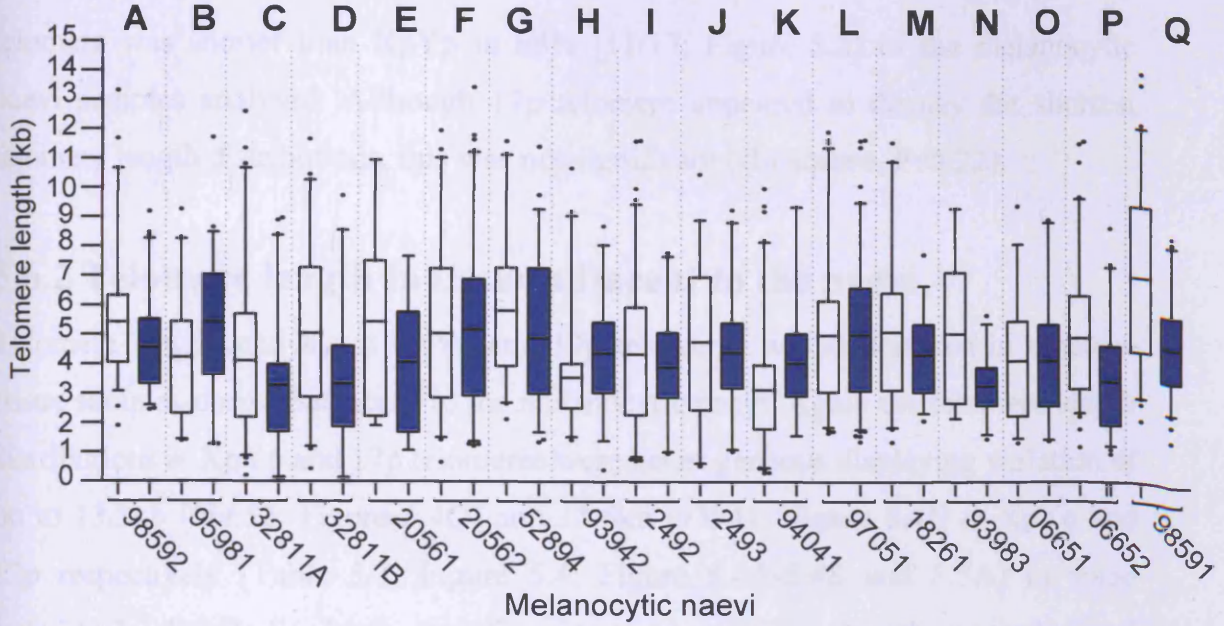


Figure 5.3. Summary of the telomere length distributions as determined by STELA at telomeres of XpYp (open boxes) and 17p (blue boxes) in 17 melanocytic naevi samples. The boxes represent lower and upper quartiles of the telomere measurements with the telomeres <5% and >95% percentile measurements considered outliers (indicated by dots). The line inside the box represents the mean telomere lengths.

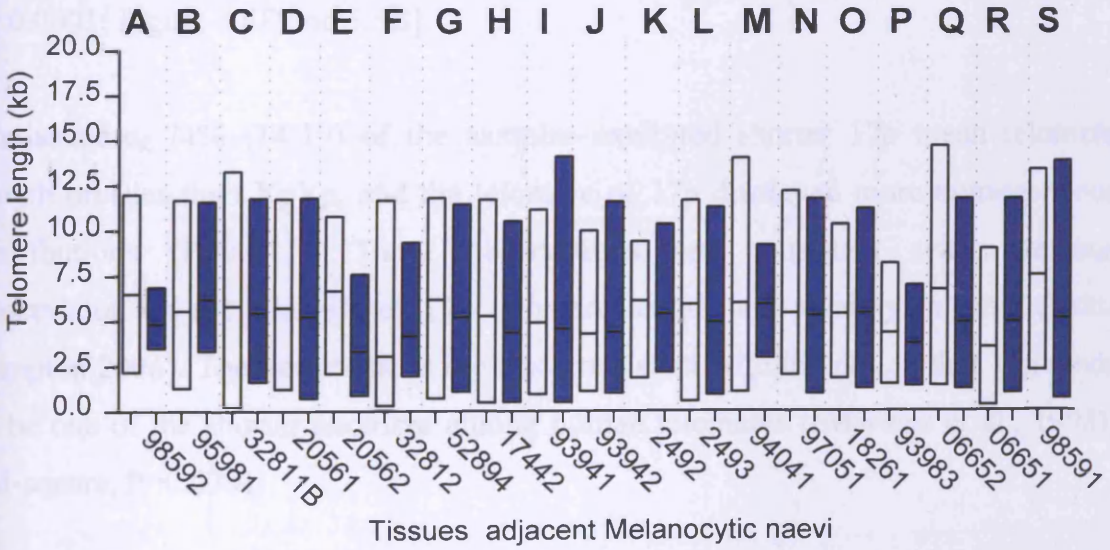


Figure 5.4. Histograms summarising the telomere length distributions at telomeres of XpYp (open bars) and 17p (blue bars) in tissues derived adjacent to melanocytic naevi from 19 individuals (identified by a number). Telomere length was determined by STELA in which products were detected by Southern hybridisations with a TTAGGG-containing probe. The line in the middle of the bar is the mean, while the top and bottom of the bars represents the largest and shortest telomere respectively.

telomere was shorter than XpYp in 65% [11/17; Figure 5.3) of the melanocytic naevi samples analysed. Although 17p telomere appeared to display the shortest telomere length distributions, this was not significant (chi square, P=0.22).

5.6.2 Telomere length in tissue adjacent to the naevi

Telomere length analysis at XpYp and 17p telomeres was undertaken in nineteen tissue samples derived adjacent to the melanocytic naevi. Again the telomere length distributions at XpYp and 17p telomeres were heterogeneous displaying variation of up to 13.3kb [06652; Figure 5.4Q] and 13.6kb [93941; Figure 5.4I] at XpYp and 17p respectively [Table 5.3; Figure 5.4; Figure 5.4A-5.4S and 5.5A] in some individuals. Similarly, large variation in mean telomere length was observed between individuals in these samples (Figure 5.4A-S), with the combined means (±SD) of 5.3±1.2 (XpYp) and 4.9±0.7 (17p).

In addition to large inter-individual variation in mean telomere distributions, XpYp displayed more heterogeneous profiles than those of 17p (P=0.047). This was more apparent in individual 32812 [XpYp, mean±SD=3.1±2.2; 17p, mean±SD=4.2±1.7; P<0.0001; Figure 5.4F and 5.5B].

Furthermore, 74% (14/19) of the samples exhibited shorter 17p mean telomere length profiles than XpYp, and the telomere of 17p displayed more homogeneous distributions (P=0.047). These observations are consistent with previous observation on the analysis of 17p telomere length and stability *in vitro* (Britt-compton 2006). The observations are also consistent with the notion that 17p tends to be one of the shorter telomere among human telomeres [(Martens et al., 1998); chi-square, P=0.039).

Outlying short or long telomeres than the rest of the telomeres were also apparent in these distributions; these events are consistent with sporadic telomere events resulting in elongation or deletion of telomeres [Figure 5.5B; (arrow-headed)].

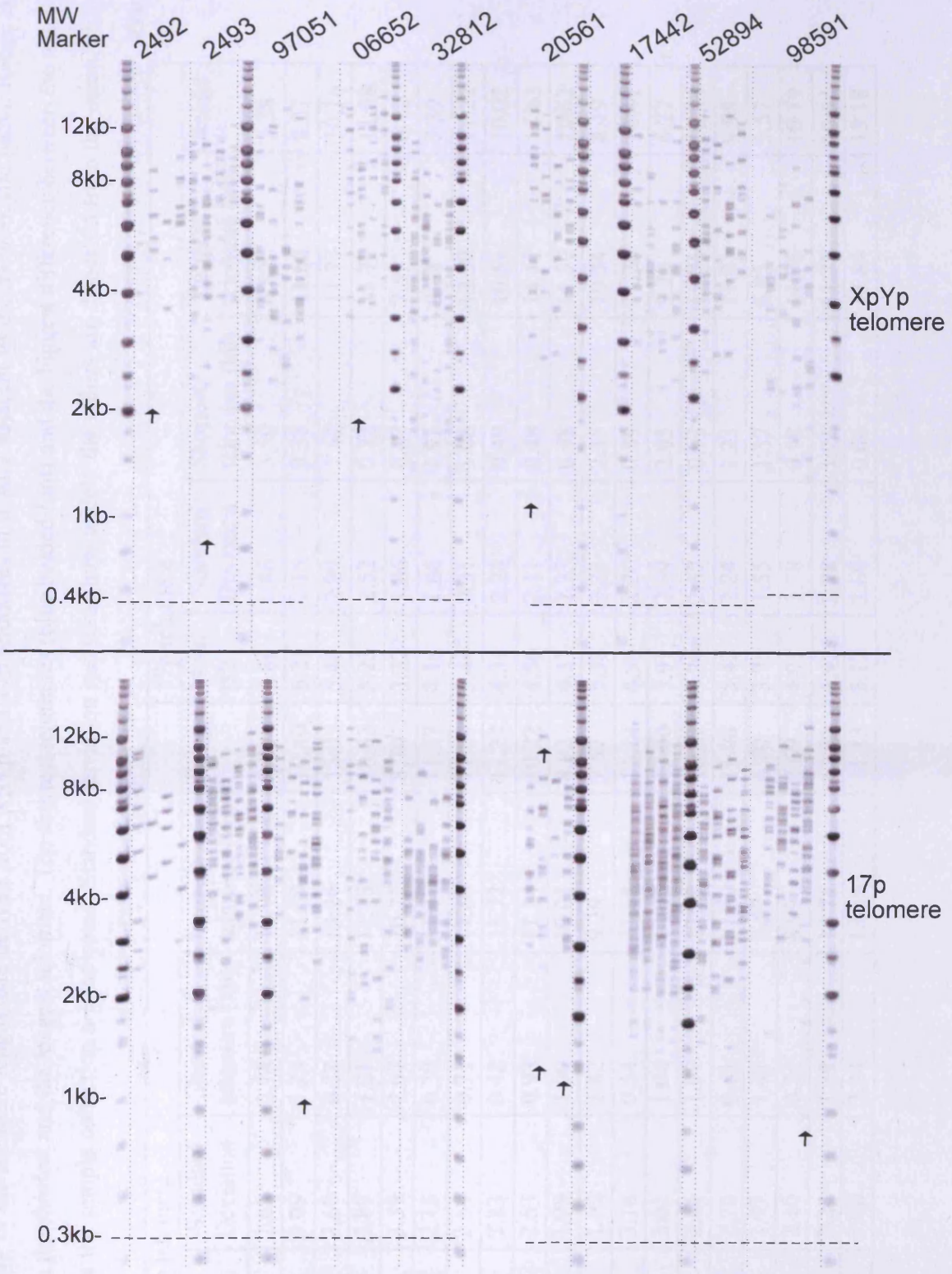


Figure 5.5. Examples of the STELA gels illustrating the telomere length data of XpYp and 17p telomeres from tissues derived adjacent to the melanocytic naevi from nine individuals. The zero limits of the distributions for each telomere are indicated by dashed horizontal line at the bottom of the gels. The molecular weight marks are shown on the left. The telomeres typical of stochastic deletion/ elongation events are indicated (arrow-headed).

Table 5.3. Summary of the telomere length data derived from XpYp and 17p telomeres in tissues adjacent to the melanocytic naevi. Range is the difference between the shortest and the longest telomere. The shortest telomeres (blue bolded font) and the biggest difference between the telomeres (red bolded) across the sample collection are indicated. Standard deviation measures how widely the values are dispersed from the mean telomere length.

Sample id	XpYp telomere					17p telomere				
	Mean (kb)	Standard Deviation	Shortest telomere (kb)	Longest telomere (kb)	Range	Mean (kb)	Standard Deviation	Shortest telomere (kb)	Longest telomere (kb)	Range
98592	6.50	2.65	2.18	11.98	9.80	4.86	0.86	3.50	6.87	3.38
95981	5.53	3.09	1.29	11.69	10.40	6.21	2.15	3.33	11.60	8.27
32811B	5.80	3.60	**0.22**	13.26	13.05	5.46	2.29	1.62	11.77	10.16
20561	5.70	2.89	1.21	11.73	10.53	5.23	2.52	0.72	11.79	11.08
20562	6.66	3.59	2.07	10.77	8.70	3.32	1.86	0.87	7.59	6.72
32812	3.13	2.15	0.30	11.67	11.37	4.16	1.66	1.95	9.34	7.39
52894	6.18	2.69	0.71	11.80	11.09	5.44	2.31	1.08	12.30	11.22
17442	5.25	2.83	0.48	11.72	11.24	4.34	2.24	0.49	10.51	10.02
93941	4.89	2.63	0.93	11.14	10.22	4.56	2.11	**0.48**	14.11	**13.63**
93942	4.25	1.98	1.09	10.01	8.92	4.37	2.33	0.98	11.59	10.62
2492	6.09	1.93	1.82	9.39	7.57	5.38	2.33	2.15	10.34	8.19
2493	5.90	3.16	0.54	11.68	11.14	4.94	2.20	0.89	11.30	10.41
94041	5.97	3.02	1.04	14.05	13.00	5.91	2.39	2.93	9.20	6.27
97051	5.40	2.84	1.97	12.19	10.22	5.29	2.57	0.93	11.76	10.83
18261	5.04	2.70	0.61	11.69	11.08	5.42	2.24	1.23	11.21	9.98
93983	4.30	1.97	1.47	8.17	6.70	3.75	1.55	1.37	6.95	5.57
06651D	3.50	2.10	0.31	8.99	8.69	4.91	2.78	0.98	11.76	10.79
06652D	6.71	3.24	1.39	14.71	**13.32**	4.92	2.78	1.21	11.75	10.54
98591	7.49	3.06	2.24	13.36	11.13	5.12	2.60	0.66	13.84	13.18

At the XpYp telomere, 25% (4/16) of individuals exhibited telomere length distributions that were significantly shorter and different from those of the dermis (Figures 5.6, 5.7A and 5.7B). For example, individual 06652, displayed a bimodal distribution in the melanocytic naevi sample but a single distribution in the dermis (Figure 5.6A and 5.7A; P=0.0001). Similarly, individual 95981 displayed heterogeneous telomere profiles in melanocytic naevi and what appeared like bimodal distribution in dermis (Figures 5.6A and 5.7D; $P<0.05$). In addition, in two other individuals 2492 and 94041, both tissues exhibited, heterogeneous profiles (Figures 5.6A, 5.7B and 5.7C; $P<0.001$).

The analysis of the 17p telomere revealed that in 56% (9/16) of the individuals, melanocytic naevi exhibited shorter telomere length profiles than the adjacent tissues (Figures 5.6B). Interestingly, a subset (3/9) of those individuals displaying shorter 17p telomeres also displayed shorter telomere profiles at XpYp; namely 06652 (Figures 5.7A, 5.6B; P=0.001); 2492 (Figure 5.7B; 5.6B; P=0.018) and 94041 (Figures 5.7C, 5.6B; P=0.013). The polymorphism at the 17p telomere was observed in one individual; individual 94041 exhibited fewer (<95%) 17p molecules compared to XpYp telomere (Figure 5.7C); this is consistent with large-scale structural polymorphism at chromosome ends which was observed previously [Chapter 3 of this thesis; (Britt-Compton et al., 2006)].

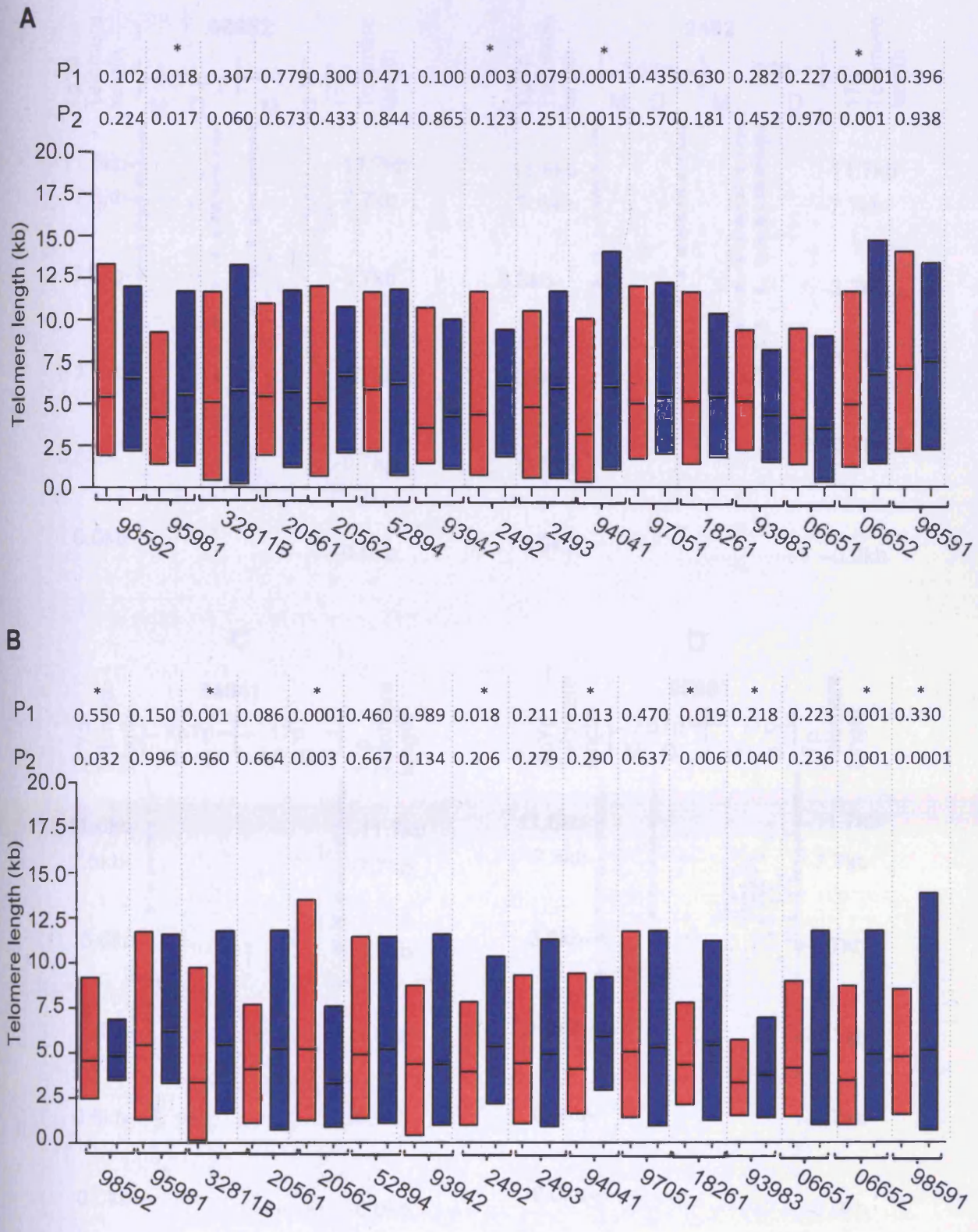


Figure 5.6. Illustrates the telomere distribution at XpYp (A) and 17p (B) telomeres for melanocytic naevi (red bars)-adjacent tissue (blue bars) matched samples. Unpaired t-test (P_1) and F-test (P_2) were used to compare means (line inside the bar) and variances respectively. *denotes the individualswho display different telomere distributions between melanocytic naevi and adjacent tissue.

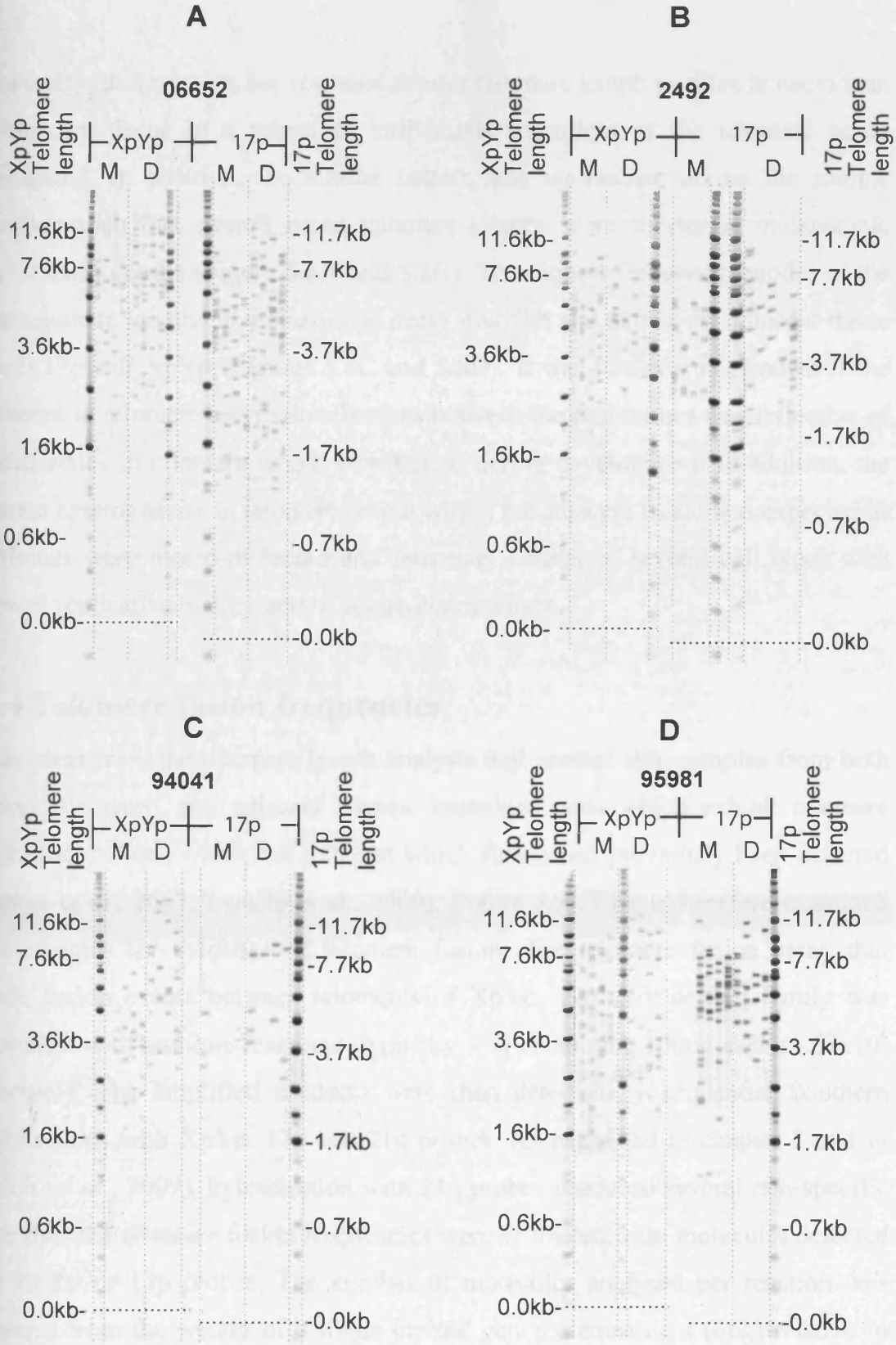


Figure 5.7. Telomere distributions at telomeres of XpYp and 17p in melanocytic naevi (M) and adjacent tissues (D). Mean telomere lengths are shown below each distribution. A-B. Both XpYp and 17p were shorter in naevi (M). C. XpYp telomere length distribution significantly shorter in naevi. >95% amplifiable molecules at 17p possible due to allelic variation. D. XpYp shorter in naevi but no significant difference at 17p telomere in both tissues.

In summary, this analysis has revealed shorter telomere length profiles in naevi than the adjacent tissue in a subset of individuals regardless of the telomere being investigated. In addition, the similar pattern was maintained across the sample collection such that overall mean telomere lengths were shorter in melanocytic naevi than in dermis (Figure 5.8A and 5.8B). The highest frequency (mode) of the mean telomere lengths in melanocytic naevi was 5kb and 6kb in the adjacent tissue at both 17p and XpYp (Figures 5.8C and 5.8D). It was however not known if the difference in telomere length distributions between the two tissues was reflective of the difference in telomere length distribution during development. In addition, the apparent heterogeneity in telomere length within the adjacent tissue was expected as the tissues were macro-dissected and thus may consist of several cell types with different replicative history and telomere distributions.

5.6.4 Telomere fusion frequencies

It was clear from the telomere length analysis that normal skin samples from both melanocytic naevi and adjacent tissues, contained cells which exhibit telomere length distributions within the range at which fusion had previously been detected [(Capper et al., 2007; Letsolo et al., 2009); Figure 5.9]. I have therefore examined these samples for evidence of telomere fusion. The telomere fusion assay that detects fusion events between telomeres of XpYp, 17p and/ or 21q family was undertaken with multiple reactions [typically 7-8] containing 100ng each ($1.67x10^4$ molecules). The amplified products were then detected by sequential Southern hybridisations with XpYp, 17p and 21q probes. As explained in chapter 3 and in (Letsolo *et al.*, 2009), hybridisation with 21q probes produced several non-specific bands thus the telomere fusion frequencies were estimated from molecules detected with XpYp or 17p probes. The number of molecules analysed per reaction was estimated from the weight of a single diploid genome equivalent (6pg) relative to the amount of input DNA. A total of $1.02x10^8$ diploid cell equivalents were analysed using multiple reactions. In melanocytic naevi, the data was collected for 15 samples only as naevi from two individuals did not yield sufficient DNA for

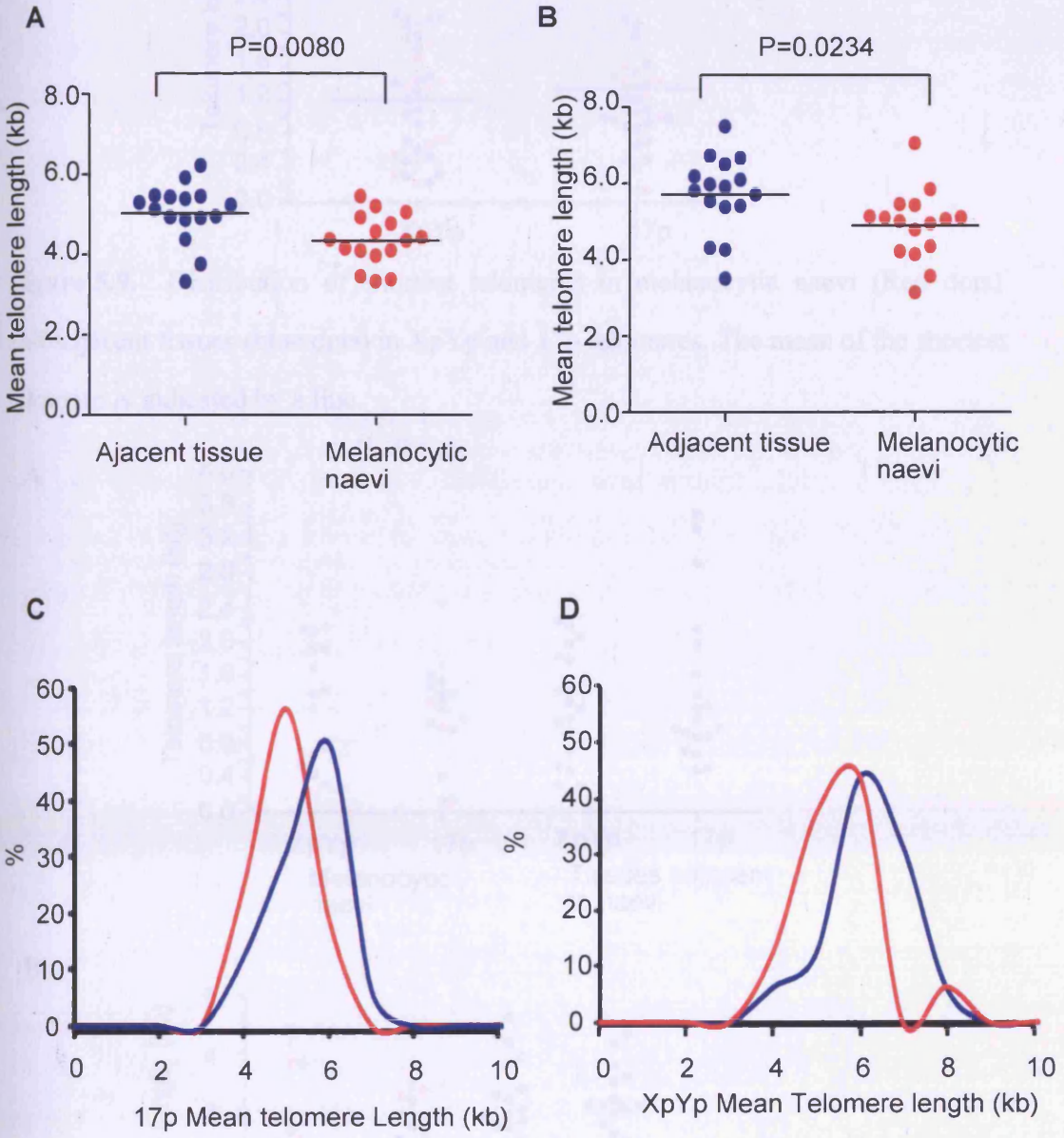


Figure 5.8. Summarising the mean telomere length distributions at telomeres of XpYp (B, D) and 17p (A, C) from 16 patient-matched melanocytic naevi (red dots and red lines) and adjacent tissue (blue dots and blue lines). A-B. Distribution of mean telomere lengths, the black line is the mean of all mean telomere lengths. C-D. The modal distribution of the mean telomere lengths indicating.

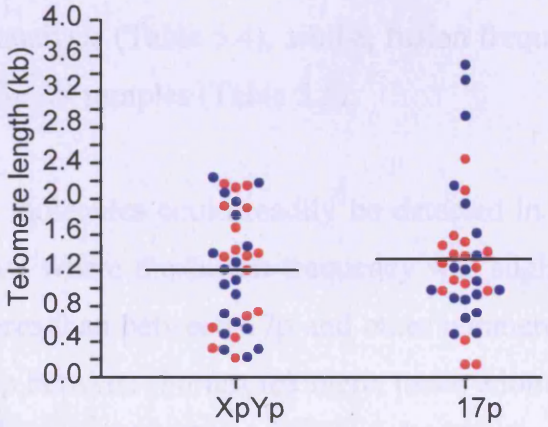


Figure 5.9. Distribution of shortest telomeres in melanocytic naevi (Red dots) and adjacent tissues (blue dots) in XpYp and 17p telomeres. The mean of the shortest telomere is indicated by a line.

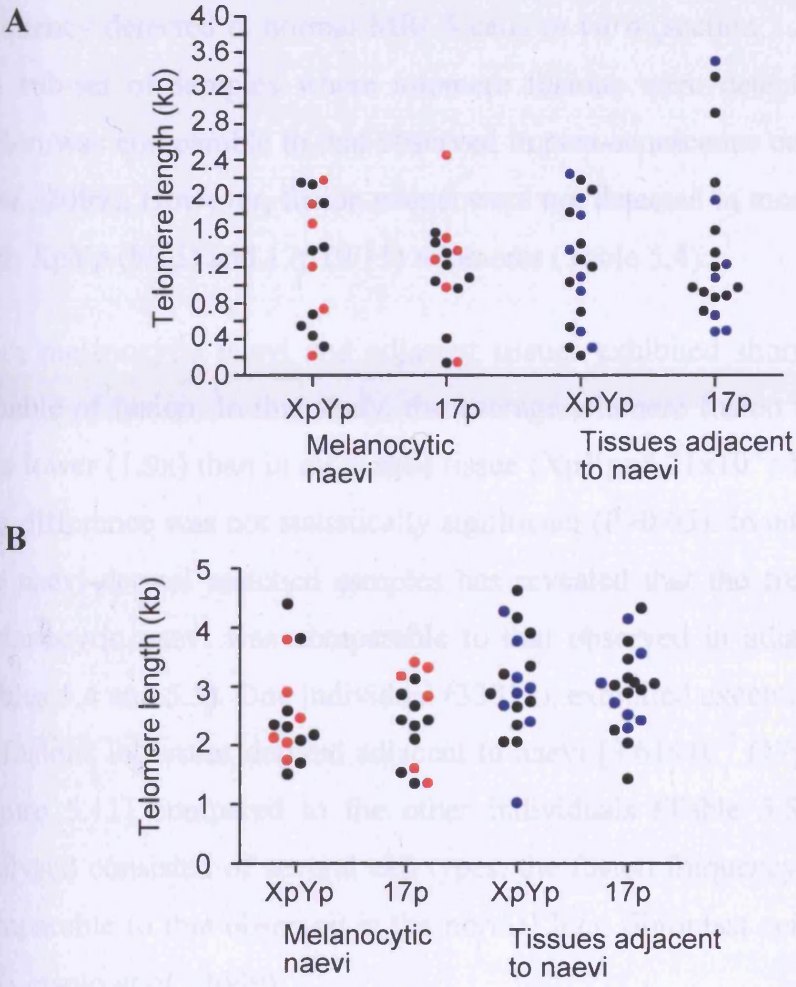


Figure 5.10. Illustrates the samples in which telomere fusions were detected in melanocytic naevi (Red dots) and adjacent tissues (blue dots) at XpYp and 17p telomeres. A. shortest telomeres with samples in which fusion were detected highlighted. B. 20th percentile of telomere distribution with samples in which fusion was detectable highlighted.

telomere fusion analysis (Table 5.4), whilst, fusion frequency for the dermal tissue was determined for 19 samples (Table 5.5).

Telomere fusion molecules could readily be detected in the samples derived from melanocytic naevi, where the fusion frequency was slightly higher between XpYp and other telomeres than between 17p and other telomeres (P=0.03). There was no direct relationship between shortest telomere, mean telomere length at XpYp or 17p or the lower 20th percentile of the telomere distribution and the frequency of the fusions detected with either probe (Figure 5.10 A; 5.10B; Table 5.4). The average telomere fusion frequency in melanocytic naevi was $3x10^{-6}$ and 4.21 $x10^{-6}$ with 17p and XpYp probes respectively [Figure 5.11]; this is comparable to the fusion frequency detected in normal MRC5 cells *in vitro* (section 3.3.4.2 in chapter 3). In the sub-set of samples where telomere fusions were detected, the frequency of fusion was comparable to that observed in post-senescence cells in culture (Letsolo *et al.*, 2009). However, fusion events were not detected in most of the individuals at both XpYp (9/15) and 17p (9/15) telomeres (Table 5.4).

Both melanocytic naevi and adjacent tissues exhibited shorter telomeres that are capable of fusion. In this study, the average telomere fusion frequency in all naevi was lower (1.9x) than in all dermal tissue (XpYp=$8.21x10^{-6}$; 17p $5.6x10^{-6}$) however this difference was not statistically significant (P>0.05). In addition, the analysis of the naevi-dermal matched samples has revealed that the frequency of fusions in melanocytic naevi was comparable to that observed in adjacent tissues (P=0.25; Tables 5.4 and 5.5). One individual (32812), exhibited exceptionally high frequency of fusions in tissues derived adjacent to naevi [$3.61x10^{-5}$ (17p); $6.22x10^{-5}$ (XpYp); Figure 5.11] compared to the other individuals (Table 5.5). Whilst the tissues analysed consisted of several cell types, the fusion frequency in dermal tissue was comparable to that observed in the normal lung fibroblast cell line MRC5 [chapter 3; (Letsolo *et al.*, 2009)].

Consistent with shorter telomeres observed at telomere of XpYp than at 17p in 25% of individuals, the frequency of fusions detected with XpYp probe was higher than

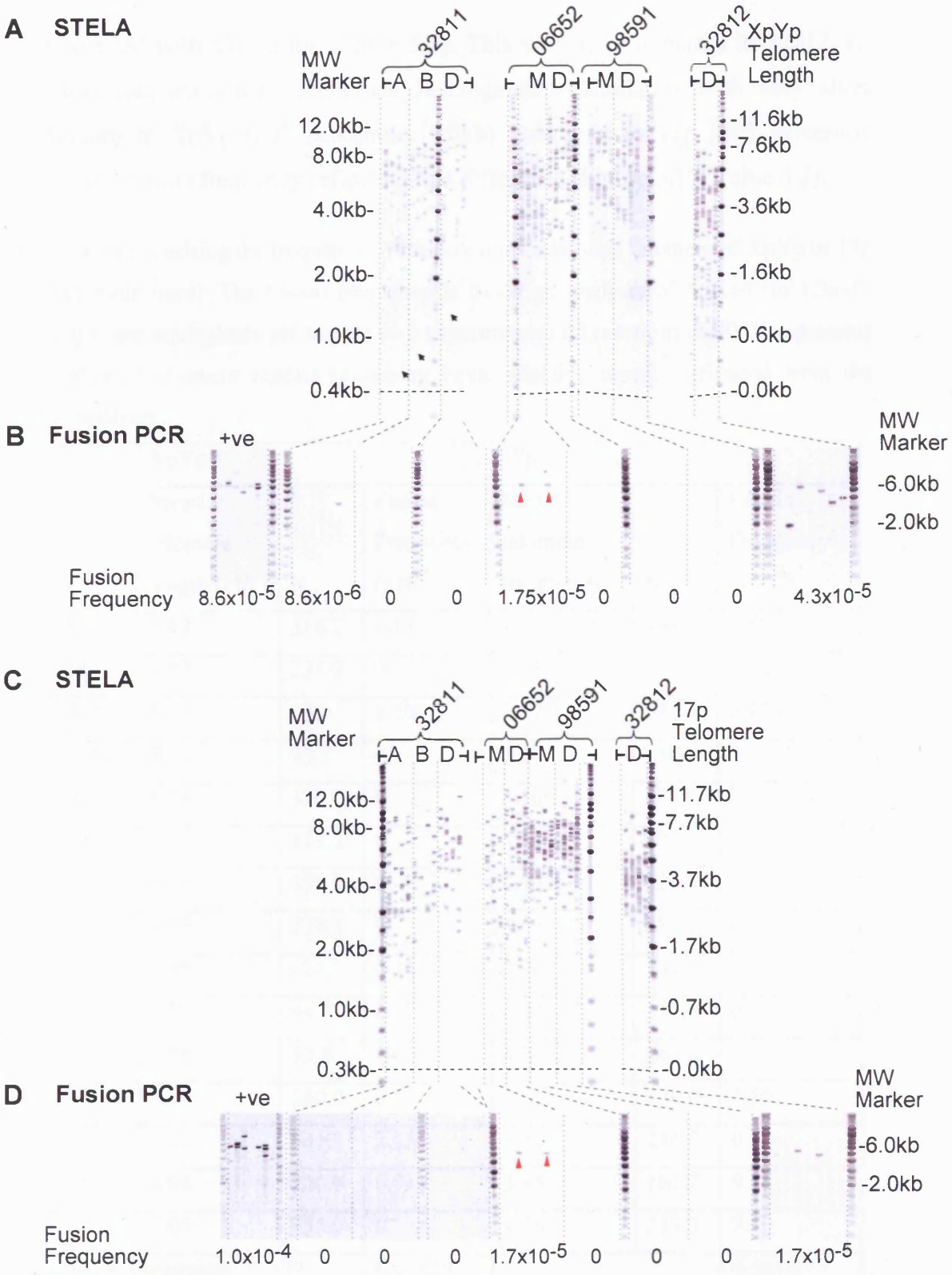


Figure 5.11. Telomere length distribution and fusion frequency in naevi (M) and adjacent tissue (D) derived from four individuals. A and C. STELA gels for XpYp and 17p telomeres respectively. B and D. Fusion PCR between telomeres of XpYp, 17p and /or 21q family detected by XpYp and 17p probes respectively. HEK293 was used as a positive (+ve) control for fusion PCR. The red arrow indicates the clonal fusion events.

those detected with 17p probe (Table 5.5). This was more apparent in 32812, an individual that exhibited exceptional heterogeneous telomeres with very short distributions at XpYp (20th percentile, 1.0kb) compared to 17p (20th percentile 2.7kb); with fusion frequency reflecting this difference (Figure 5.11; Table 5.5).

Table 5.4. Summarising the frequency of fusion events involving telomere of XpYp or 17p in melanocytic naevi. The fusion frequency is based on analysis of $5.8x10^5$ to $1.3x10^6$ diploid genome equivalents per sample (4-5 experiments of 8 reactions each). N represents the number of telomere repeats (assuming hexanucleotide repeat) estimated from the shortest telomere.

	XpYp			17p		
Sample	Mean telomere length (kb)	N	Fusion Frequency ($x10^{-5}$)	Mean telomere length (kb)	N	Fusion Frequency ($x10^{-6}$)
98592M	5.42	316.7	1.13	4.57	408.3	7.50
95981M	4.22	231.7	0	5.45	213.3	1.86
32811A-M	4.13	35.0	1.50	3.27	23.3	0.779
32811B-M	5.10	73.3	0	3.37	23.3	0
20561M	5.45	320.0	0	4.10	181.7	0
20562M	5.06	113.3	0	5.20	205.0	0
52894M	5.84	358.3	0	4.92	226.7	0
93942M	3.57	238.3	0	4.37	68.3	0
2492M	4.35	121.7	0.375	3.96	161.7	0
2493M	4.79	91.7	0	4.42	175.0	0
94041M	3.15	53.3	0	4.09	265.0	0
97051M	5.00	280.0	0.156	5.05	230.0	1.56
93983M	5.12	361.7	2.25	3.33	246.7	0
06652M	4.94	200.0	0.911	3.45	161.7	9.11
98591M	7.05	355.0	0	4.76	253.3	7.50
Total fusion Frequency			$6.32x10^{-5}$			$4.5x10^{-5}$
Average fusion Frequency			$4.21\ x10^{-6}$			$3x10^{-6}$

Table 5.5. Summarising the frequency of fusion events involving telomeres of XpYp or 17p in tissues derived adjacent to melanocytic naevi. The fusion frequency is based on analysis of $5.8x10^5$ to $1.3x10^6$ diploid genome equivalents per sample (4-5 experiments of 8 reactions each). N represents the number of telomere repeats (assuming hexa-nucleotide repeat) estimated from the shortest telomere.

	XpYp			17p		
Sample	Mean telomere length (kb)	N	Fusion Frequency ($x10^{-5}$)	Mean telomere length (kb)	N	Fusion Frequency ($x10^{-5}$)
98592	6.5	363.3	3.93	4.86	583.3	2.75
95981	5.53	215	0	6.21	555	0
32811B	5.8	36.6	0	5.46	270	0
20561	5.7	201.6	0	5.23	120	0
20562	6.66	345	0	3.32	145	0
32812	3.13	50	6.22	4.16	325	3.61
52894	6.18	118.3	0	5.44	180	0
17442	5.25	80	1.53	4.34	81.6	0.613
93941	4.89	155	1.12	4.56	80	1.12
93942	4.25	181.6	0.678	4.37	163.3	0.678
2492	6.09	303.3	0	5.38	358.3	0
2493	5.9	90	0	4.94	148.3	0
94041	5.97	173.3	0	5.91	488.3	0
97051	5.4	328.3	0.313	5.29	155	0
18261	5.37	296.6	1.45	5.42	205	1.07
93983	4.3	245	0	3.75	228.3	0
06651	3.5	51.6	0	4.91	163.33	0
06652	6.71	231.6	0.311	4.92	201.6	0
98951	7.49	373.3	0	5.12	110	0.75
Total fusion frequency			$1.56x10^{-4}$			$1.06x10^{-4}$
Average fusion frequency			$8.21x10^{-6}$			$5.6x10^{-6}$

5.6.5 Molecular structure of telomere fusions

We have previously characterised telomere fusion events between telomeres of XpYp, 17p and 21q family *in vitro* (Letsolo *et al.*, 2009). Thus, I have extended that study to investigate telomere fusion events between these telomeres *in vivo.* The molecular structure of the fusion events was determined from putative fusion events by semi-nested PCR amplification and direct sequencing analysis. However, the fusion assay techniques cannot re-amplify the sister-chromatid type fusions as these have potential to form palindromic structures that can be refractory to PCR. Thus putative sister chromatid-type fusion events detected during this study could not be verified as true fusion by direct sequence analysis.

Forty-five putative fusion molecules were successfully isolated and reamplified for characterisation with direct sequence analysis. Of the 45 isolated putative fusion events, seven did not produce readable sequences while 14 were sequenced 5-6 times but appeared too large to obtain the fusion junction, thus require additional sequencing reactions for future analysis.

Consistent with our *in vitro* (Capper *et al.*, 2007; Letsolo *et al.*, 2009) and other *in vivo* studies (Lin et al., 2010), all of the twenty-four fusion events that were fully sequenced involved the deletion of at least one of the telomeres. The deletion events involved the 1-2kb proximal part of the telomere that contained telomere variant repeats interspersed with TTAGGG repeats (Baird *et al.*, 1995), and extended into the subtelomeric DNA close to the limit of the assay at telomeres of 21q family (3.5kb).

The sub-telomeric deletion size for all the telomeres involved was plotted together due to small sample size [n=24; Figure 5.12]. The mean deletion size was 1253bp, whilst the smallest and largest deletion were 91bp (XpYp) and 3436bp (21q) from the start of the repeat arrays respectively (Figure 5.12A). Interestingly, 65% of the deletion events were clustered within 1.2kb of the telomere repeat array, with the distribution beyond 1.2kb being random. However, these data should be interpreted

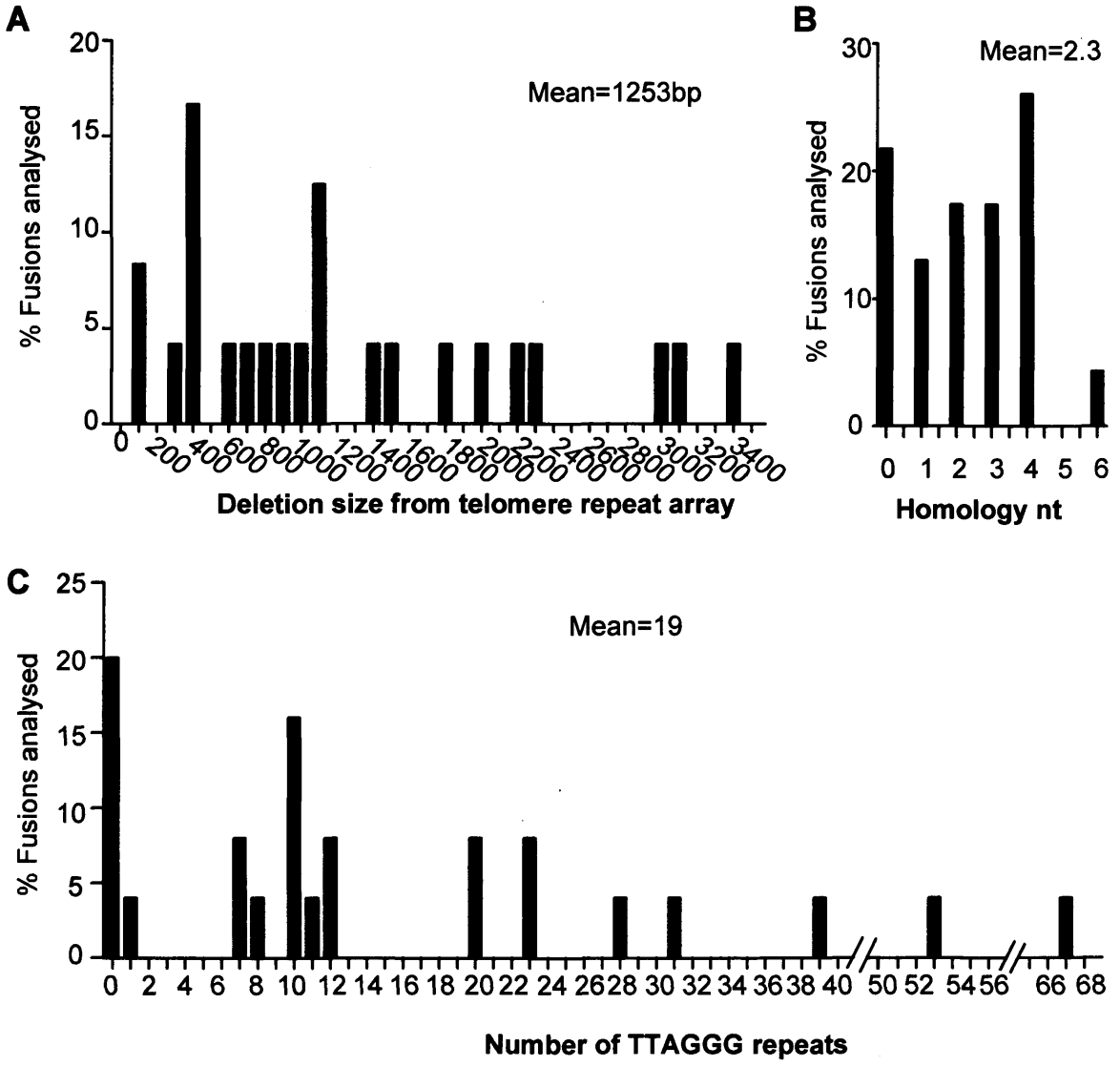


Figure 5.12. Histograms summarising the telomere fusion data from melanocytic naevi and adjacent tissue. A. Deletion size from telomeres of 17p, XpYp and 21q family: 1q, 2q, 5q, 6p, 6q, 8p, 10q, 13q, 17q, 19p, 19q, 21q, 22q and interstitial 2q13. B. The number of perfect microhomology nucleotides at fusion point. C. The number of perfect TTAGGG repeats adjacent to the fusion point. Means are shown for each distribution.

with caution, as the small sample size (n=24) might not be an accurate representation of the distribution of deletion events. Consistent with our previous studies (Capper et al., 2007; Letsolo et al., 2009; Lin et al., 2010), these data indicate that the telomeric deletion can be very extensive.

In addition to sub-telomeric deletions, 72% of the fusion events were characterised by short patches of micro-homology (1-6 nucleotides) at fusion point [Figures 5.12B and 5.13A-D; Mean=2.3 nucleotides]. Although the data did not reveal a clear GC bias (chi-square, P=0.07), analysis of a large sample collection could reveal the GC bias similar to that we observed in the large (n=308) dataset of fusion sequences obtained from cells in culture [(Letsolo *et al.*, 2009); chapter 4].

Furthermore, 20% of the fusion events were characterised by lack of telomere (TTAGGG) repeats but contained extensive number of telomere variant repeats at a fusion junction (Figure 5.12C). Although these events lacked TTAGGG repeats at fusion point, the telomere variant repeats at the fusion junction were preceded by long tracts of TTAGGG repeats (Figure 5.13A). The remaining 80% of fusion events contained TTAGGG repeats at the fusion point that were longer than those we have observed in cells *in vitro* with the mean number of TTAGGG repeats in skin samples being 3.3x [mean 19; chi square, P=0.004] that we observed in HEK293 [mean, 5.2] and MRC5 cells that have bypassed senescence [mean 5.2]. In addition, the largest number of telomere repeats at the fusion point was 67.18 (403bp) perfect TTAGGG repeats. This is the largest number of repeats we have detected so far with these assays [except for MCF7 TRF2 knockdown; (Figures 5.12C and 5.13B)]; indicating that *in vivo,* longer telomeres may become uncapped and initiate fusion compared to that observed *in vitro*.

The fusion molecules are sequenced from individual cells, therefore sequencing of the same-sized fusion molecule which displays same DNA sequence more than once from the same individual (Figure 5.11B and 5.11D; 06652M), would imply that the fusion event is clonal. Of the 24 fusion events, six fusion events represented

three different clonal fusion events; one clonal event detected in 06652M [Figure 5.13C] while two were detected in 32812, an individual that exhibited exceptionally short telomere profiles at XpYp such that 20% of the distributions lie within the TVR region [20^{th} percentile (1kb); Figures 5.11 and 5.13D).

In addition, these events and other events that involved XpYp and 17p telomeres, were characterised by a deletion of the XpYp telomere fused to long tracts of telomere repeats from 17p. Consistent with our study with MRC5 clone 1 that displayed loss of XpYp allele prior to loss of 17p (Capper *et al.*, 2007), these events were derived from individuals in which 17p exhibited longer telomere length profiles than XpYp telomere (Figures 5.13A and 5.13C). This was more apparent in 32812, where XpYp telomere distributions were significantly shorter than those of 17p telomere [$P<0.001$; 20^{th} percentile, (XpYp, 1kb; 17p, 2.7kb); Figures 5.11A and 5.11C, rightmost gels].

A subset of fusion events (3/24) contained an insertion of sequences from elsewhere in the human genome. One of these events contained simple insertion comprising three nucleotides (ACA; Figure 5.13E). The other two events involved an insertion of sequences near documented fragile sites [14q32 (280bp); 9q22.3 (367bp); Figure 5.13F]. Importantly, the locus 9q22.3 is often deleted in sebaceous naevi which have potential to develop into basal skin carcinoma (Xin et al., 1999). These fusion events were both derived from naevi.

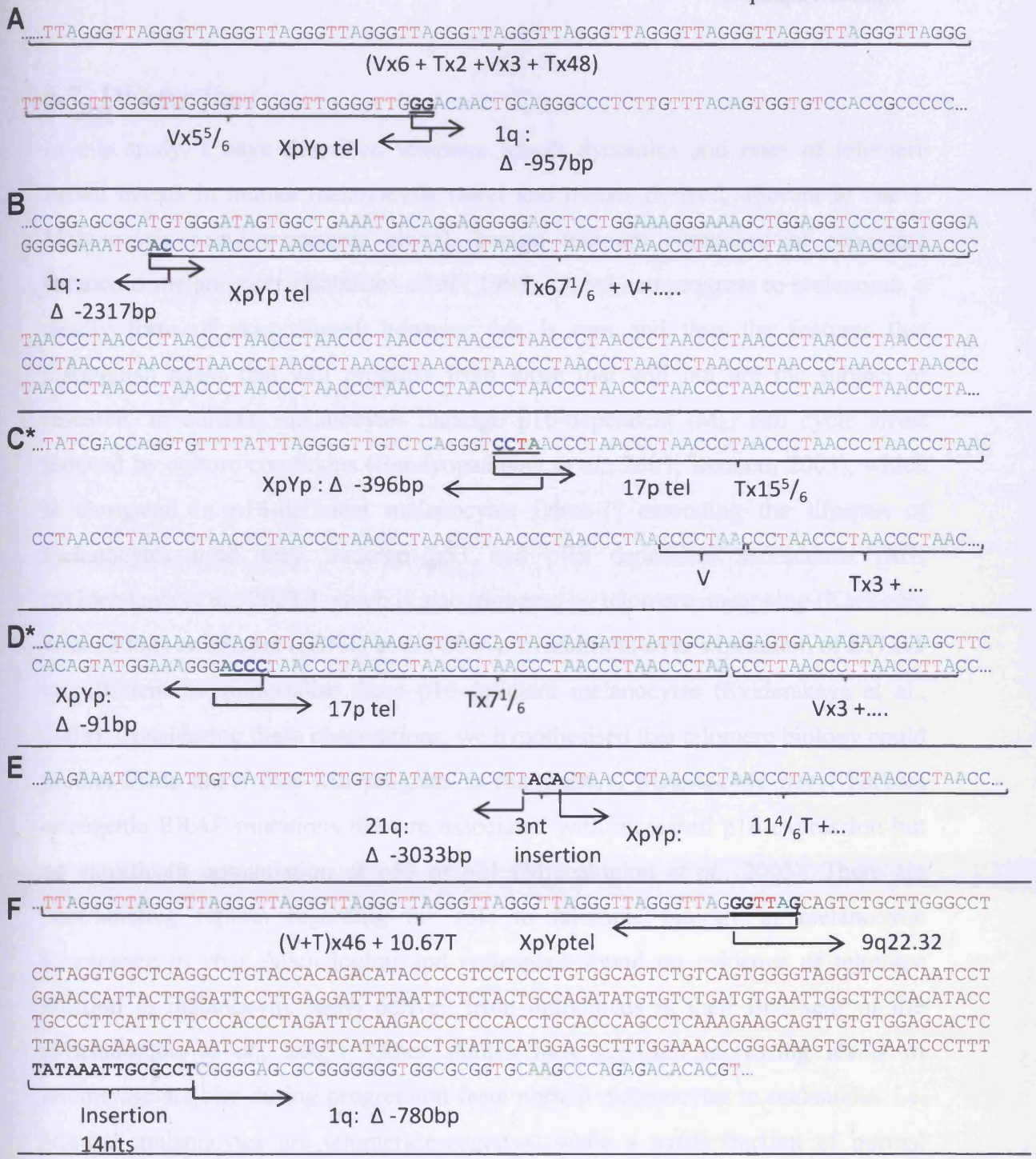


Figure 5.13. Example of DNA sequences for telomere fusion events in melanocytic naevi and associated dermis. A. 1q:XpYp fusion, involving the telomere variant repeats (denoted V: TVRs) and long tracts of TTAGGG (T) prior to TVRs at the fusion junction. B. 1q:XpYp fusion with the longest TTAGGG repeat array so far. C-D. XpYp:17p fusion events which were detected twice in same individual, thus represent 2 different clonal events (*). Short patches of micro-homology (underlined and bolded). E. 21q:XpYp fusion event, with insertion of three nucleotides at fusion point (black emboldened font). F. Complex fusion event involving 1q:XpYp and insertion of 9q22.32. The deletion size as estimated from start of telomere repeat array is indicated by delta (Δ).

5.7 Discussion

In this study, I have described telomere length dynamics and rates of telomere fusion events in human melanocytic naevi and tissues derived adjacent to naevi. Melanocytic naevi represents clonal, benign tumours composed of senescent cutaneous melanocytes (Robinson *et al.*, 1998). Naevi can progress to melanoma, a deadly form of skin cancer, however this is rare and thus the features that distinguish naevi that will progress from those that will not are the subject of research. In culture, melanocytes undergo p16-dependent (M_0) cell cycle arrest induced by culture conditions (Bandyopadhyay *et al.*, 2001; Bennett, 2003), which is abrogated in p16-deficient melanocytes [Hme-1] extending the lifespan of melanocytes until they undergo p53 and pRb dependent senescence [M1; (Sviderskaya et al., 2003)] which is also triggered by telomere uncapping (Karlseder *et al.*, 2002) or erosion (Herbig et al., 2004). In addition, over-expression of hTERT is sufficient to immortalise these p16-deficient melanocytes (Sviderskaya et al., 2003). Considering these observations, we hypothesised that telomere biology could predict those naevi that will progress to malignancy. Melanocytic naevi harbors oncogenic BRAF mutations that are associated with increased p16 expression but no significant upregulation of p53 or p21 (Michaloglou *et al.*, 2005). There are contradicting reports regarding the role to telomere biology in melanocytic senescence *in vivo*. Michaloglou and colleagues found no evidence of telomere attrition in melanocytic naevi derived from individuals in their first year of life (Michaloglou *et al.*, 2005). Other studies have reported increasing levels of telomerase activity during progression from normal melanocytes to melanoma. i.e. Normal melanocytes are telomerase-negative, while a small fraction of normal naevi are telomerase-positive. All atypical naevi and melanomas exhibit elevated telomerase activity (Glaessl *et al.*, 1999; Parris *et al.*, 1999; Taylor *et al.*, 1996). It has been demonstrated that progressive telomere attrition did indeed trigger senescence in normal melanocytes and the ectopic expression of the telomerase catalytic subunit extended the lifespan of melanocytes (Bandyopadhyay et al., 2001; Sviderskaya et al., 2003). Thus the role that telomere biology plays in the progression of melanoma is still unclear.

5.7.1 Large Variation in telomere length

In this study we have utilised our high resolution technologies to re-address the role of telomere dynamics in melanocytic naevi. Using STELA, I have demonstrated that telomere lengths in naevi are shorter than those described in some senescent fibroblasts (Britt-Compton *et al.*, 2006) and telomerase-negative melanocytes in culture (Bandyopadhyay *et al.*, 2001).

The telomere lengths in melanocytic naevi were shorter than those observed in adjacent tissue. These differences could be explained by inherent telomere length difference within each cell type as majority of common naevi and associated peri-lesion tissues are telomerase-negative (Glaessl *et al.*, 1999; Parris *et al.*, 1999). Indeed, several studies have demonstrated that telomere length in epidermis is shorter than in the dermis but both erode with chronological age at the similar rate of 9-75bp/year (Friedrich *et al.*, 2000; Lindsey *et al.*, 1991; Nakamura *et al.*, 2002; Sugimoto *et al.*, 2006). These differences may also be due to differences in the replicative histories of the different cells constituting these tissues (Allsopp et al., 1992).

In addition, large variation in telomere length was observed in both tissues implicating that the telomere length variation at each telomere may be due to inter-individual variation which is probably predetermine in the zygote and maintained throughout life (Graakjaer *et al.*, 2006). Indeed, *in vitro* and *in vivo* studies have revealed inter-allelic variation at the same telomere as well as inter-individual variation across the telomeres of different individuals (Baird *et al.*, 2006; Baird *et al.*, 2003; Britt-Compton *et al.*, 2009; Britt-Compton *et al.*, 2006).

All naevi (except for one sample) exhibited heterogeneous telomere length similar to adjacent tissue. Considering the clonal nature of melanocytic naevi, one would expect that the telomere length profiles would be homogeneous, typical of those of clonal senescent cells in culture. These differences could be accounted for by presence of several sub-clones within the melanocytic naevi that display different

telomere length profiles. In support of this view, other studies have reported the existence of mosaicism in the expression of other genetic markers such as p16 within the same naevi. In addition the fact that some melanocytes that express $BRAF^{-E600}$ do not exhibit any detectable p16, in spite of cells having undergone senescence (Michaloglou *et al.*, 2005), could indicate that other factors such as telomere dysfunction could induce senescence in a subset of melanocytes. Furthermore, the presence of normal melanocytes within the naevi could account for longer telomere lengths. Another possible explanation could be the quality of the sample analysed. The naevi were macro-dissected, thus it is possible that samples contained significant proportion of other cell types either from the epidermis such as keratinocytes or dermis (DMB personal comm.), all contributing to the apparent heterogeneity in telomere lengths. Thus for future investigation, the pure melanocytic naevi preparations would reveal if the large heterogeneity is due to existence of several sub-clones with each naevi.

A subset of individuals exhibited shorter telomere lengths at 17p compared to XpYp. The short arm of chromosome 17 (17p) carries p53, a tumour suppressor protein, which is often deleted in human cancers, including skin cancer and its telomere is one of the shorter in human genome (Martens *et al.*, 1998; Zou *et al.*, 2004). The fact that 17p telomere was longer than XpYp in some individuals suggest that 17p telomere being shorter is not absolute but a random event, consistent with previous studies *in vitro* (Britt-Compton *et al.*, 2006). LOH at 17p has been reported in dysplastic naevi, the naevi which carry increased risk to melanoma (Healy et al., 1995; Park et al., 1998). We have also demonstrated that LOH at 17p telomere was linked to LOH at p53 in B-cells of CLL patients (Lin et al., 2010). The loss of p53 function allows cells to bypass cell cycle arrest and continued telomere erosion, which could generate large-scale rearrangements typical of cancers (Artandi *et al.*, 2000; Rudolph *et al.*, 2001). Thus if telomere dysfunction triggers cell cycle exit in melanocytes *in vivo*, the cells that have lost p53 may exhibit a growth advantage and escape senescence and through accumulation of additional mutations become malignant.

5.7.2 Telomere fusion in normal tissues is similar to that observed *in vitro*.

We have demonstrated that the shortest telomeres are subjected to fusion in cells undergoing crisis in culture and in chronic lymphocytic leukaemia [(Capper et al., 2007; Letsolo et al., 2009; Lin et al., 2010)]. This study has revealed that short telomeres in normal and senescent cells *in vivo* are subjected to fusion. Telomere fusion represents a significant mutational event that has potential to fuel large-scale rearrangements that can lead to deactivation of tumour suppressor gene or oncogenic activation (Artandi *et al.*, 2000; Murnane, 2006; Murnane and Sabatier, 2004).

The study revealed no significant difference in fusion frequencies between the naevi and adjacent tissues; suggesting that the same background rates of fusion occur in both tissues and that this may be independent of replicative telomere erosion but instead more consistent with sporadic telomere deletion. Indeed as illustrated in Figure 5.10, there was no association between shortest telomere, mean telomere length or lower distribution of the telomere profile and fusion frequency. The telomere fusion events that were consistent with sporadic deletion events have been described in Chapter 4 of this thesis as well as in our recent publications in which fusion events that involve longer and shorter length alleles could be distinguished (Capper et al., 2007; Letsolo et al., 2009).

In this study, the frequency of fusion events in dermis (tissue adjacent to naevi) was comparable to that documented from lung fibroblast cell line, MRC5. The dermis consists of several cell types of which fibroblasts constitute the larger portion. Thus the observed fusions could be contributed mainly by the fibroblasts.

The molecular structure of the fusion events was similar to that we have described in human cells *in vitro* (Capper *et al.*, 2007; Letsolo *et al.*, 2009) and *in vivo* (Lin et al., 2010), characterised by large deletions extending into sub-telomeric DNA, patches of DNA sequence homology and a lack on TTAGGG on one side of the

fusion junction. Although the mechanism that underlies these events is not known, it is error-prone and may be indicative of the DNA PKcs/ku independent end-joining processes (Maser *et al.*, 2007; Wang *et al.*, 2003; Yu and Gabriel, 2003) as discussed in chapter 4.

The fusion events that display longer arrays of TTAGGG than those described in cells undergoing crisis were detected. It is possible that different cell types cannot withstand the telomere uncapping to a specific length. Indeed in fibroblast cells that have just bypassed senescence, we observed longer tracts of repeats compared to crisis cells, which indicate that the *in vitro* analysis is physiologically relevant. Another explanation for long tracts of repeats in fusion events that are consistent with sporadic deletion would be the mutational mechanism that the skin is exposed to. First, skin is exposed to UV radiation which is known to target the dithymidines causing breakage, thus it is possible that the UV radiation induces telomere breaks resulting in uncapped telomeres that are capable of fusion (Jin and Ikushima, 2004; Kawanishi and Oikawa, 2004). Similarly UV radiation could create reactive oxygen species which target the triguanine structures and induce telomere erosion (Kawanishi and Oikawa, 2004; Oikawa and Kawanishi, 1999). In support of this, oxidative stress has been implicated as one of the mechanisms that has potential to accelerate telomere erosion (Houben et al., 2008; Kurz et al., 2004; von Zglinicki, 2002). Another possible explanation could be telomere uncapping due to insufficient telomere binding proteins such that certain cell types may harbour mutations in shelterin components. It has been demonstrated that disruption of the shelterin components such as loss of TRF2, Rap1 and Pot1 result in telomere fusions while loss of TRF1 function result in telomere deletion (Bae and Baumann, 2007; He et al., 2006; Sarthy et al., 2009; Sfeir et al., 2009; van Steensel et al., 1998). It is therefore possible that any mutations that results in tethering of shelterin components may generate telomeric deletions resulting in telomere capable of fusion.

Similar to our previous studies (Capper et al., 2007; Letsolo et al., 2009; Lin et al., 2010), there was evidence of clonal fusion events, characteristic of solid tumours (Gisselsson *et al.*, 2001). This data indicates that some of these lesions occur early in premalignant tissues. Clonal events have potential to dominate the cell population and result in clonal expansion, thus creating an environment in which more abnormalities that could drive malignant progression may arise. Clonal expansion has been reported in early-stage, premalignant conditions (Galipeau et al., 1999; Maley et al., 2006).

The telomere fusion analysis also revealed insertion of non-telomeric sequences, two of which were close to fragile sites. The involvement of loci near fragile sites that are often deleted in basal skin cancer could indicate that telomere biology may play a role in chromosome instability that could drive progression to malignancy. Of these fragile sites, 9q22.3 was of interest because LOH at 9q22.3 is frequent in sporadic basal cell carcinoma (Xie *et al.*, 1998). Within 0.3Mb of the fusion junction, lies the PTCH, a gene responsible for nevoid basal cell carcinoma syndrome, a condition that predisposes to basal cell carcinoma. Interestingly, the properties of the naevi are some of the important features in nevoid basal cell carcinoma syndrome (Xin *et al.*, 1999). Thus, the involvement of this locus in telomere fusion in an early premalignant lesion is tantalising. However it is not known if terminal deletion involving 9q22.3 that is frequent in skin cancer is a result of telomere dysfunction. It is thus a subject of next investigation through 9q telomere-specific length analysis in these samples.

In conclusion, the data described in this chapter is consistent with previous studies from human cells in culture which revealed that telomere dysfunction may generate uncapped telomeres that are capable of fusion. It is therefore exciting to observe that telomere erosion, instability and fusion observed *in vitro* is physiologically relevant.

5.8 Key findings

- Telomere length at 17p telomere was shorter than that of XpYp in 65% of melanocytic naevi.

- Similarly, 17p telomere is shorter than XpYp in 74% of skin adjacent to melanocytic naevi.

- In addition, 17p telomere is shorter in naevi than adjacent tissue (56% of individual).

- In a subset of individuals, both XpYp and 17p telomeres were shorter in naevi than adjacent tissue.

- Clonal telomere length distributions at 17p were observed in both naevi and adjacent tissue in different individuals. i.e. homogenous telomere length in one tissue and heterogeneous in another.

- Although the telomere length profiles were shorter in melanocytic naevi compared to the adjacent dermis, there was no difference in the frequency of fusion events using our assays that detect fusion events between telomeres of XpYp, 17p and 21q family.

- For those individuals whose 17p telomere was longer than XpYp, the fusion events involving the two telomeres contained a deletion from XpYp and long arrays of telomere repeats from 17p.

- Clonal telomere lengths and fusion events were observed in naevi and adjacent tissue.

- Fusion frequencies in both naevi and adjacent tissue were similar to those estimated from normal cells *in vitro*. However, the frequency was lower than estimated from cells undergoing crisis.

- Consistent with studies in cells undergoing crisis, the telomere fusion events were characterised by deletions extending into the sub-telomeric DNA, short patches of micro-homologies and lack of TTAGGG repeats on one side of the fusion events.

- In addition, complex fusion events involving insertion of DNA sequences near documented fragile sites were observed. Interestingly, some of these sites are often deleted in skin cancer.

6 Chapter 6: Telomere instability and fusion in Barrett's oesophagus

6.0 Summary

The aim of the work described in this chapter was to investigate telomere dynamics in Barrett's oesophagus. Barrett's oesophagus is a premalignant metaplastic condition in which the normal squamous epithelium lining the distal oesophagus is replaced by columnar epithelium with goblet cells due to chronic reflux disease (Haggitt, 1994; Spechler, 2002). Although, it predisposes patients to development of adenocarcinoma of the oesophagus, only a fraction of Barrett's oesophagus patients progress to adenocarcinoma (Flejou and Svrcek, 2007). Thus, it is essential to determine bio-molecular markers that may predict those individuals that will progress.

A detailed analysis of telomere length at the telomeres of XpYp, 17p, 11q and 9p has revealed that the transformation of native squamous tissue into Barrett's metaplasia is accompanied by telomere erosion ($P<0.001$). In addition, telomere length dynamics was related to zonal segments of oesophageal tissues derived from Barrett's oesophagus patients, such that segments physically separated by distance of 2cm displayed large variation in telomere length ($P<0.01$). Some segments displayed short telomere profiles with narrow variance characteristic of clonal cell populations in culture [(Baird et al., 2003; Britt-Compton et al., 2006); $P<0.0001$)]. The cells displaying such clonal telomere length profiles have potential to dominate the tissue and result in clonal expansion, which could confer selective advantage for other abnormalities that may drive progression to oesophageal adenocarcinoma.

Preliminary analysis of telomere fusion events between telomeres of XpYp, 17p, 16p and 21q families revealed that tissue samples that displayed short telomere length profiles also displayed increased frequency of telomere fusions, characterised by large deletions extending into the sub-telomeric DNA and DNA sequence homology at the fusion point. This mutational profile is similar to that we described in human cells *in vitro* and *in vivo*.

6.1 Terminology

Z-line is the oesophageal squamo-columnar junction: an arbitrary line at which the columnar epithelium changes to squamous epithelium in Barrett's oesophagus [Figure 6.1; (Spechler, 2004)].

Gastro-oesophageal junction [also known as cardia-oesophageal junction (C-O) or oesophago-gastric (O-G) junction is an anatomical region that marks the end of the oesophagus and the start of the stomach. It is often characterised by gastric fold during upper endoscopy [Figure 6.1; (Hayward, 1961; Wild and Hardie, 2003)].

Upper Endoscopy is the procedure in which a flexible fibre-optic lens or a thin scope with a light and camera is passed through the mouth to visualise the upper gastrointestinal tract, oesophagus, stomach or upper part of small intestines (Wild and Hardie, 2003).

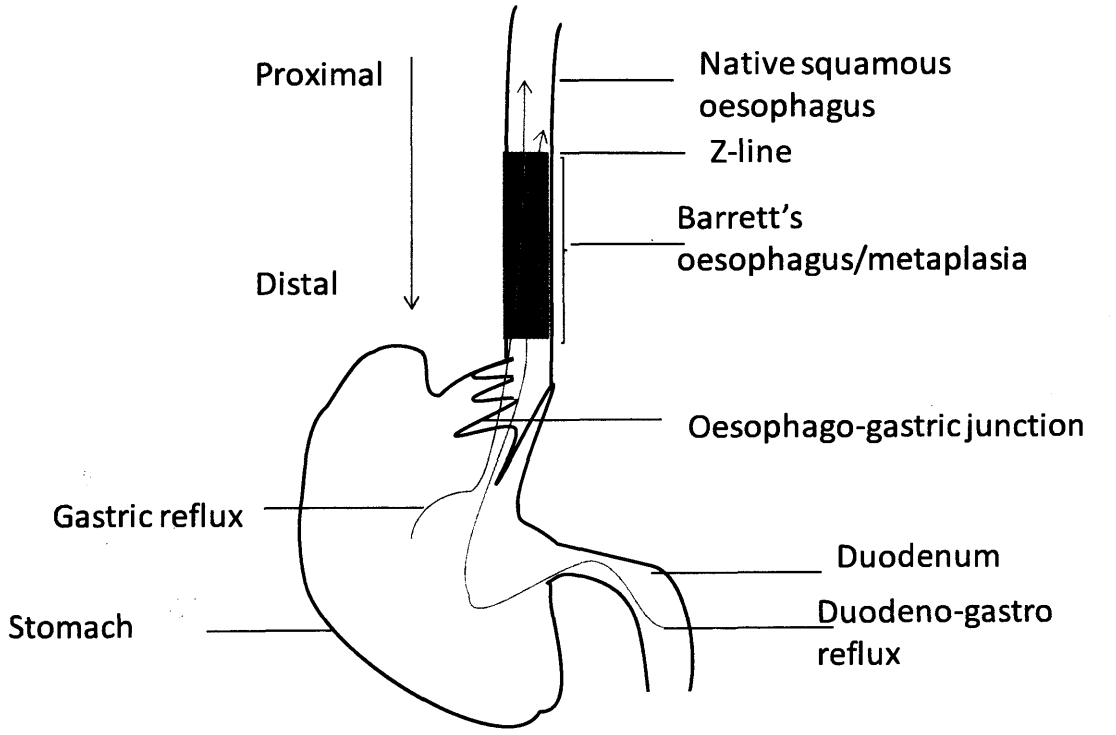


Figure 6.1. Diagrammatic outline of the part of the human digestive system illustrating the normal oesophagus, Barrett's oesophagus, stomach and duodenum.

6.2 Brief introduction

Oesophageal cancer is the eighth [8th] most common cancer worldwide (Parkin, 2001; Parkin et al., 2005). The two types, squamous cell carcinoma and adenocarcinoma of the oesophagus differ histologically but are diagnosed, treated and managed in the similar way. Squamous cell carcinoma tends to occur on proximal part of the oesophagus (Parkin, 2001; Parkin et al., 2005) and its geographical distribution varies widely with exceptionally high occurrences of the malignancy in the Shanxi province of north central china, whose cause is not known (He et al., 2008; He et al., 2003).

Oesophageal adenocarcinoma, on the other hand is found at the lower/distal part of the oesophagus [Figure 6.1 for distal oesophagus]. It is the predominant form of oesophageal cancer in western countries (Parkin, 2001; Parkin et al., 2005). This study will be limited to the discussion of oesophageal adenocarcinoma with little reference to squamous cell-carcinoma whenever required. The incidence of oesophageal adenocarcinoma has been increasing dramatically over the past three decades in the western world (Devesa et al., 1998; Newnham et al., 2003). The risk factors include: age of 65 or older, being male, smoking, heavy alcohol consumption, poor nutrition that is low in vitamins and mineral but high in fats and salts, obesity, acid reflux disease and Barrett's oesophagus (Devesa et al., 1998; Lagergren et al., 1999; Wild and Hardie, 2003).

6.2.1 Barrett oesophagus and Gastro-oesophageal reflux disease (GORD)

Gastro-oesophageal reflux disease (GORD; referred to hereafter as reflux disease) is a complication due to reflux of stomach and possibly duodenual contents into the oesophagus causing symptoms or damage to the mucosa [Figure 6.1; (Fox and Forgacs, 2006)]. The most commonly recognised manifestation of reflux is frequent heartburn or a burning sensation in the chest after meals and/or on bending (Klauser et al., 1990; Lagergren et al., 1999). Other symptoms

include regurgitation, hoarseness, chronic cough, bleeding and weight loss (Klauser et al., 1990).

The progression from reflux disease to cancer is believed to be a stepwise (Flejou, 2005; Miros et al., 1991) process arising from the prolonged exposure of oesophageal tissue to duodena-gastric contents (bile, acid, pepsin) thus damaging the oesophageal tissue. The damaged tissue then heals possibly through a metaplastic replacement of native squamous epithelium by columnar intestinal-like epithelium with goblet cells, a condition termed Barrett's oesophagus. Such intestinal metaplasia can then progress through accumulation of genetic abnormalities to dysplasia and ultimately cancer (Flejou, 2005; Jenkins et al., 2002; Spechler, 2002).

Barrett's oesophagus is an acquired, hyper-proliferative and premalignant lesion that predisposes to oesophageal and oesophago-gastric adenocarcinomas; the malignancies on the increase in western world (Blot et al., 1991; Drewitz et al., 1997). It is defined as a change of any length [long segment (>3cm) or short segment (<3cm)] that can be observed during endoscopy (Sampliner, 2002; Sharma et al., 1998). However, it excludes internal metaplasia of the cardia but includes any intestinal metaplasia at the oesophagus (Sampliner, 2002).

The risk of developing cancer in Barrett's oesophagus patients is estimated to be 30-125 times more than the general population (Cameron et al., 1985; Jenkins et al., 2002); whilst the incidences of adenocarcinoma are 0.5-1% per patient year i.e. 1 incidence per 100-200 patient years of observation (Koppert et al., 2005; Shaheen et al., 2000). However, the prevalence of Barrett's oesophagus in the general population is not known, as some patients are asymptomatic (Gerson et al., 2002; Shalauta and Saad, 2004). Nonetheless, those individuals with symptomatic Barrett's oesophagus, exhibit symptoms, typical of reflux disease. It is not known whether reflux disease predisposes to Barrett's oesophagus or whether it promotes carcinogenesis in patients with Barrett's oesophagus.

Nevertheless, it is estimated that 5-15% of patients with gastro-oesophageal reflux disease also have Barrett's oesophagus (Flejou, 2005; Lagergren et al., 1999).

Like oesophageal adenocarcinoma, risk factors for Barrett's oesophagus include being a Caucasian male, alcohol and drug-abuse, obesity and older age greater than 60 years (Cameron and Lomboy, 1992; Prach et al., 1997). It is uncommon in Asia and Far east, with an estimated 0.3-0.6% cases reported in Japan (Nakamura et al., 1999). The mean age at diagnosis is 55-60 years, however, studies have reported the increasing risk of Barrett's oesophagus with progressive increase in age from 30 to 70 years (Conio et al., 2001). Nonetheless, Barrett's oesophagus is rarely diagnosed during childhood. In addition, individuals with family history of Barrett's oesophagus, oesophageal or oesophago-gastric junction adenocarcinoma are likely to have Barrett's oesophagus (Chak et al., 2004; Chak et al., 2002; Chak et al., 2006). However, no specific gene has been identified so far that predisposes individuals to either Barrett's oesophagus or oesophago-gastric cancers.

6.2.2 Common genetic changes in Barrett's oesophagus

Several genetic changes have been associated with clonal evolution in precancerous Barrett's metaplasia and hence neoplastic progression (Jankowski et al., 1999; Reid et al., 2003). However, this discussion will be limited to tumour suppressor loci and some of the important cell cycle proteins.

The CDKN2A gene, located at 9p21 encodes for a CDK inhibitor, p16. Loss of p16 (a product of CDKN2A) function, is reported in 85% of Barrett's oesophagus patients and is detectable very early in Barrett's oesophagus (Maley et al., 2004b; Wong and Fitzgerald, 2005). The allelic losses of p16 through promoter hyper-methylation, loss of heterozygosity and/or sequence mutations in metaplastic and dysplastic Barrett's tissue are more prevalent than losses of p53,

and precedes aneuploidy (Barrett et al., 1996a; Galipeau et al., 1999; Hardie et al., 2005).

The TP53 gene at chromosome 17p13, encodes for a cell cycle protein p53, which monitors the integrity of the genome. The loss of p53 function through mutations and LOH is a common feature of human cancers (Hollstein et al., 1991; Lane, 1994). Similarly, losses of p53 appear to be an early event during oesophageal carcinogenesis, with losses of p53 reported in 60% of metaplastic and 40-66% of dysplastic Barrett's tissues and 40-88% of oesophageal adenocarcinomas. However, in Barrett's metaplasia, loss of p53 is preceded by loss of p16 thus exhibiting selective advantage for clones that have lost p16 (Doak et al., 2003; Jenkins et al., 2003). Indeed, loss of p53 has been associated with increased risk of progression to cancer. For instance, according to Reid and colleagues, patients with inactive p53 have 16x increased risk of progressing to cancer than their counterparts with functional p53 (Reid et al., 2000; Reid et al., 2003).

Allelic losses at the adenomatosis polyposis coli gene (APC; 5q21), loci for deleted in colorectal cancer (DCC;18q) and deleted in pancreatic cancer (DPC4;18q) tumour suppressor genes are all common in Barrett's oesophagus (Barrett et al., 1996b; Bektas et al., 2000; Zhuang et al., 1996).

Cyclin D1, which is located on 11q13 is a cell cycle protein that phosphorylates pRb thereby inactivating it and thus facilitating G_1-S phase cell cycle progression (Lukas et al., 1996). Cyclin D1 is often over-expressed in Barrett's oesophagus and has been reported to be associated with increased risk of disease progression to cancer (Arber et al., 1996; Bani-Hani, 2000). In contrast, the loss or alteration in function of pRb genes occurs late during oesophageal carcinogenesis, with no losses detected in Barrett's metaplasia (Maley et al., 2006; Sarbia et al., 2001).

6.2.3 Clonal evolution

Neoplastic progression is an evolutionary process characterised by genomic instability. During progression, cells in neoplasm acquire heritable genetic and epigenetic lesions that affect their survival and reproduction, typically generating clonal heterogeneity (Barrett et al., 1999). If these cells are selected for during evolution, they could dominate the population and lead to clonal expansion. Barrett's metaplasia is recognised as a neoplasm (Maley et al., 2004b; Wong et al., 2001) as clones carrying CDKN2A mutation and methylation, 9p LOH, TP53 mutations and 17p LOH are usually highly selected for clonal expansion during oesophageal carcinogenesis (Galipeau et al., 1999). Evidently, the sizes of clones with 17p LOH or DNA content tetraploidy and aneuploidy increases the risk of progression from Barrett's oesophagus to oesophageal adenocarcinoma (Maley et al., 2006; Maley et al., 2004a).

6.2.4 Telomere dynamics in Reflux disease and Barrett's oesophagus

The gastro-oesophageal reflux disease is known to increase the proliferation of squamous cells lining the oesophagus. In addition, contents of reflux could produce reactive oxygen species, which also cause oxidative DNA damage, which is believed to accelerate telomere shortening (Olliver et al., 2003; von Zglinicki, 2002; Zhang et al., 2001). It would be expected that telomeres in reflux disease undergo progressive telomere shortening. Indeed Souza and colleagues have reported short telomeres in tissues derived from the distal oesophagus of reflux disease sufferers (Souza et al., 2007). In addition, telomere erosion has been implicated in the evolution of chromosome instability in Barrett's oesophagus, resulting in gains and losses of specific chromosomes (Finley et al., 2006; Meeker et al., 2004). Telomere erosion has also been detected in tissue samples derived from Barrett's oesophagus patients, with telomere erosion being associated with increased chromosome instability (Finley et al., 2006). In contrast, the inverse association between the frequency of chromosome anaphase

bridges, surrogate markers of chromosome instability induced by telomere dysfunction and telomere lengths have been reported in tissues derived adjacent to Barrett's metaplasia (Kammori et al., 2006; Kammori et al., 2007). Short telomeres in leucocytes blood from Barrett's oesophagus patients have also been reported to predict risk of oesophageal cancer (Risques et al., 2007). Whilst increasing levels of hTERT expression are associated with progression to cancer, such that minimal telomerase activity is detectable in Barrett's metaplasia with a further increase in high-grade dysplasia (HGD) and adenocarcinoma (Going et al., 2004).

It is clear from these studies, cited previously that telomere shortening and chromosome instability occur early in neoplastic progression of reflux disease and Barrett's metaplasia. However, these studies have not defined the actual telomere length in tissues derived from corresponding patients. Similarly, the molecular structure of telomere-related chromosome fusions has not been studied. Thus, this study will investigate these aspects; telomere length and telomere fusions in details.

6.3 This study

Barrett's oesophagus is a good model for human neoplastic progression, as progression from Barrett's oesophagus is believed to follow multi-step, histological stages of metaplasia, low-grade and high-grade dysplasia to cancer, with high-grade dysplasia carrying the elevated risk of progression (Barrett et al., 1999; Flejou, 2005; Jankowski et al., 1999). However, inter-observer variation during diagnosis (Reid et al., 1988) coupled with difficulty in identifying stages due to chronic inflammation and possibility of regression in putative high-risk dysplasia (Conio et al., 2003; Weston et al., 2000), complicates the prediction for those individuals who will progress. Thus, there is a need for development of reproducible biomarkers that could identify those individuals that will progress.

The development of Barrett's oesophagus involves the hyper-proliferative and chronic inflammatory state (Spechler, 2002), thus we considered that the generation of reactive oxygen species (ROS) from acid and bile could drive telomere attrition and dysfunction in Barrett's metaplasia, resulting in evolution of clones with increased chromosome instability and hence initiation of oesophageal cancer. This study was therefore aimed at investigating the role of telomere biology in the transformation of normal squamous oesophageal epithelium to Barrett's metaplasia.

In addition, chromosome instability has been implicated to initiate and drive genomic instability in pre-neoplastic diseases such as ulcerative colitis (Chin et al., 2004; Meeker et al., 2004; O'Sullivan et al., 2002). Similarly, chromosome instability is one of the common events during progression from Barrett's metaplasia to adenocarcinoma (Finley et al., 2006; Jenkins et al., 2002). The chromosome instability could be a result of telomere dysfunction which has been shown to drive tumour progression through among others breakage-fusion-bridge cycles (Artandi et al., 2000; Rudolph et al., 2001). Therefore, another objective of the study was to investigate the telomere instability and fusion in tissues derived from Barrett's oesophagus patients.

To achieve these objectives, STELA and telomere fusion assays were employed to study telomere length distribution at the telomeres of XpYp, 17p, 11q and 9p; these were selected because LOH at 17p, and 9p, and gains of 11q are frequent events in Barrett's oesophagus (section 6.2.2). Telomere fusion events between telomeres of XpYp, 17p, 21q and 16p family were investigated in patient-matched samples consisting of gastric tissues, Barrett's metaplasia and native squamous epithelium. These data will define the extent of the telomere erosion, instability and dysfunction. In addition, whilst the chromosome instability is common in Barrett's oesophagus during disease progression, the data collected from this study will provide insights into the nature of telomere-induced chromosome instability (in the form of telomere fusion sequences).

6.4 Sample collections and Statistical tests

Tissue biopsies, obtained from 14 patients undergoing periodic endoscopy at the Morrison's hospital in Swansea were treated with the tissue rupture homogeniser (Qiagen) prior to DNA extraction with standard methods (Sambrook et al., 1989). The tissue samples were composed of 14 gastric mucosae, 13 Barrett's metaplasia and 9 squamous epithelia. It is worth noting that biopsies derived from only eight individuals consisted of all the three tissues.

Another set of tissue biopsies was obtained from patients undergoing periodic endoscopy for Barrett's oesophagus at Glasgow Royal Infirmary (Going et al., 2004). Tissues samples were supplied from 24 patients consisting of samples cut every 2cm from the normal squamous epithelium of the oesophagus, oesophageal squamo-columnar junction ("Z line"); through Barrett's metaplasia, cardia or oesophago-gastric (O-G) junction, then the gastric body and antrum (Figure 6.2). Of these, two were classified as minimal and severe dysplasia respectively (Table 2.3 in chapter 2). An additional two sets of samples (making a total of 26 patients) were obtained from one patient in which the gastric and oesophageal tumours were detected as well as another patient that had an unclassified tumour (by collaborators). The telomerase activity of these samples was documented previously (Going et al., 2004); telomerase activity was absent in the gastric tissues; body and antrum. Whereas, all the oesophageal mucosa samples were telomerase-positive and this increased moving distally within the Barrett's mucosa (Going et al., 2004).

The tissue biopsies derived from patients at Swansea and Glasgow were analysed independently as biopsies collected from each individual comprised of different number of samples.

Statistical methods employed were as described in chapter 5 of this thesis.

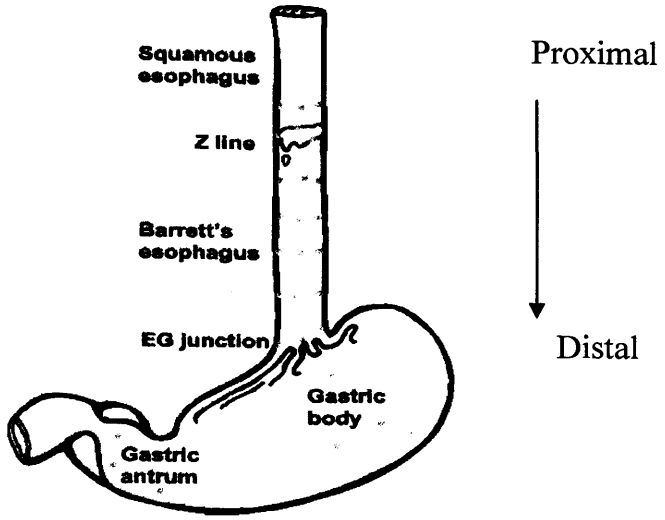


Figure 6.2. Anatomy of the oesophagus and Stomach, with biopsy sites indicated by grey dots. The normal oesophagus is lined by the squamous epithelium, however when the distal oesophagus become damaged partly due to gastric reflux the cells changes to columnar-like, a condition called Barrett's oesophagus/metaplasia. The end of oesophagus and start of the stomach is characterised by the folds, made of cardia tissue and is termed the oesophago-gastric (EG) junction. The proximal and distal parts of the stomach are called gastric body and gastric antrum respectively [obtained from (Going et al., 2004)].

6.5 Results

6.5.1 Telomere length in Squamous, Barrett's and Gastric Mucosa.

The telomere length was investigated at the telomeres of XpYp, 17p, 11q and 9p in squamous epithelium, Barrett's and gastric tissues collected from patients at Morrison hospital Swansea (referred to hereafter as first dataset). The analysis of all samples revealed no difference in mean telomere length distributions at all the four telomeres in squamous tissues of the nine individuals (P=0.36). However, within each individual, the XpYp telomere was the shortest in 4 out of 9 individuals (P<0.05; Figure 6.3i). Similarly, the mean telomere lengths were not different in Barrett's (P=0.96; Figure 6.3ii) and gastric mucosa (P=0.64; Figure

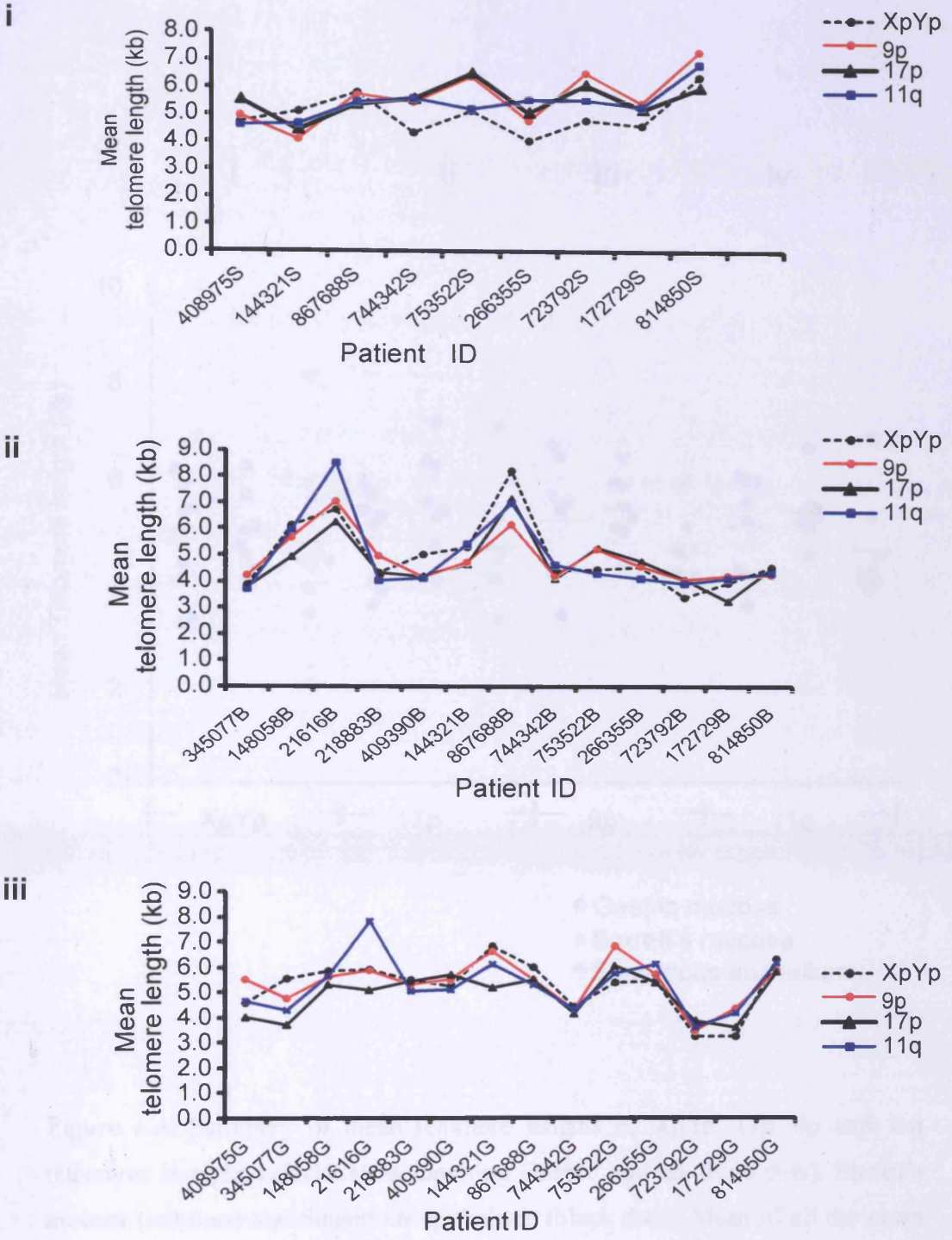


Figure 6.3. Summary of the mean telomere length at telomeres of XpYp, 17p, 9p, 11q in normal squamous oesophagus (i), Barrett's metaplasia (ii) and gastric mucosa (iii). The mean telomere length distributions were not different in any of the individuals.

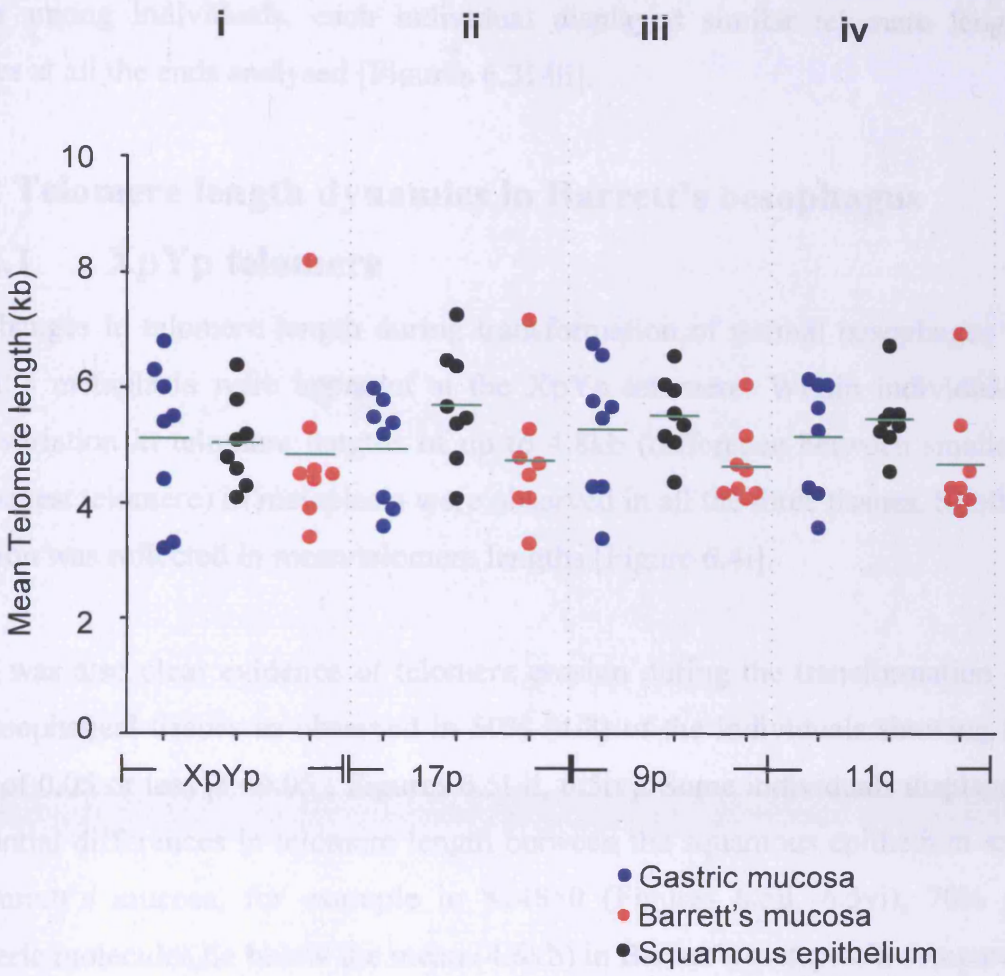


Figure 6.4. Summary of mean telomere lengths of XpYp, 17p, 9p and 11q telomeres in eight individuals comprising Gastric mucosa (blue dots), Barrett's mucosa (red dots) and Squamous epithelium (black dots). Mean of all the mean within sample population are indicated (Green line).

6.3iii), with no apparent telomere being the shortest. Interestingly there was large inter-individual variation in mean telomere length in Barrett's mucosa in all telomeres; with variation of up to 4.8kb (difference between smallest and longest means) at XpYp telomeres. Whilst there is considerable variation in telomere length among individuals, each individual displayed similar telomere length profiles at all the ends analysed [Figures 6.3i-iii].

6.5.2 Telomere length dynamics in Barrett's oesophagus

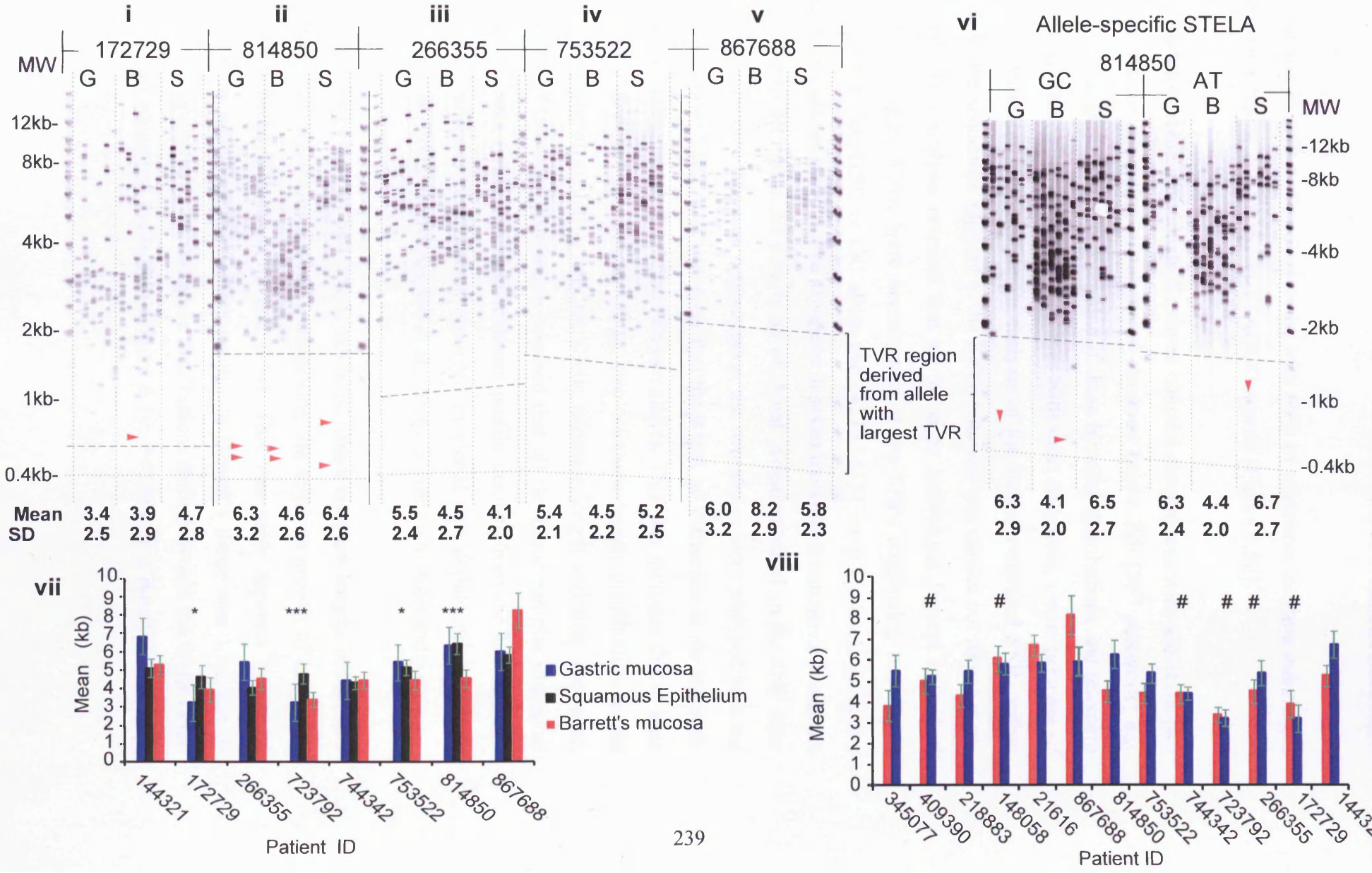
6.5.2.1 XpYp telomere

The changes in telomere length during transformation of normal oesophagus to Barrett's metaplasia were apparent at the XpYp telomere. Within individuals, large variation in telomere lengths of up to 4.8kb (difference between smallest and longest telomere) in metaplasia were observed in all the three tissues. Similar variation was reflected in mean telomere lengths [Figure 6.4i].

There was also clear evidence of telomere erosion during the transformation of the oesophageal tissues as observed in 50% (4/8) of the individuals showing P-value of 0.05 or less [$P<0.05$; Figures 6.5i-ii, 6.5iv]. Some individuals displayed substantial differences in telomere length between the squamous epithelium and the Barrett's mucosa, for example in 814850 (Figures 6.5ii, 6.5vi), 70% of telomeric molecules lie below the mean (4.6kb) in Barrett's metaplasia compared to 45% of molecules greater than 6.4kb (mean) in squamous epithelium (Figure 6.5ii).

It was also apparent that telomere erosion could result in severe telomere shortening with telomere profiles extending close to the length range at which telomere fusion has previously been detected (Capper et al., 2007). The evidence for this came from the analysis of the telomere variant region (TVR) using TVR-PCR [Figures 6.5i-vi; (Baird et al., 1995)] where telomere length data was corrected for the presence of the non-functional telomere variant repeat region. It was clear that telomere length in Barrett's oesophagus were similar to that

Figure 6.5 Telomere distribution at XpYp telomere in squamous (S, black bars), gastric (G, blue bars) and Barrett's mucosae (B, red bars). STELA products were detected by Southern hybridisation with TTAGGG-containing probe. i Bimodal telomere distribution in Barrett's and gastric tissue. ii. Individual with two alleles could be distinguished by allele-specific STELA (vi). iv. Telomeres in Barrett's metaplasia are shorter than squamous. iii, v. Barrett's oesophagus length is longer than in squamous tissue. Mean telomere length and standard deviations (SD) are shown below each distribution vii. Summary of the mean telomere distributions in eight individuals. For $P<0.0001$ [***] and $P<0.05$ [*] are indicated. Viii. Summary of the mean distributions at XpYp between gastric (blue bars) and Barrett's mucosae (red bars). The individuals in which there was no significant difference between the mean telomere distributions are shown by #. Error bars (green) represent 95% confidence intervals.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vi
MW
172729
814850
266355
753522
867688
G B S
12kb-
8kb-
4kb-
2kb-
1kb-
0.4kb-
Mean 3.4 3.9 4.7 6.3 4.6 6.4 5.5 4.5 4.1 5.4 4.5 5.2 6.0 8.2 5.8
SD 2.5 2.9 2.8 3.2 2.6 2.6 2.4 2.7 2.0 2.1 2.2 2.5 3.2 2.9 2.3
Allele-specific STELA
814850
GC
AT
TVR region derived from allele with largest TVR
-12kb
-8kb
-4kb
-2kb
-1kb
-0.4kb
6.3 4.1 6.5 6.3 4.4 6.7
2.9 2.0 2.7 2.4 2.0 2.7
vii
Mean (kb)
Gastric mucosa
Squamous Epithelium
Barrett's mucosa
144321 172729 266355 723792 744342 753522 814850 867688
Patient ID
viii
Mean (kb)
345077 409390 218883 148058 21616 867688 814850 753522 744342 723792 266355 172729 144321
Patient ID

observed in cells undergoing crisis, with 60% of telomeres in some individuals clustered below 2kb of perfect TTAGGG repeats [Figure 6.5ii].

In addition, some individuals displayed bimodal distributions with one of the two distributions consisting of telomeres clustered below 2kb [30th percentile; e.g. 172729; Figure 6.5i]. To determine if these bimodal distributions and telomere erosion represented evolution of clones with short telomeres, or the presence of allelic variation, direct sequence analysis of the well-documented SNPs within the 850bp sequences adjacent to the XpYp telomere was carried out (Baird et al., 1995). This analysis revealed that all the eight individuals [except individual 814850; Figure 6.5vi] were homozygous at the SNPs comprising two XpYp alleles (-427G/-415C or GC allele and -427A/-415T or AT allele). Thus, the telomeric alleles could not be separated in seven individuals and hence it was not possible to determine the possibility of clonal evolution based on the SNP data. In one individual that was heterozygous, the two alleles were analysed with the allele-specific STELA. It was clear that there was no difference in the telomere length distributions derived from the two alleles. This data indicates that at least in this individual the shorter of the two telomere length distributions could represent clonal evolution and not allelic telomere length variation. However, analysis of telomere distribution revealed that all the tissue samples displayed heterogeneous (wide variance) telomere profile distinct from the homogenous (narrow variance) distributions observed in clonal cell population in culture [(Baird et al., 2003; Britt-Compton et al., 2006); Figures 6.6i, 6.6ii and 6.6iii].

Twenty-five percent of individuals exhibited longer telomere lengths in Barrett's mucosa than squamous ($P<0.01$), suggesting the selective growth of cells with long telomeres (Figures 6.5iii and 6.5v). This was more apparent in patient 867688, in which mean telomere lengths in Barrett's tissue was 1.5x that of squamous epithelium, with telomere distributions shifted towards the upper range relative to squamous epithelium (Figure 6.5v; $P<0.05$). It is possible that these

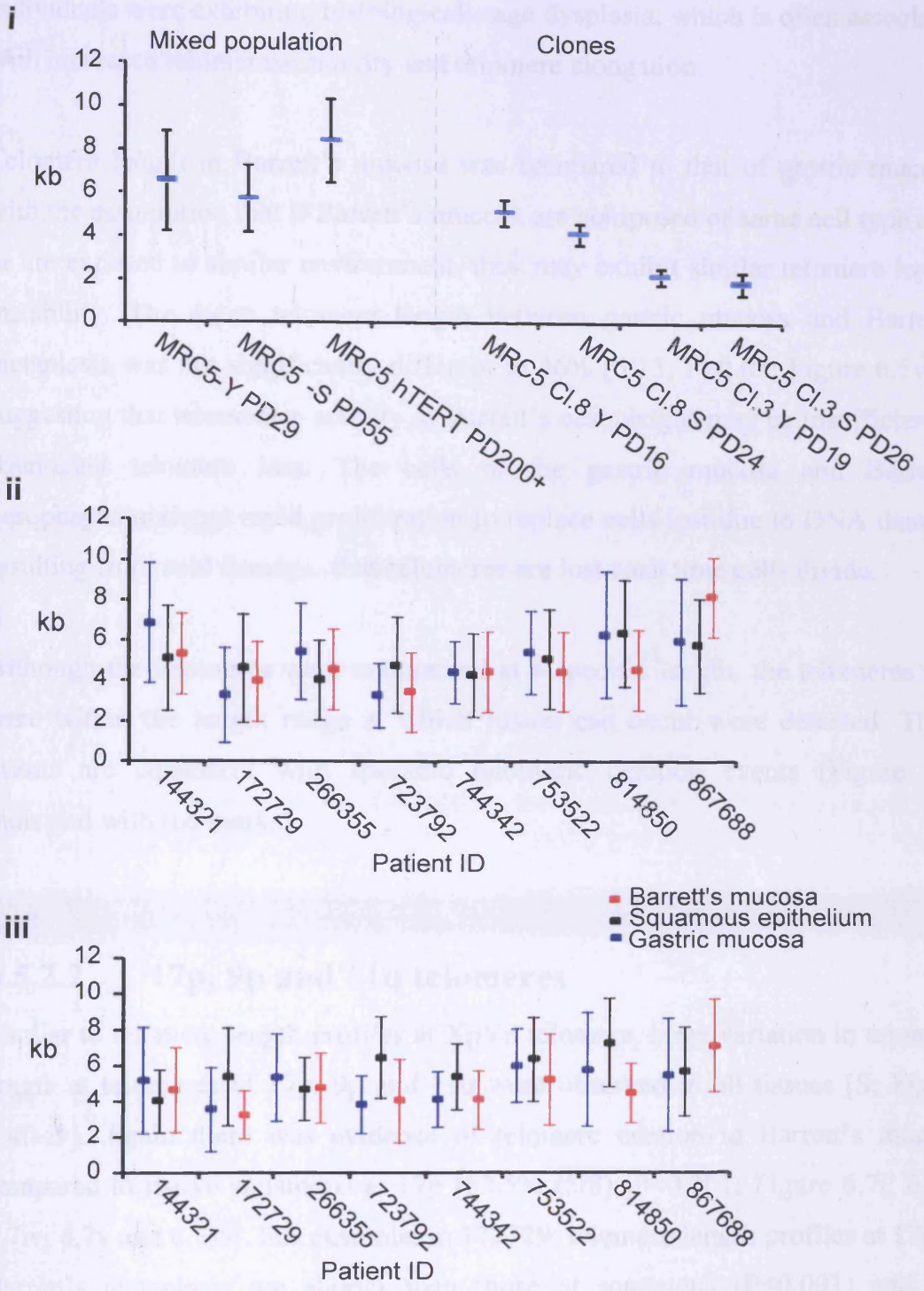


Figure 6.6. Summary of mean telomere length distribution (middle bar) and Standard deviations (error bars). (i) Mixed population and clonal (Cl.) cell proliferations derived from human lung fibroblast MRC5. Y and S represents young and senescent cells respectively (data derived from Baird et al 2003, supplementary data). (ii) and (iii) distributions in gastric (blue), Barrett's (red) and squamous epithelium of the oesophagus (black) in XpYp (i) and 17p (ii) telomeres respectively.

individuals were exhibiting histological stage dysplasia, which is often associated with increased telomerase activity and telomere elongation.

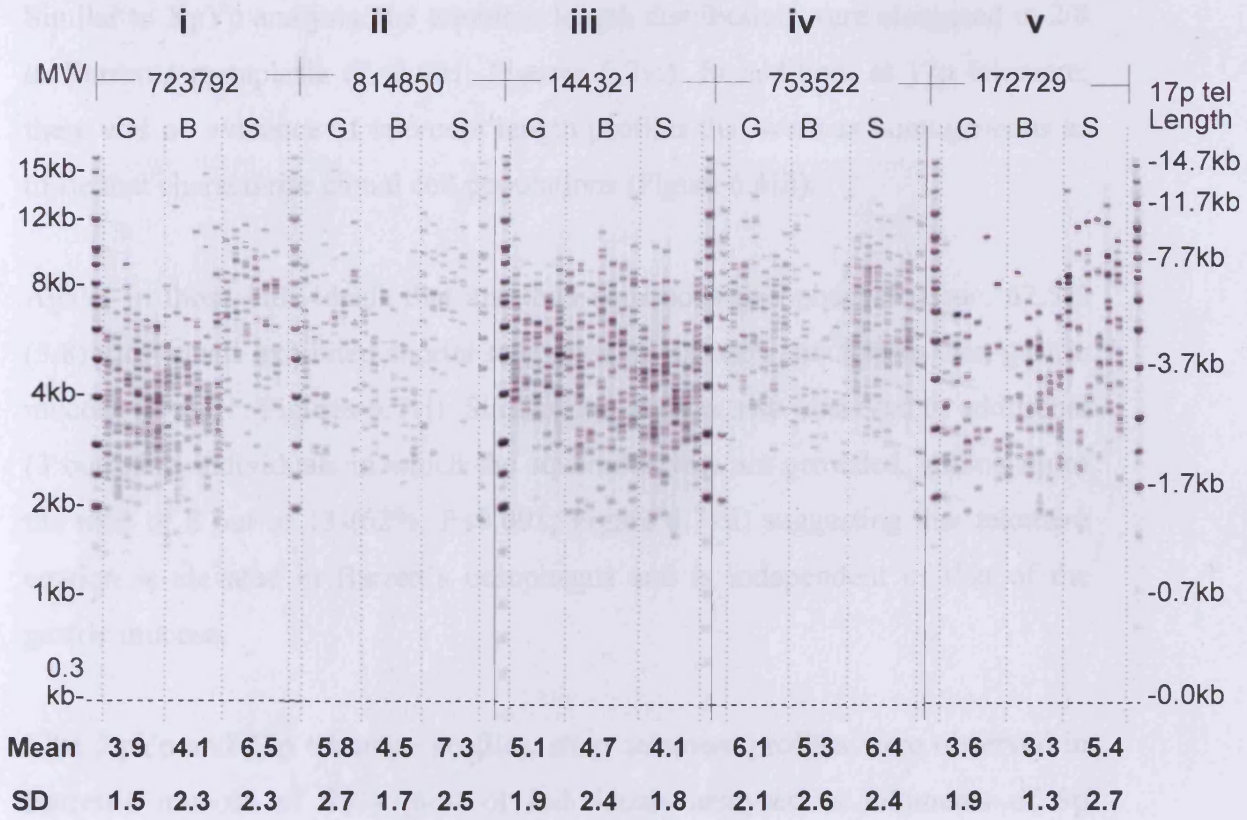
Telomere length in Barrett's mucosa was compared to that of gastric mucosa, with the assumption that if Barrett's mucosa are composed of same cell type and/ or are exposed to similar environment, they may exhibit similar telomere length instability. The mean telomere length between gastric mucosa and Barrett's metaplasia was not significantly different in 46% [6/13; P>0.05; Figure 6.5viii], suggesting that telomerase activity in Barrett's oesophagus may be insufficient to counteract telomere loss. The cells of the gastric mucosa and Barrett's oesophagus undergo rapid proliferation to replace cells lost due to DNA damage resulting from acid damage, thus telomeres are lost each time cells divide.

Although the telomeres were maintained at a specific length, the telomeres that were within the length range at which fusion can occur were detected. These events are consistent with sporadic telomeric deletion events (Figure 6.5, indicated with red marks).

6.5.2.2 17p, 9p and 11q telomeres

Similar to telomere length profiles at XpYp telomere, large variation in telomere length at telomeres of 17p, 9p and 11q were observed in all tissues [S; Figure 6.4ii-iv]. Again there was evidence of telomere erosion in Barrett's mucosa compared to native squamous at 17p [62.5% (5/8); P<0.001; Figure 6.7i; 6.7ii; 6.7iv; 6.7v and 6.7vi]. For example, in 172729: telomere length profiles at 17p in Barrett's metaplasia are shorter than those at squamous (P<0.001) and not different from the gastric tissue (P=0.64). In addition, there was clear presence of short telomeres; however, it was not possible to include the TVR region at these telomeres.

Figure 6.7. Telomere length analysis at 17p telomere in squamous (S, black bars), gastric (G, blue bars) and Barrett's mucosae (B, red bars). STELA products were detected by Southern hybridisation with TTAGGG-containing probe. i-ii, iv-v. Barrett's oesophagus telomere lengths are shorter than those of native squamous epithelium. iii. Telomeres are longer in Barrett's oesophagus than squamous. Mean telomere lengths and standard deviation (SD) are shown for each distribution vi. Summary of the mean telomere distributions in eight individuals. For $P<0.0001$[***] and $P<0.05$ [*] are indicated. vii. Summary of the mean distributions at XpYp between gastric (blue bars) and Barrett's mucosae (red bars). The individuals in which there was no significant difference between the mean telomere distributions are shown by #. Error bars (green) represent 95% confidence intervals.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MW
723792
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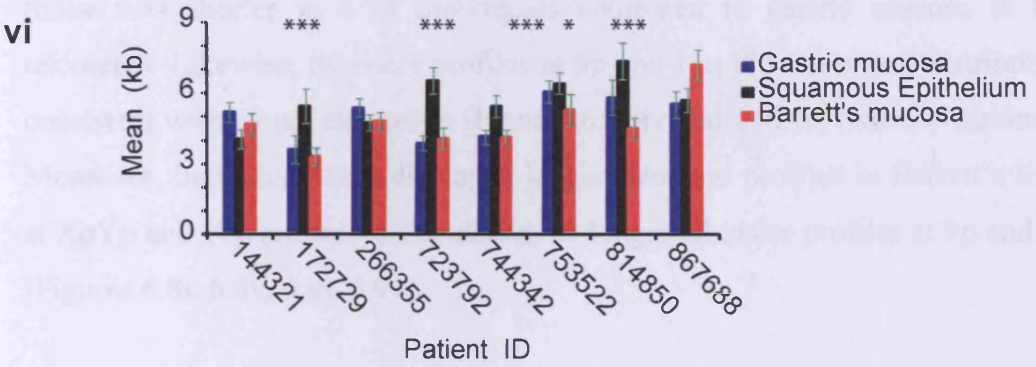
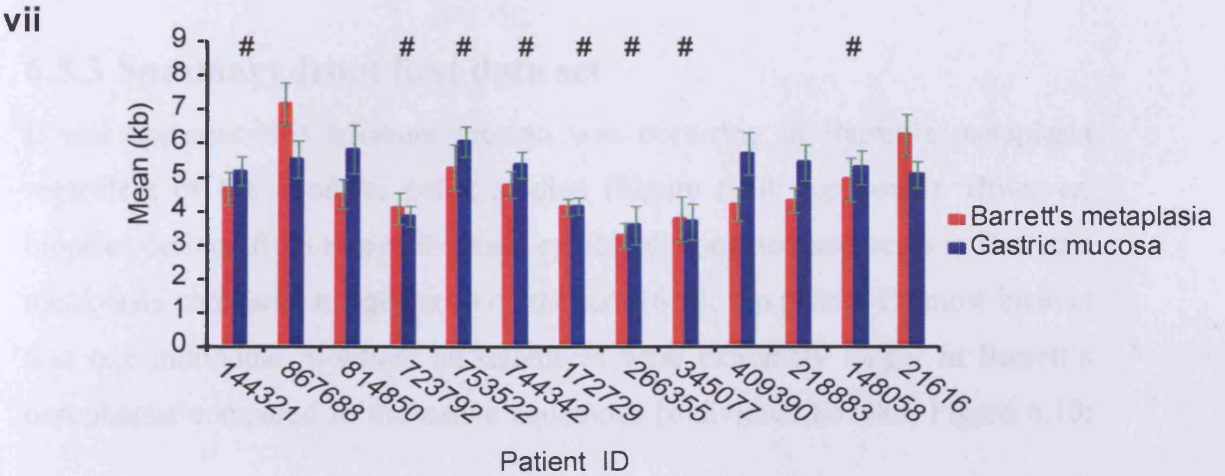
144321
753522
172729
17p tel Length
G B S G B S G B S G B S G B S
15kb-
12kb-
8kb-
4kb-
2kb-
1kb-
0.3 kb-
-14.7kb
-11.7kb
-7.7kb
-3.7kb
-1.7kb
-0.7kb
-0.0kb
Mean 3.9 4.1 6.5 5.8 4.5 7.3 5.1 4.7 4.1 6.1 5.3 6.4 3.6 3.3 5.4
SD 1.5 2.3 2.3 2.7 1.7 2.5 1.9 2.4 1.8 2.1 2.6 2.4 1.9 1.3 2.7


vi
Mean (kb)
Patient ID
Gastric mucosa
Squamous Epithelium
Barrett's mucosa
144321
172729
266355
723792
744342
753522
814850
867688


vii
Mean (kb)
Patient ID
Barrett's metaplasia
Gastric mucosa
144321
867688
814850
723792
753522
744342
172729
266355
345077
409390
218883
148058
21616

Similar to XpYp analysis, the telomere length distribution were elongated in 2/8 in Barrett's metaplasia (P<0.001; Figures 6.7vi). In addition, at 17p telomere, there was no evidence of telomere length profiles that were as homogeneous as those that characterise clonal cell populations (Figure 6.6iii).

Again, in those individuals that also had squamous oesophageal tissue, 62.5% (5/8) individuals exhibited shorter telomeres in Barrett's metaplasia than gastric mucosa (P<0.01; Figures 6.7vi). Similar pattern was also observed in additional (3 out of 5) individuals in which the squamous was not provided, adding up to the total of 8 out of 13 (62%; P<0.001; Figure 6.7vii) suggesting that telomere erosion is elevated in Barrett's oesophagus and is independent of that of the gastric mucosa.

Like XpYp and 17p telomere profiles, short telomere profiles were observed in Barrett's mucosa of 75% (6/8) of individuals analysed at telomeres of 9p [P<0.01; Figure 6.8i-v] and 11q (P<0.01; Figure 6.9i-v). Again the Barrett's tissue was shorter in 8/13 individuals compared to gastric mucosa at both telomeres. Likewise, telomere profiles at 9p and 11q did not reveal distributions consistent with clonal expansion [Figures 6.8i-iv and 6.9i-iv; (narrow variance)]. Moreover, individuals that displayed longer telomere profiles in Barrett's tissue at XpYp and 17p telomeres also displayed longer telomere profiles at 9p and 11q [Figures 6.8i, 6.8v, 6.9i, 6.9v].

6.5.3 Summary from first data set

It was apparent that telomere erosion was occurring in Barrett's metaplasia regardless of the telomere being studied (Figure 6.10; top panel). However, biopsies derived from two individuals exhibited elongated telomeres in Barrett's metaplasia compared to squamous epithelium (6.10; top panel). Of most interest was one individual in whom all telomeres were extremely longer in Barrett's oesophagus compared to the native squamous [individual 867688, Figure 6.10;

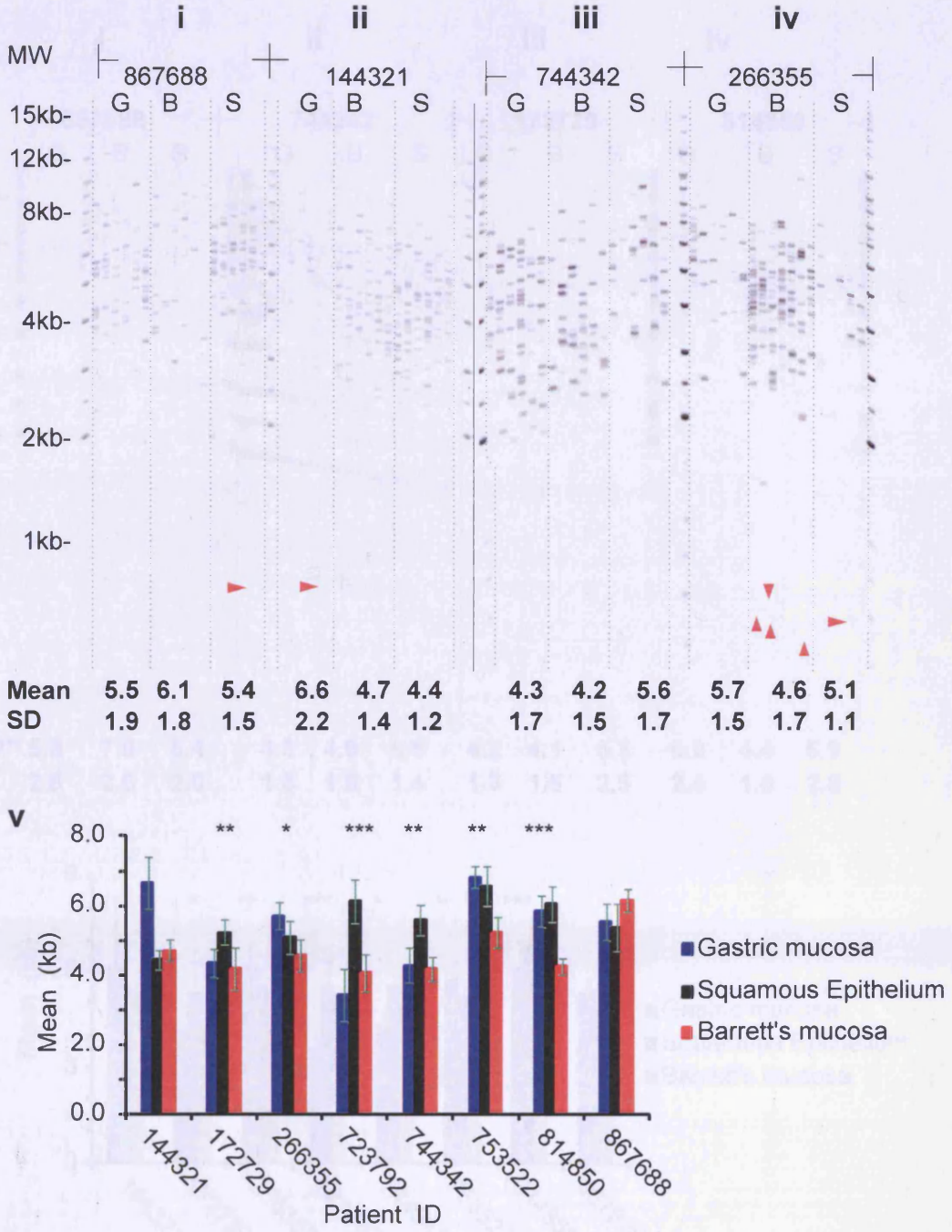


Figure 6.8. Telomere length analysis at 9p telomere in squamous (S, black bars), gastric (G, blue bars) and Barrett's mucosa (B, red bars). STELA products were detected by Southern hybridisation with TTAGGG-containing probe. i-ii Barrett's oesophagus length longer than in squamous epithelium. iii-vi. Barrett's oesophagus telomere lengths are shorter than those of native squamous epithelium. Mean telomere lengths and standard deviation (SD) are shown for each distribution v. Summary of the mean telomere distributions in eight individuals. For $P<0.0001$[***], $P<0.01$ [**] and $P<0.05$ [*] are indicated. Error bars (green) represent 95% confidence intervals.

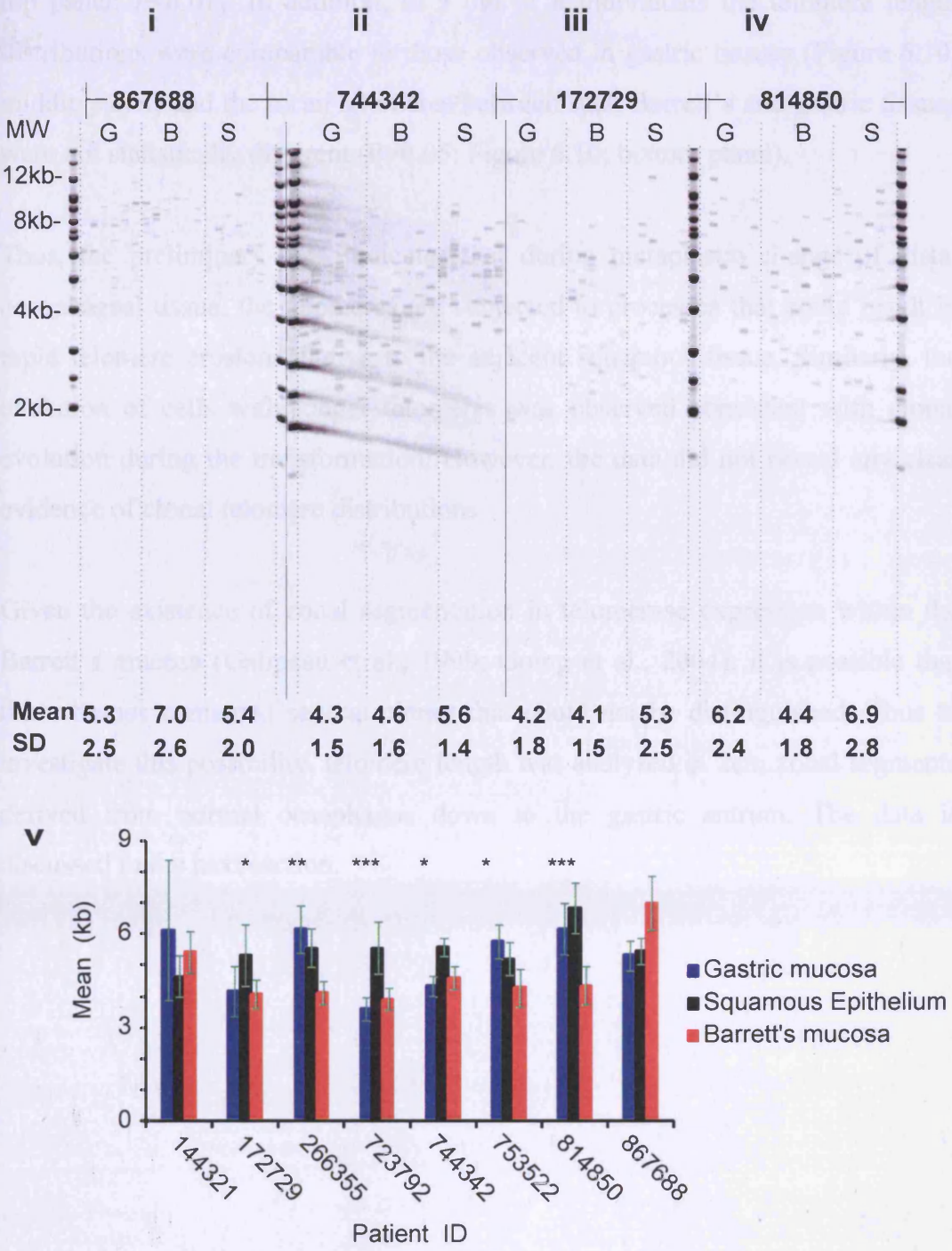


Figure 6.9. Telomere length analysis at 11q telomere in squamous (S, black bars), gastric (G, blue bars) and Barrett's mucosae (B, red bars). i. Barrett's oesophagus length longer than in squamous epithelium. ii-iv. Telomere length shorter in Barrett's oesophagus than in squamous. Mean telomere lengths and standard deviation (SD) are shown for each distribution v. Summary of the mean telomere distributions in eight individuals. For P<0.0001[***], P<0.01 [**] and P<0.05 [*] are indicated. Error bars (green) represent 95% confidence intervals

top panel; $P<0.01$]. In addition, in 3 out of 8 individuals the telomere length distributions were comparable to those observed in gastric tissues (Figure 6.10; middle panel) and the mean telomeres between such Barrett's and gastric tissues were not statistically different ($P>0.05$; Figure 6.10; bottom panel).

Thus, the preliminary data indicates that during metaplastic change of distal oesophageal tissue, the telomeres are subjected to processes that could result in rapid telomere erosion relative to the adjacent squamous tissue. Similarly, the evolution of cells with longer telomeres was observed consistent with clonal evolution during the transformation. However, the data did not reveal any clear evidence of clonal telomere distributions.

Given the existence of zonal segmentation in telomerase expression within the Barrett's mucosa (Galipeau et al., 1999; Going et al., 2004), it is possible that these tissues contained several clones that could not be distinguished. Thus to investigate this possibility, telomere length was analysed in 2cm zonal segments derived from normal oesophagus down to the gastric antrum. The data is discussed in the next section.

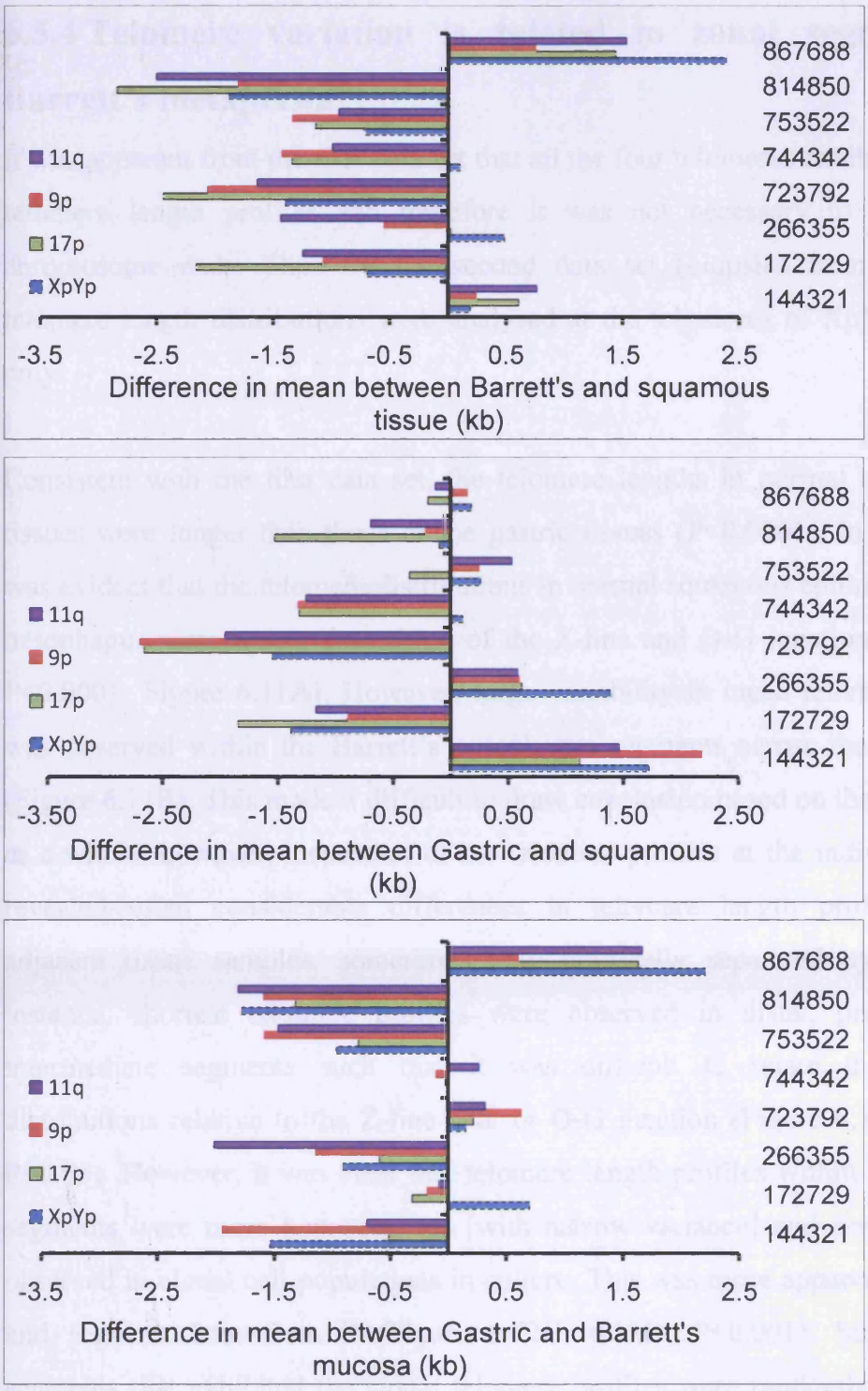


Figure 6.10. Illustrating the difference in mean telomere length at XpYp, 17p, 9p and 11q between: Barrett's and squamous oesophageal tissues (top panel), gastric and squamous mucosa (middle panel); gastric and Barrett's mucosa (bottom panel). The positive number shows an increase in telomere length while negative number shows decline in telomere length in the two tissues under comparison.

6.5.4 Telomere variation is related to zonal segments in Barrett's metaplasia

It was apparent from the first data set that all the four telomeres exhibited similar telomere length profiles and therefore it was not necessary to analyse all chromosome ends. Thus for the second data set (biopsies from Glasgow), telomere length distributions were analysed at the telomeres of XpYp and 17p only.

Consistent with the first data set, the telomere lengths in normal oesophageal tissues were longer than those of the gastric tissues ($P<0.0001$). In addition, it was evident that the telomere distributions in normal squamous epithelium of the oesophagus were longer than those of the Z-line and O-G junction [ANOVA; $P<0.0001$; Figure 6.11A]. However, large variability in mean telomere lengths was observed within the Barrett's oesophagus segments across the population (Figure 6.11B). This made it difficult to draw conclusion based on the population as a whole; however, inspection of the telomere profiles at the individual level revealed often considerable differences in telomere length profiles within adjacent tissue samples, sometimes only physically separated by 2cm. For instance, shortest telomere profiles were observed in distal, proximal and intermediate segments such that it was difficult to relate the telomere distributions relative to the Z-line and/ or O-G junction (Figure 6.12A, 6.12B; $P<0.01$). However, it was clear that telomere length profiles within some tissue segments were more homogeneous [with narrow variance] and similar to that observed in clonal cell populations in culture. This was more apparent in Z-line, and segment 2cm from it (Figure 6.12C, 6.12D; $P<0.001$). Similarly, the segments that exhibited the clonal telomere profiles were randomly distributed within the Barrett's oesophagus with no clear relation between distance from Z-line and/ or O-G junction and the telomere length distribution. These clonal distributions consisted of short telomere profiles some of which were within the range at which fusions can occur (Figure 6.13; $P<0.001$). These clonal distributions were observed at both XpYp and 17p, indicating that evolution of

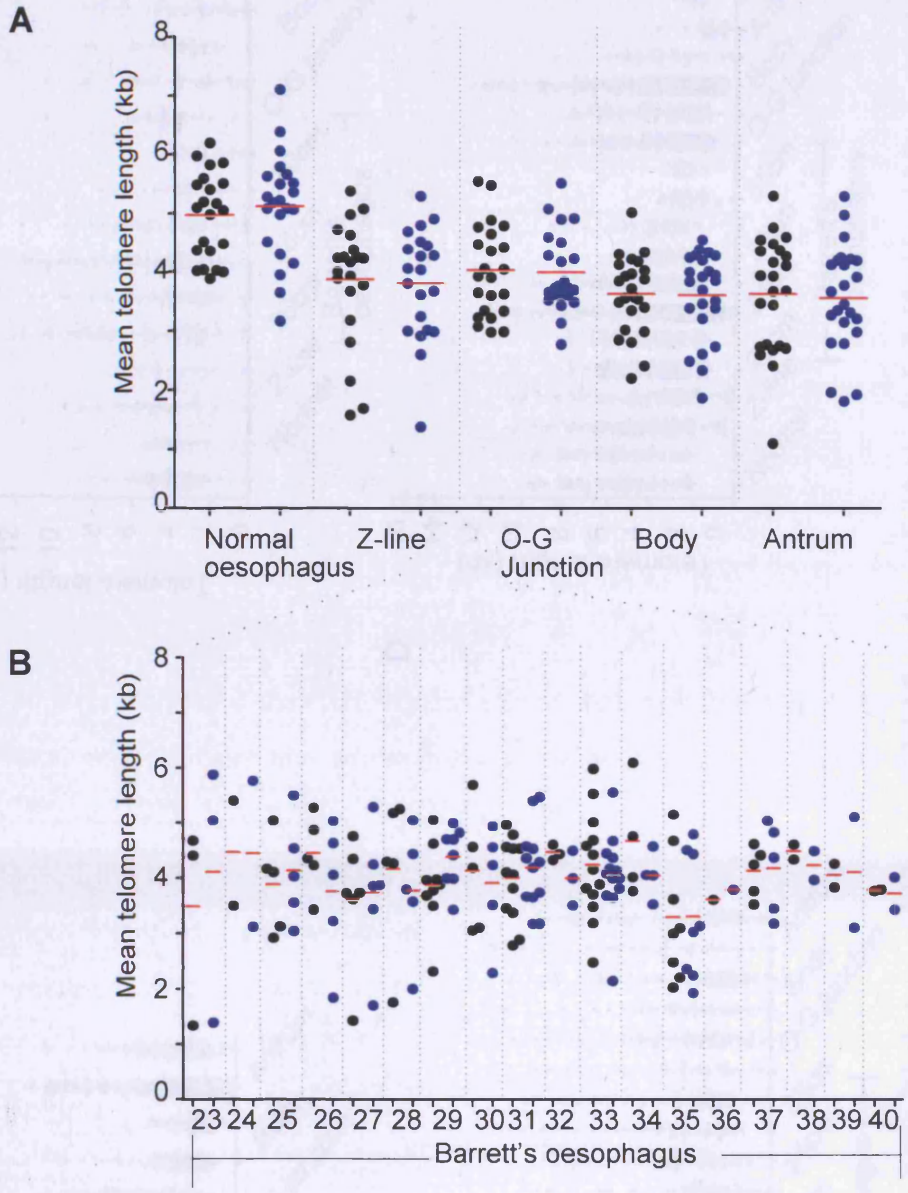


Figure 6.11. Summarising the combined data of from telomere distributions at XpYp (black dots) and 17p (blue dots). A. Mean telomere distributions in normal oesophagus, Z-line, oesophago-gastric junction (O-G), gastric body and antrum. B. Mean telomere distributions in different segments of Barrett's oesophagus cut every 2cm from 23cm down to the 40cm.

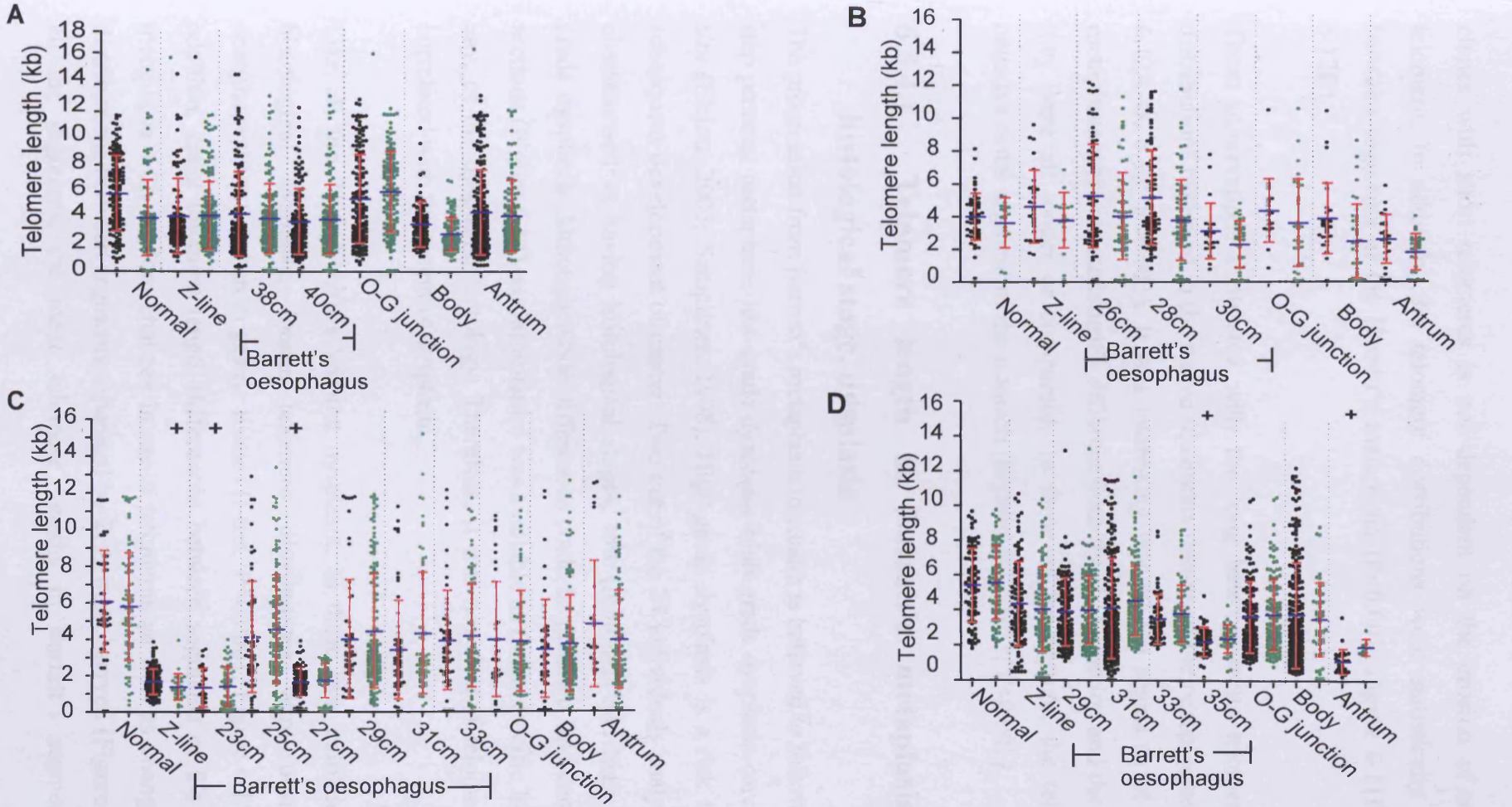


Figure 6.12. Telomere length distribution at XpYp (black dots) and 17p (green dots) from two individuals. A. Individual in which telomere length decreases from normal to Z-line and Barrett's oesophagus. B. Individual in which telomere length decreases from normal oesophagus (Normal) to Z-line and Barrett's oesophageal segments then decreasing in the distal most segment of Barrett's segment. C-D. The telomeres in different segments are similar to those observed in clonal cells in culture (indicated with a cross +). The distance of each segment of Barrett's from start of oesophagus are shown in centimetres (cm). Telomeres in gastric tissues, the body and antrum are shorter than normal oesophagus. C-O (O-G) junction represents oesophago-gastric or cardia-oesophageal junction.

clones with short telomeres is not dependent on the erosion of a specific telomere. In addition, the telomere distributions were statistically different between segments of the Barrett's metaplasia ($P<0.001$; Figure 6.11B, 6.12A, 6.12B).

These observations contrasted with the long heterogeneous telomere length distributions observed in the native squamous tissues of the oesophagus [Figures 6.12A-D, 6.13; $P<0.001$). It was interesting that all the segments of Barrett's metaplasia were not statistically different from the C-O junction and the body but they were all longer or comparable to those of the antrum, the telomerase-negative distal segment of the stomach [Figures 6.12, 6.13; $P<0.001$].

6.5.4.1 Telomere length in Barrett's metaplasia with histological stage, dysplasia

The progression from Barrett's metaplasia to cancer is believed to follow a multi-step process; metaplasia-low-grade dysplasia–high-grade dysplasia-carcinoma *in situ* (Flejou, 2005; Sampliner, 1998). High-grade dysplasia is a risk factor for subsequent development of cancer. Two out of the 24 individuals analysed were characterised as having histological stages, low (minimal) and high (severe)-grade dysplasia. Although subtle differences could be detected between adjacent sections (Figure 6.14), no information was available as to the specific location, if any, of the dysplastic histology. Therefore, it was not clear if telomere length correlated with the extent of dysplasia.

Like all the 24 individuals without dysplasia, in these two individuals with histological dysplasia, longer telomere distributions were observed in oesophageal tissues than in gastric tissues [t-test, $P<0.0001$; Figure 6.14A-B]. In addition, zonal telomere length differences between segments of the Barrett's metaplasia with random changes in mean telomeres as well as changes in the distributions between segments separated by 2cm was observed (Figure 6.14). In all the segments, the mean telomere lengths in Barrett's segments were

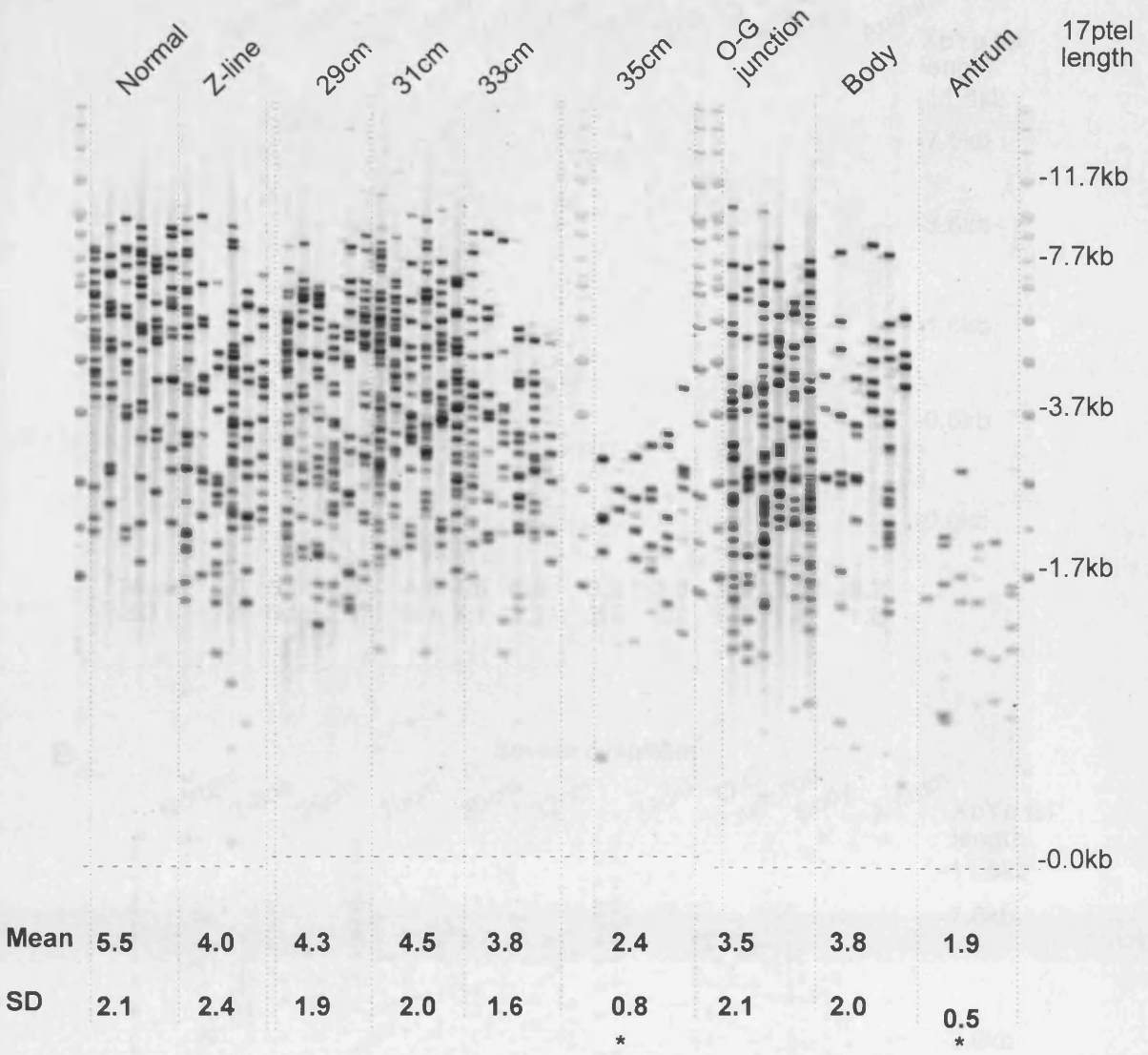


Figure 6.13. Telomere distribution at 17p telomere in individual (same as Figure 6.12D) in which there is evidence of clonal expansion in Barrett's oesophagus (35cm). STELA gels for all the tissues from normal oesophagus to stomach antrum. The mean telomere lengths and standard deviations are shown below each distribution. The distribution that similar to those seen in clonal cell cultures are indicated with the asterisk (*).

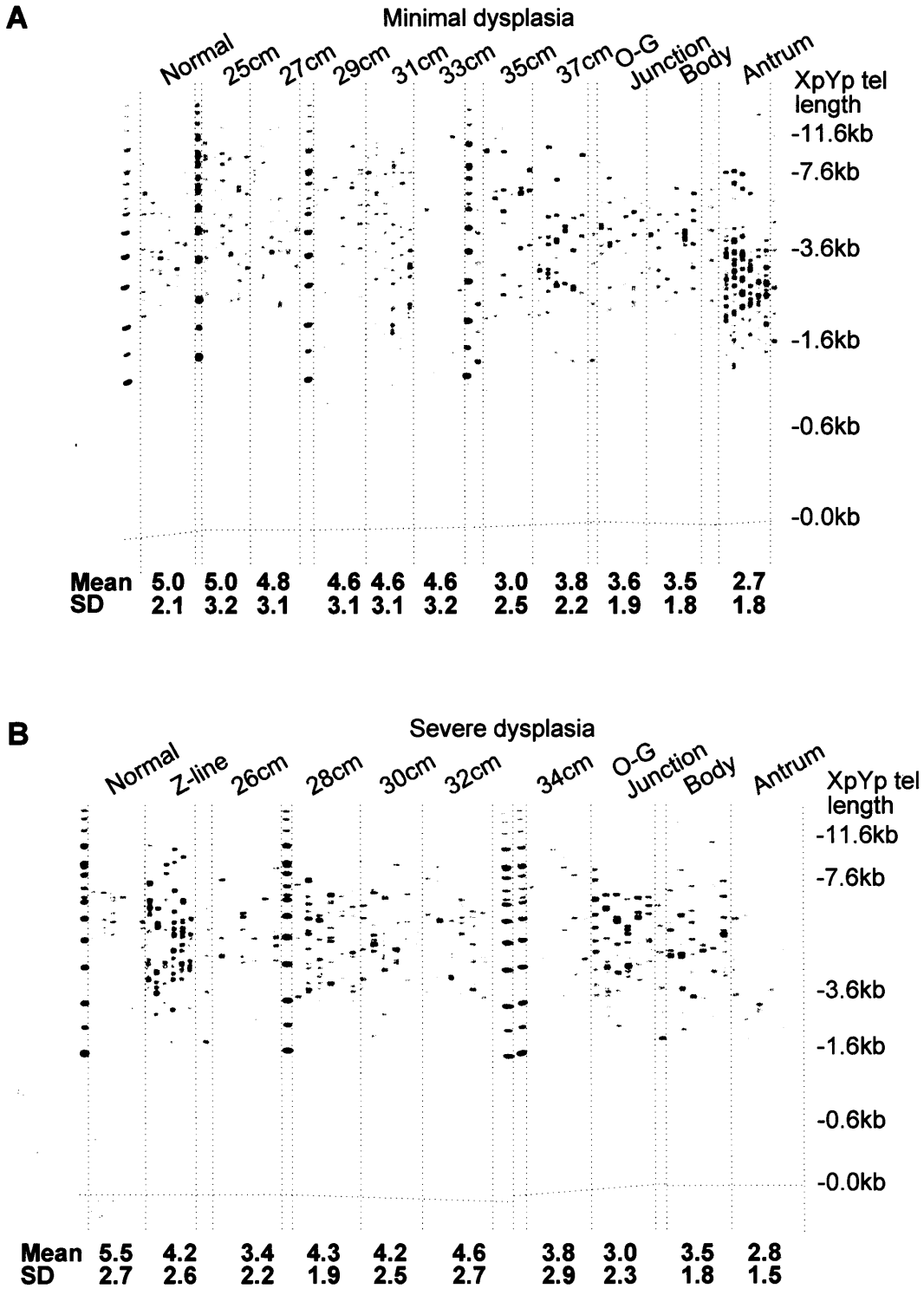


Figure 6.14. Telomere distribution at XpYp telomere in individual with minimal (A) and severe (B) dysplasia. STELA products were detected by Southern hybridisation with telomere probe. The mean telomere length and standard deviations (SD) are shown for each distribution.

significantly shorter than those of the normal oesophagus, an observation inconsistent with the report that telomerase over-expression in Barrett's oesophagus with dysplasia was accompanied by telomere elongation [(Finley et al., 2006); P<0.0001]. However, this data is uninformative as it is based on two individuals. It is therefore possible that analysis of several individuals with histological stage dysplasia could yield informative telomere length profiles.

6.5.5 Telomere distributions in Tumours

In a sample of three tumours comprising oesophageal and gastric from one individual and an unclassified tumour from another individual, there was no consistent pattern in telomere distributions between the tumour and adjacent tissue at both XpYp (P=0.51) and 17p (P=0.96; Figure 6.15). In an individual with two tumours, the telomere length in normal oesophagus was longer than that of the tumour at both XpYp and 17p (P<0.001; Figure 6.15). Whilst telomeres in the gastric tumour were longer than those of gastric body (t-test, P=0.006; F-test, P=0.002; Figure 6.15), the mean telomeres in the gastric body were also shorter than those of the antrum (t-test, P=0.001; F-test, P<0.0001; Figure 6.15).

6.5.6 Telomere fusions in premalignant Barrett's oesophagus

Telomere dysfunction can promote chromosome instability through anaphase bridge-breakage-fusion cycles in ulcerative colitis (O'Sullivan et al., 2002), and mouse models (Artandi et al., 2000). In addition, increased levels of aneuploidy have been reported in Barrett's oesophagus (Jenkins et al., 2002; Koppert et al., 2005). It was clear from the telomere length analysis, that short telomeres within the length range at which fusion can occur were readily detected in these samples. Thus, to examine if telomere dysfunction could contribute to chromosome instability observed in Barrett's oesophagus, the frequency of telomere fusion events was investigated in patient-matched oesophagus and gastric tissues derived from Barrett's oesophagus patients. This was facilitated by using the more sensitive telomere fusion assay between the telomeres of XpYp,

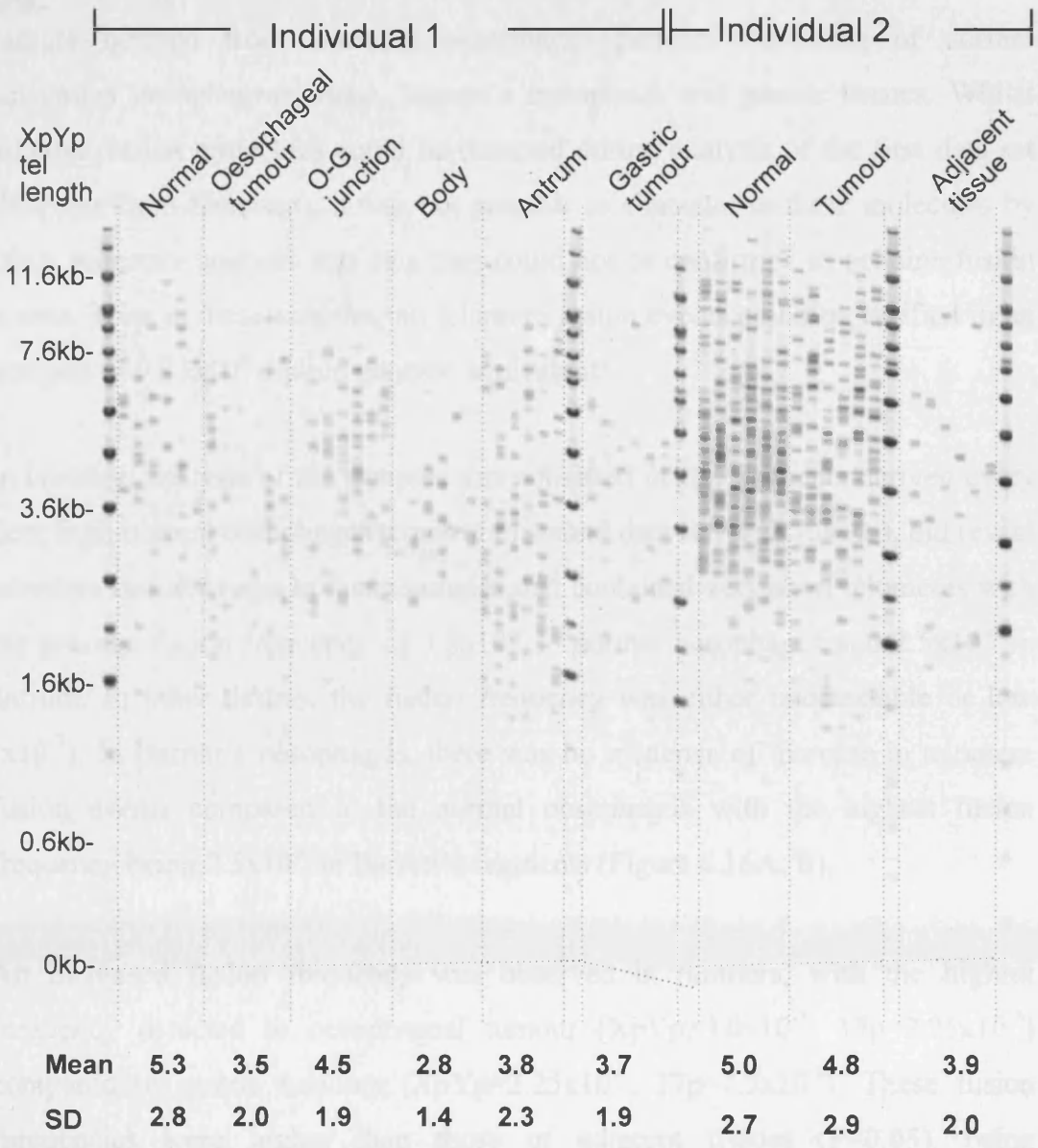


Figure 6.15. Telomere distribution at XpYp telomeres in three tumours and adjacent tissues derived from two individuals that had oesophageal and gastric tumours. STELA products were detected by Southern hybridisation with TTAGGG-containing probes. The mean telomere lengths and standard deviations (SD) are shown below each distribution.

17p, 21q family and/ or 16p family described in chapter 3. Multiple reactions (typically 7-8) containing 50-100ng per reaction were carried out with each DNA sample derived from Barrett's oesophagus patients consisting of normal squamous oesophageal tissue, Barrett's metaplasia and gastric tissues. Whilst putative fusion molecules could be detected during analysis of the first data set (biopsies from Swansea), it was not possible to characterise these molecules by DNA sequence analysis and thus they could not be confirmed as genuine fusion events. Thus in these samples, no telomere fusion events could be verified in an analysis of $9.33x10^6$ diploid genome equivalents.

In contrast, analysis of the samples that consisted of the segments derived every 2cm from normal oesophagus to antrum [second data set; $n=2.73x10^7$), did reveal telomere fusion events in those samples that contained very short telomeres with the average fusion frequency of $3.8x10^{-6}$ in normal oesophagus and $2.5x10^{-6}$ in antrum. In other tissues, the fusion frequency was either undetectable or low ($x10^{-7}$). In Barrett's oesophagus, there was no evidence of increase in telomere fusion events compared to the normal oesophagus with the highest fusion frequency being $7.5x10^{-6}$ in Barrett's segments (Figure 6.16A, B).

An increased fusion frequency was observed in tumours, with the highest frequency detected in oesophageal tumour ($XpYp=3.0x10^{-5}$; $17p=2.25x10^{-5}$) compared to gastric tumours ($XpYp=2.25x10^{-5}$; $17p=7.5x10^{-6}$). These fusion frequencies were higher than those of adjacent tissues ($P<0.05$), being undetectable in normal oesophagus and $7.5x10^{-6}$ in normal gastric tissues (data not shown).

As explained in chapter 3 and 4, putative fusion molecules were isolated using nested PCR primers and molecular structure confirmed by direct sequence analysis. Whilst 98 putative telomere fusion events could be readily re-amplified prior to sequencing, only six of these could be fully characterised. The remaining 92 could not be fully characterised because they were either too large (23) and it

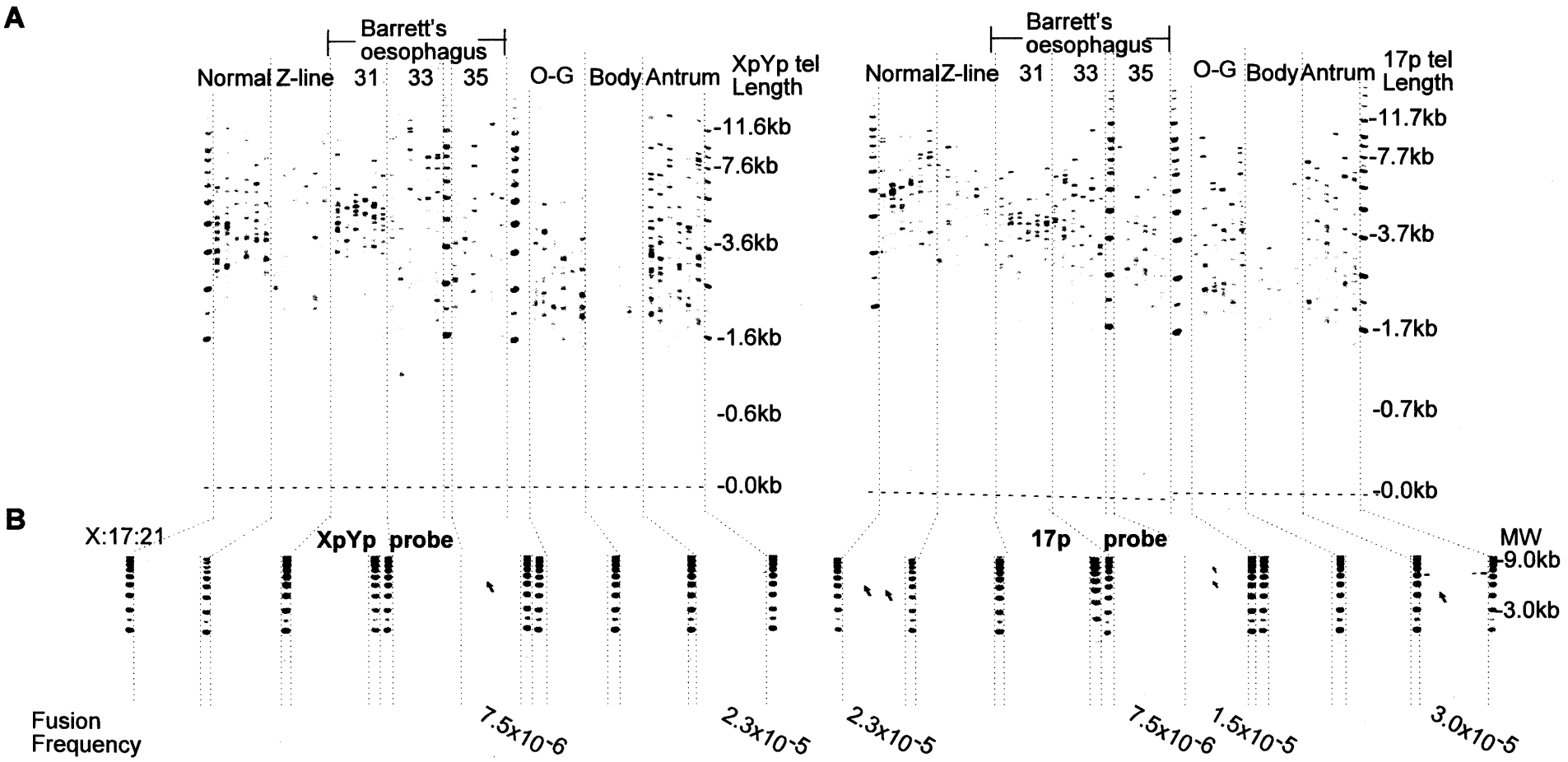


Figure 6.16. Example of telomere length distribution and telomere fusion assay in Barrett's oesophagus patient. A. XpYp (left) and 17p STELA gels. B. Telomere fusion assay between telomeres of XpYp, 17p and 21q family (X:17:21). The fusion products were detected by XpYp (left) and 17p probes. The fusion frequency for those tissues that was detectable is shown below gels. The molecules that may be difficult to see with this resolution are indicated by arrows. The molecular weight (MW) marker is shown on the right.

was not possible to identify the fusion point or in others, the involvement of related telomeres created inverted repeats that resulted in uninterpretable sequence information (69).

The preliminary data indicated that the fusion events were characterised by large sub-telomeric deletions, a lack of TTAGGG repeats at the fusion point as well as short patches of microhomology, similar to those described in cells *in vitro* and human tissues *in vivo* (Chapter 4-5; Figure 6.17 A-D).

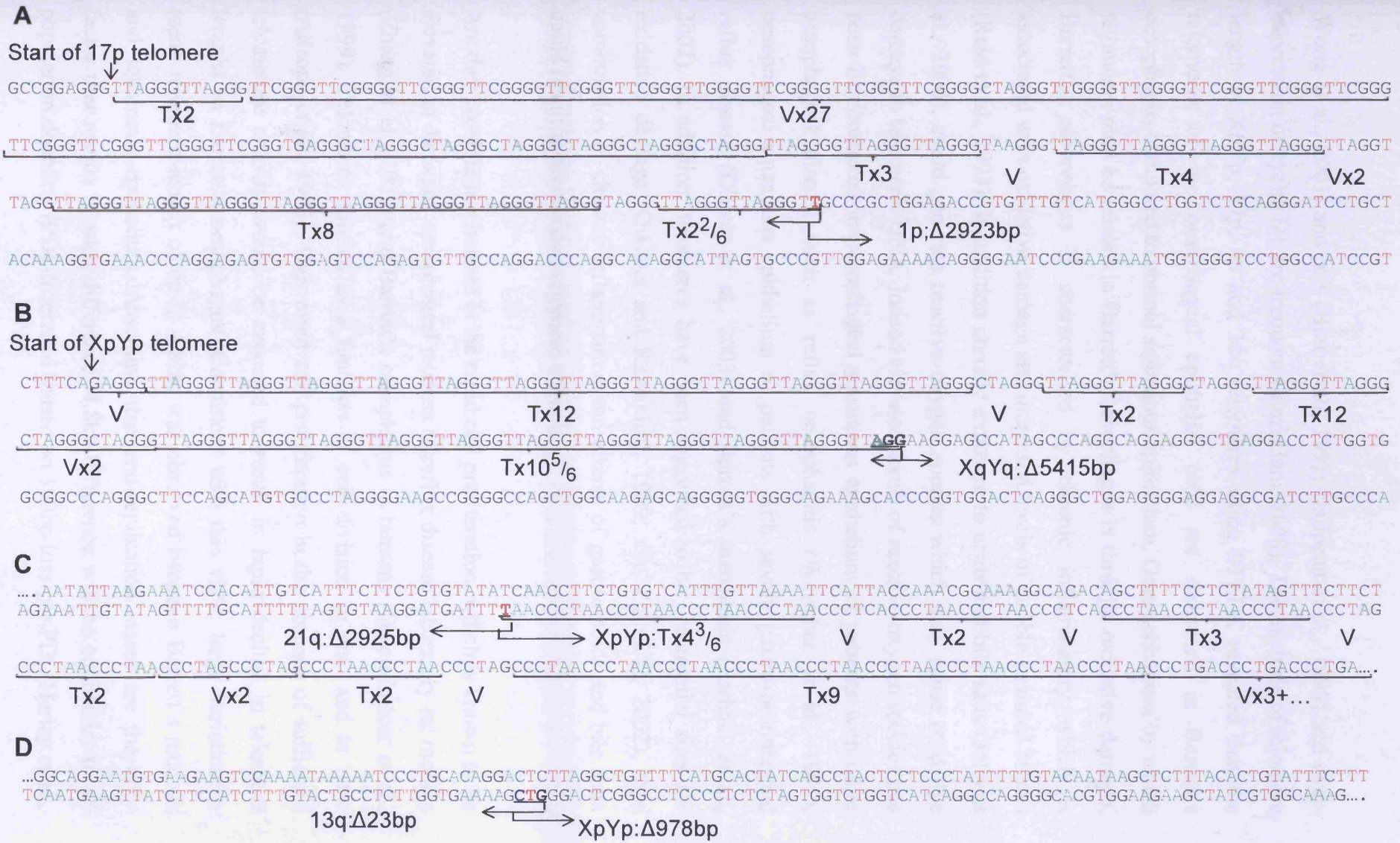
6.6 Discussion

6.6.1 Telomere length distribution

Barrett's oesophagus is a complex premalignant condition in which the cells of the distal oesophagus are subjected to physical, thermal and chemical trauma, which could result in rapid cell proliferation and accumulation of genetic changes such as inactivation of tumour suppressor genes in particular p16, p53 and over-expression of cyclin D1 (Arber et al., 1996; Bani-Hani, 2000; Galipeau et al., 1996; Hardie et al., 2005; Jenkins et al., 2002). These events could allow cells with damaged DNA to progress through the cell cycle and facilitate progression to cancer. Loss of p16 function is associated with late G_1 cell cycle arrest and is one of the early events during Barrett's oesophagus progression thus could confer selective advantage (Barrett et al., 1996a; Galipeau et al., 1999; Wong et al., 2001). Additional loss of p53, the multifunctional protein that mediates cell cycle arrest or apoptosis in response to several cellular and environmental stresses could facilitate the bypassing of checkpoints such as senescence and apoptosis that may be induced by telomere attrition (Galipeau et al., 1996; Galipeau et al., 1999).

In this study, telomere length analysis at telomeres of XpYp, 9p, 11q and 17p has been described. Three telomeres of 11q, 9p and 17p were selected for analysis because these chromosome arms are known to be involved in the early stages of Barrett's oesophagus. i.e. losses of p16 (Barrett et al., 1996a; Maley et al., 2004b;

Figure 6.17. Examples of the sequences of the fusion events detected in tissues derived from Barrett's oesophagus patients. A. 17p:1p fusion event illustrating large deletion size B. XpYp:XqYq fusion events, has potential to form ring chromosomes. C. 21q:XpYp fusion event. D. 13q:XpYp fusion showing deletion on both sides of the fusion point. The short patches of micro-homology nucleotides are boldened and underlined while deletion (Δ) from start of telomere tracts are shown in base pairs (bp). V represents telomere variant repeats while T represents TTAGGG repeats.


A
Start of 17p telomere
Tx2
Vx27
Tx3
V
Tx4
Vx2
Tx8
Tx2 2/6
1p;Δ2923bp
B
Start of XpYp telomere
V
Tx12
V
Tx2
Tx12
Vx2
Tx10 5/6
XqYq:Δ5415bp
C
21q:Δ2925bp
XpYp:Tx4 3/6
V
Tx2
V
Tx3
V
Tx2
Vx2
Tx2
V
Tx9
Vx3+…
D
13q:Δ23bp
XpYp:Δ978bp

Wong et al., 2001) and p53 (Blot et al., 1991; Galipeau et al., 1996) and over-expression of cyclin D1 are frequent (Bani-Hani, 2000). The analysis of telomere length at XpYp, 17p, 9p and 11q telomeres using STELA revealed that the telomeres of the oesophageal epithelial cells are shortened in Barrett's oesophagus compared to normal squamous epithelium. One mechanism by which telomeres may be eroded in Barrett's oesophagus is through oxidative damage. Barrett's oesophagus is characterised by chronic inflammatory which is associated with oxidative damage and increased levels of double stranded breaks (Reid et al., 1993). In addition chronic exposure to acids and bile salts (Jolly et al., 2004), could generate reactive oxygen species which may cause oxidative damage to telomeric DNA. Indeed elevated levels of reactive oxygen species has been demonstrated in oesophageal squamous epithelium of patients with other oesophageal disease such as reflux oesophagitis (Wetscher et al., 1995); oesophageal squamous epithelium of patients with severe gastro-oesophageal reflux disease (Olliver et al., 2003) and Barrett's metaplasia (Farhadi et al., 2002). In addition, telomeres have been suggested to be preferential sites for oxidative damage (Oikawa and Kawanishi, 1999; von Zglinicki, 2002), thus combination of chronic inflammation and effects of gastric acids and bile salts could result in accelerated telomere erosion.

Another possible mechanism is the rapid cell proliferation, which is known to be elevated in damaged oesophageal mucosa of reflux disease laboratory rat models (Zhang et al., 2001) and Barrett's oesophagus in humans (Ouatu-Lascar et al., 1999). Telomeres shorten as a function of cell division *in vivo* and *in vitro* (Allsopp et al., 1995). Thus rapid cell proliferation in the absence of sufficient telomerase activity would be expected to result in rapid decline in telomere lengths in Barrett's oesophagus. Consistent with this view, large variation in mean telomere length of up to 4.8kb, was observed between Barrett's mucosa and squamous epithelium. Assuming that end-replication losses are the only factor that results in such difference, 4.8kb difference will be equivalent to 48 population doubling (PD) difference [based on 100bp loss per PD; (Harley et al.,

1990)] in the absence of telomerase activity. Since both tissues express telomerase activity, such differences in mean telomere lengths could represent many more PDs.

Consistent with other studies, telomeres in Barrett's oesophagus are eroded (Finley et al., 2006; Meeker et al., 2004; Souza et al., 2007) despite expression of telomerase (Going et al., 2004; Lord et al., 2000; Morales et al., 1998; Souza et al., 2007). In addition, it has been reported by others that telomerase expression increases with increasing histological grade during oesophageal carcinogenesis resulting in increased telomere length within the high-grade dysplasia (Finley et al., 2006; Lord et al., 2000). Telomerase expression compensates for telomere loss that could activate DNA damage checkpoints (Blackburn, 1990). However, it appears that the telomerase activity in Barrett's metaplasia is insufficient to counteract telomere erosion. In support of this, it has been shown that telomerase extends the lifespan of human cells without net telomere lengthening (Zhu et al., 1999) and inhibition of telomerase in Barrett's cancer cells *in vitro* leads to senescence and apoptosis (Shammas et al., 2005; Shammas et al., 2004).

In a subset of individuals, the telomere lengths in Barrett's oesophagus were similar to those in gastric mucosa, suggesting that the damage to telomeres by gastric contents result in cells of the oesophagus mimicking those of the gastric mucosa. In addition, shorter telomere length profiles than those of the gastric tissues were observed. These observations are of interest because oesophageal tissues are telomerase-positive but gastric tissues are not, thus it would be expected that despite increased proliferation, the telomere erosion could be counteracted by telomerase. However, given the rapid turnover in both gastric and Barrett's metaplasia, it is possible that telomerase expression in Barrett's oesophagus allowed the extended proliferation and thus further erosion. An alternative explanation could be the differences in apoptotic ability of the cells in these tissues. i.e. apoptosis is less frequent in Barrett's metaplasia compared to gastric tissues (Chen et al., 2002), thus, it is possible that whilst cells with short

telomeres exit cell cycle through apoptosis to maintain tissue homeostasis, high proliferation in Barrett's metaplasia is not counteracted by cell death thus resulting in further telomere erosion, and possibly clonal growth.

There was evidence of clonal telomere length profiles in some segments of Barrett's oesophagus at all telomeres analysed. It has been suggested that clonal expansion of cells that have lost p53 and p16 could subvert cell cycle checkpoints, thus conferring selective advantage for these clones to divide uncontrollably (Barrett et al., 1999; Galipeau et al., 1999; Maley et al., 2006). Loss of p16 function could confer selective advantage to cells during neoplastic progression while loss of p53 gives cells the proliferative advantage by bypassing cell cycle checkpoints such as senescence and apoptosis that can be activated by short telomeres (Jenkins et al., 2002). Thus it is possible that the clonal proliferation could lead to telomere attrition in Barrett's metaplasia, consistent with this and other studies (Finley et al., 2006), which could in turn initiate chromosome instability once telomeres have eroded to critical lengths. Once telomeres have eroded to within length ranges at which telomere fusion can occur, they may initiate large-scale chromosome instability, a hallmark of human cancers. However, substantial variation among individuals in telomere lengths coupled with the small samples made it difficult to conclude whether telomere biology can be used to predict those individuals that will progress. It will be interesting to observe how these telomere length distributions correlate with genomic complexity.

As described in Chapter 5, there was a subset of telomeres that were several orders of magnitude shorter than the bulk of the distribution. These telomere profiles are consistent with stochastic telomere deletions that generate truncated telomeres, which are capable of fusion. These events could drive background chromosome instability, which would activate onco-proteins or inactivate tumour suppressor proteins (Callen et al., 2002; Capper et al., 2007; Murnane et al., 1994).

Evidence of telomere elongation in cells of Barrett's oesophagus were also observed in a subset of biopsies analysed implicating that in these individuals other factors that select for cells with longer telomeres could play a role in Barrett's oesophagus. This may be a consequence of the over-expression of telomerase resulting in telomeric elongation. Indeed, elevated telomerase expression during oesophageal carcinogenesis as early as in Barrett's metaplasia being highest in dysplasia and adenocarcinoma has been documented (Lord et al., 2000). Although these individuals were not characterised as having histological stage, dysplasia, it is possible that dysplasia was present at the distal oesophagus. In support of this, several studies has demonstrated that increase in telomerase expression in dysplastic biopsy of Barrett's oesophagus is associated with telomere elongation (Finley et al., 2006; Lord et al., 2000; Morales et al., 1998).

6.6.2 Telomere fusion events

It was clear from both data sets that, the telomeres can erode to within the length range at which fusion was detected. Fusions could readily be detected in all the samples analysed in the zonal samples [second dataset]. However, there was no apparent increase in telomere fusion frequencies in Barrett's oesophagus. It is not known whether the absence of fusions is a reflection of a low-level of telomere-driven chromosome instability or whether the chromosomes being targeted with our assays were not subjected to fusion in Barrett's oesophagus. In their study, Finley and colleagues reported increased levels of chromosome fusion in high-grade dysplasia, the histological stage that is believed to have elevated risk of progressing to cancer (Finley et al., 2006), as well as in other hyper-proliferative conditions such as ulcerative colitis (O'Sullivan et al., 2002). Thus the preliminary results described in this thesis are in contrast to that study in which they observed an increased fusion frequency at 17p, 11q and 9p using FISH (Finley et al., 2006). In this study, the telomere fusion frequency in Barrett's mucosa of the two individuals that were diagnosed as having dysplasia, there was no significant difference in telomere fusion frequency. Considering the fact that only 10% of Barrett's oesophagus progress to cancer (Koppert et al., 2005), the

data reported here may not provide sufficient representation of those individuals that will progress. In addition, the fusion frequencies in different tissues may be too low to make a meaningful comparison and thus these data are uninformative for understanding disease progression in Barrett's oesophagus.

Preliminary fusion data has revealed that the molecular structure of the fusion events consists of large deletions extending into the sub-telomeric DNA, short patches of micro-homology nucleotides and lack of TTAGGG repeats at fusion junction. This profile is similar to that we have reported in cells in culture [chapter 4; (Capper et al., 2007)], as well as in normal cells (chapter 5). The occurrence of such fusion events even though at low frequency could initiate chromosome instability during successive cell division.

6.6.3 Telomere instability and fusion in tumours.

Telomere shortening has been reported to be one of the earliest and most prevalent alterations in epithelial carcinogenesis (Meeker et al., 2004) contributing to the acquisition of chromosomal instability (Finley et al., 2006; O'Sullivan et al., 2002), which in turn may promote tumour evolution (Feldser et al., 2003; Gisselsson et al., 2001). In addition, during oesophageal carcinogenesis, telomerase activity is elevated in dysplasia and adenocarcinomas (Lord et al., 2000; Morales et al., 1998). Consistent with this, increased telomere lengths and chromosome instability in the form of telomere fusion events were observed in the oesophageal tumours analysed here. It has been shown that for cancer cells to continue proliferating they require the telomere maintenance mechanism such as telomerase, which is expressed in 85-90% of human epithelial cancers (Kim et al., 1994). However, telomerase expression in tumours is known to be insufficient to completely stabilise the telomeres and prevent chromosome instability (Gisselsson et al., 2001). This is evident from the three tumours analysed here, where telomere lengths were longer in tumours than adjacent normal tissues.

6.7 Conclusion

In this study, telomere erosion, instability and fusion have been described. It is clear that telomere erosion occurs very early in premalignant condition, Barrett's oesophagus. In addition, like other biomarkers such as LOH at p53 and p16, evolution of clones with short telomeres that are capable of fusion was evident. It is clear that Barrett's oesophagus is a very complex condition consisting of multiple clones some of which carry the features that may drive neoplastic progression. However, it remains to be determined if telomere erosion and telomere-driven chromosome instability can predict individuals at risk of progression.

6.8 Key findings:

- Telomere attrition is apparent in 75% of Barrett's oesophagus individuals, regardless of the telomere under-investigation. However, telomeres were longer in Barrett's oesophagus in two individuals [first data set].
- Although it was apparent that telomeres in Barrett's tissues were shorter than those of the normal squamous epithelium, large variation in telomere distributions was observed within segments of Barrett's oesophagus within each individual as well as across the whole sample collection [second data set].
- The large telomere variation in segments separated by 2cm was evident with evidence of clonal expansion in some segments, as described by homogeneous telomere distributions similar to those described in analysis of clonal cell populations in culture.
- Preliminary data has revealed that the molecular structure of the telomere fusion events observed in these tissue samples is similar to those described in cells in culture (chapter 3 and 4) and other normal human cells (Chapter 5).
- In tumours, telomere elongation was accompanied by increase in telomere fusion frequency.

7 Chapter 7: Final Discussion and Future Work

7.1 Summary of key results

Much of the work described in this thesis has been discussed in the relevant chapters. Therefore, in this chapter, a general overview of the results, possible limitations and future directions will be considered. First, the key findings of the study are outlined:

- The telomere fusion assays have been expanded to cover at least 43% of the human genome. This will allow the understanding of the mechanistic basis of telomere fusion and how this may impact upon genomic instability.

- Using the extended and improved assays, telomere fusion events have been isolated and characterised in cells in culture, as well as in human tissue biopsies, derived from normal and diseased tissues including normal dermis, melanocytic naevi, Barrett's metaplasia, normal oesophageal and gastric tissues.

- The analysis of the molecular structure of fusion events has revealed that fusion of short dysfunctional telomeres is accompanied by large deletions extending into the sub-telomeric DNA up to 5.6kb (close to the limit of the assay at 6kb). The fusion events were also characterised by short patches of homology nucleotides with a GC bias at the fusion junction. In addition, complex fusion events involving insertion of DNA sequences near loci that have been implicated in human cancers were detected.

- Clonal telomere length distributions and fusion events were observed *in vitro* and *in vivo*. These events may suggest that genomic rearrangements arising as a consequence of telomere erosion, instability and fusion could

confer selective advantage for other abnormalities that may drive neoplastic progression.

- The telomere length profiles were shorter in melanocytic naevi compared to the adjacent dermis; however there was no difference in the frequency of fusion events using our assays that detect fusion events between telomeres of XpYp, 17p and 21q family.

- For those tissue samples in which 17p telomere length distributions were longer than those observed at XpYp telomere, the fusion events involving the two telomeres contained a deletion from XpYp and long arrays of telomere repeats from 17p.

- The fusion frequencies in both melanocytic naevi and adjacent tissue were comparable to those of normal cells *in vitro*. However, the frequency was lower than estimated from cells undergoing crisis in culture.

- Telomere attrition was apparent at all telomeres analysed in Barrett's mucosa. Telomere erosion was also related to zonal segments of Barrett's oesophagus such that large variation in telomere length profiles was evident in segments that are physically separated by only 2cm.

- In tumours, telomere elongation was accompanied by increase in telomere fusion frequency.

7.2 Technological development of STELA and Fusion assay

The data described in this thesis examines telomere erosion, instability and fusion in human cells and tissues using single-molecule approaches, STELA (Baird et al., 2003) and telomere fusion assays (Capper et al., 2007).

The sequence composition of the sub-telomeric DNA immediately adjacent to the telomeres is highly variable consisting of duplications, low-copy number repeats as well as multiple repeat families (Brown et al., 1990; Mefford and Trask, 2002; Riethman et al., 2005). The repetitive nature of the sub-telomeric DNA has allowed the development of the telomere fusion assays that detect fusion events between multiple chromosomes simultaneously. The telomere fusion assay has been extended to include additional telomeres comprising at least 43% of the human telomeres (Letsolo et al., 2009). The improved assays have enabled the detection and isolation of fusion events in human cells *in vitro* and tissues *in vivo*. It has also been employed by colleagues to study telomere instability and fusion in solid and haematological tumours as well as in experimental systems testing the mechanistic basis of fusion of short dysfunctional telomeres [DMB personal comm.]. The development of this assay has revealed that telomere instability and fusion that we previously described between XpYp and 17p (Capper et al., 2007) was not unique to these ends but a feature of critically eroded, uncapped telomeres.

Development of STELA assays at additional telomeres proved to be technically challenging due to high sequence homology between the sub-telomeric DNA of multiple chromosomes (Brown et al., 1990). Nevertheless, STELA could still be developed at additional telomeres using the single nucleotide differences. These STELA assays revealed that properties of XpYp and other four autosomal telomeres analysed previously were not unique (Baird et al., 2003; Britt-Compton et al., 2006). Thus taking advantage of the single nucleotide sequence differences, STELA can potentially be extended to other chromosome ends.

7.2.1 Possible limitations

Current fusion assays are restricted to detection of fusion events between heterologous chromosomes as well as sister-chromatid type fusion events that involved the deletion of one of the participating telomeres. However, sister-

chromatid fusions that include long arrays of telomere repeats on both sides of the fusion junction cannot be detected as these have potential to form palindromic structures that may be refractory to PCR (Capper et al., 2007; Letsolo et al., 2009). If these types of events occur they would not be represented in these telomere fusion assays. However, fusion analysis following the knockdown of TRF2 revealed telomere fusion events between heterologous chromosomes that contained long arrays of TTAGGG on either side of the fusion junction (Capper et al., 2007), indicating that these types of events could be detected with our fusion assays. However, as it was apparent from the data presented in this thesis, the telomere fusion containing TTAGGG repeats on either side of the fusion point is rare. It is possible that this also applies to sister chromatid-type fusion events. It would be informative to develop the fusion assay to allow the detection of sister chromatid fusion events that contain TTAGGG repeats on either side of the fusion point. This would allow an assessment to be made about contribution that these events make to telomere mutation.

The presence of repetitive sequences such as satellites immediately adjacent to the telomere (Blouin et al., 1995; Brown et al., 1990; de Lange et al., 1990), also limits the detection of fusions within those regions, as repetitive DNA sequences may be refractory to PCR.

Despite the mentioned limitations, the current telomere fusion assay appeared to be a robust methodology. Unlike other technologies that can detect large deletions and complex chromosomal fusions (Gisselsson et al., 2000; Takubo et al., 2010), the telomere fusion assay cannot detect fusion events comprising larger deletions than those detected with our assays (maximum deletion of 6kb). However, the assay is robust in giving the actual molecular structure of the fusion events. The study of the molecular structure of fusion events will give inside into the mechanistic basis of telomere fusion of critical dysfunctional telomeres. Again, unlike other methods that could not be utilised for analysis of

tissue biopsies as the method requires proliferating cells for metaphase spreads (McNeil and Ried, 2000), the telomere fusion assay has been used in this study and by colleagues to study telomere-related chromosome instability in human tissues. Furthermore, unlike other methods that study frequency of anaphase bridges which may not be a consequence of telomere dysfunction (Gisselsson et al., 2000), the telomere fusion assay detects fusion events between the dysfunctional telomeres (Capper et al., 2007; Letsolo et al., 2009).

The sequence homology within sub-telomeric DNA poses a challenge for development of STELA at specific telomeres. This makes it difficult to identify sufficient sequence difference to differentiate one telomere within the family from its close relatives. Indeed it has been reported that some chromosome ends such as 6p contain telomere adjacent repeat sequences that hybridise to more than one repeat family, telbam 11 and telbam 3.4 (Brown et al., 1990). However, it has become increasingly apparent from the data presented in this thesis, as well as that from this laboratory, that telomere length dynamics appeared to be conserved within the cell strain analysed (Baird et al., 2003; Britt-Compton et al., 2006). Thus it may not be necessary to develop STELA at additional chromosome ends. To compensate for above limitations, the STELA technique has been improved by others in a method called "universal STELA." The "universal STELA" estimates telomere length from almost all telomeres simultaneously. Although robust for detecting very short telomeres, "universal STELA" may not be widely applicable as it suffers the amplification efficiency for detection of molecules greater than 7kb (Bendix et al., 2010). In contrast chromosome-specific STELA can detect the full spectrum of human telomeres [0-25kb; (Baird et al., 2006)]. Again like TRF analysis, telomere length estimated by "universal STELA" contains unknown and varying length of subtelomeric DNA. Furthermore, Unlike chromosome-specific STELA which displays linear correlation [r=0.91; (Baird et al., 2003)] with mean telomere lengths estimated by TRF analysis, "universal STELA" shows non-linear correlation with TRFs (Bendix et al., 2010). Thus chromosome-specific STELA is the ideal method for

detecting telomere length at specific telomeres as it does not suffer hybridisation threshold like TRF analysis and also does not give the mean telomere length or ratio like other methods described in chapter 1 of this thesis (Baird, 2005; Saldanha et al., 2003).

7.3 Telomere dynamics *in vitro* is a reflective of that *in vivo*.

Human malignancies are characterised by complex cytogenetic profiles typically highlighted by presence of complex rearrangements such as non-reciprocal translocations that occur early in tumour development (Hanahan and Weinberg, 2000; Maser and DePinho, 2002). One of the mechanisms that may initiate genomic instability is the state of the telomere, which if "uncapped", could initiate genomic instability during early stages of carcinogenesis (Artandi et al., 2000; Rudolph et al., 2001). Whilst in advanced stages the genome is partially stabilised by reactivation of telomere maintenance mechanism such as telomerase (Kim et al., 1994; Meyerson et al., 1997). Telomere function depends on the telomere length and intact telomere binding proteins which forms a scaffold that protect the chromosome ends (de Lange, 2005). However, dysfunctional telomeres are detected as double stranded breaks and activate DNA damage response checkpoints including ATM, CHK2 which induce p53-dependent cell cycle exit through cascade of events (d'Adda di Fagagna et al., 2003; Karlseder et al., 1999). If DNA checkpoints are defective as in $terc^{-/-}$ $p53^{-/-}$ mice, progressive telomere erosion leads to end-to-end fusions, which during subsequent cell division could fuel large-scale genomic instability through cycles of breakage-fusion-bridging and result in rearrangements such as non-reciprocal translocations, that typify epithelial cancers (Artandi et al., 2000; Murnane and Sabatier, 2004). The role of telomere instability in generating genomic instability and carcinogenesis is further supported by studies with late generation telomere knockout mice ($terc^{-/-}$) which show that mice displaying telomere loss and genomic instability also display an increased rate of tumour formation very early in life (O'Hagan et al., 2002; Rudolph et al., 1999).

The work described in this thesis has addressed the role of telomere erosion, instability and fusion *in vitro* and *in vivo* in human cells and tissues, and their possible role in driving malignant progression. Previous studies with mouse models have shown that fusion occur between chromosome arms bearing shortest telomeres (Hemann et al., 2001). Similarly in this study and other recent studies we have demonstrated that in human cells fusion occur between critically short telomeres *in vitro* (Capper et al., 2007; Letsolo et al., 2009). Likewise recent studies using other methods of analysis have revealed that short telomeres accumulate in senescent cultures (Bendix et al., 2010) and chromosome instability is increased in cells displaying short telomeres but not in young proliferating cells (Takubo et al., 2010; Zou et al., 2009). We have also demonstrated that telomere dysfunction is physiologically relevant as telomere instability and fusion could be detected in normal and diseased human tissues *in vivo* (chapters 5 and 6 of this thesis) and was correlated with disease progression in chronic lymphocytic leukaemia (Lin et al., 2010). Whilst, telomere erosion, instability and fusion have been described extensively in humans, our studies provide some of the first insights into the nature (DNA sequence arrangement) of the telomere-related chromosomal fusions. It would be interesting to investigate how our dataset correlates with genome-wide chromosome instability. The data will also provide a reference for experimental settings investigating the mechanistic basis of fusion of critically short dysfunctional telomeres.

Telomere fusion between sister chromatids can create inverted repeat structures which may in turn create fragile sites which can break at adjacent loci to original fusion (Lo et al., 2002) resulting in broken chromosomes and amplified segments; thus possibly leading to localised deletions and amplifications (Murnane, 2006). Indeed this study and our recent publications have described fusion events that are consistent with sister chromatid-type fusion in which one telomere is fused to telomere-adjacent DNA of same telomeric allele in the reverse orientation (Capper et al., 2007; Letsolo et al., 2009).

We have also described fusion events that involve interstitial loci near sites implicated in cancer, some of which are located near documented fragile sites. Fragile sites are gaps or breaks in chromosomes that may be induced by environmental mutagens (Sutherland et al., 1998). They are classified according to their chemistry of induction and their frequency in the population. The rare fragile sites are found in 5% of the population and are induced by folic acid-deficiency or bromodeoxyuridine (BrdU) or distamycin A (Schwartz et al., 2006). Whereas common fragile sites are found in all individuals and are induced by aphidicolin or 5-azacytidine (Schwartz et al., 2006). The interstitial loci detected in fusion during this study, were near documented common fragile sites, 8q24.3 (FRA8D), 7p22 (FRA7B), 9q22.1 (FRA9D) and Xp22.31 [FRAXB; (Debacker and Kooy, 2007)]. Common fragile sites are involved in sister chromatid exchange, deletions and translocations in human malignancies (Debacker and Kooy, 2007; Glover, 2006). If such events occur near growth control genes, they may result in rearrangements and amplification which could result in tumour suppressor gene inactivation or oncogene activation (Maser and DePinho, 2002). Indeed, common fragile site, FRA3B spans over putative tumour suppressor genes (FHIT) and is implicated in human diseases, for example, FHIT gene is often deleted in premalignant conditions such as Barrett's oesophagus [81-86%] and oesophageal adenocarcinoma (Lai et al., 2010; Michael et al., 1997).

The data presented here is consistent with the view that telomere dysfunction leads to the different types of large-scale genomic mutation (Murnane, 2006). These types of mutations have the potential to drive disease progression in early stages of cancer. This view is consistent with many studies in the field implicating telomere dysfunction in tumour progression. These include early studies showing telomere erosion in human tumour tissues compared to adjacent normal tissue (Hastie et al., 1990; Nakamura et al., 2000). In addition the adenoma-carcinoma transition is characterised by increased frequency of anaphase-bridges, surrogate marker of telomere fusion in colorectal carcinoma,

breast and oral squamous cell carcinomas (Chin et al., 2004; Gordon et al., 2003; Rudolph et al., 2001), further implicating that telomere dysfunction may drive genomic instability during progression in human cancers.

The end-to-end fusions can be induced by loss of telomere protection due to loss of TTAGGG repeats or titration of telomere binding proteins, especially TRF2 from telomeres (van Steensel et al., 1998). Several experiments have reported that telomere dysfunction resulting from loss of telomere capping activates DNA repair mechanisms that are employed for repair of double stranded breaks, non-homologous end-joining [NHEJ; (Karran, 2000)], and homologous recombination [HR; (Denchi and de Lange, 2007; Wu et al., 2006)]. NHEJ is employed at any stage of the cell cycle to repair uncapped telomeres that contain long tracts of telomere repeats (Yang et al., 2005) whilst HR is the main method for repair of sister chromatid telomeres (Rudd et al., 2007).

However, the fusion events characterised by large sub-telomeric deletions (up to 5.6kb) and short patches of homology DNA sequences at the fusion junction described in the thesis, as well as other data from the laboratory (Capper et al., 2007) implicate additional end-joining mechanisms in facilitating fusion of dysfunctional telomeres. Similarly others have shown that the fusion of dysfunctional telomeres in human fibroblasts is independent of the activity of Ligase IV, a protein involved in non-homologous recombination (Zou et al., 2009). This may include Ku-independent error-prone end-joining mechanisms such as micro-homology mediated end-joining or alternative NHEJ. MMEJ is distinguished from the other Ku/DNA-PK independent error-prone mechanisms by its utilisation of 5-25 base pair micro-homology DNA sequences to align and join the broken strands (Bentley et al., 2004; McVey and Lee, 2008). However, our dataset comprising fusions containing less that 5bp DNA sequence homology are inconsistent with micro-homology mediated end-joining described in yeast (Ma et al., 2003), plants (Heacock et al., 2007) and some human cancers (Bentley et al., 2004).

The alternative NHEJ method of repair employs shorter homology sequences. An example of the use of this mechanism is in mammalian cells during class switching recombination in the absence of NHEJ. Class switch recombination (CSR) is a biological mechanism that changes a B cell's production of antibody from one class to another. During this process, double-stranded breaks are generated in DNA at two switch (S) regions, which are upstream from gene segments that encode for the constant regions of antibody heavy chains (Stavnezer, 1996). After the removal of the intervening DNA between the S-regions, the free ends of the DNA are rejoined by NHEJ (Lieber et al., 2006; Ma et al., 2002). In the absence of non-homologous end joining components, free ends of DNA may be rejoined by an alternative pathway which is biased toward micro-homology and is independent of NHEJ-factors such as ku and ligase iv (Boboila et al., 2010; Yan et al., 2007).

Clonal expansion has been implicated as one of the early lesions that may predict progression to malignancy (Maley et al., 2006). During this study, clonal fusion events and telomere length profiles that were consistent with clonal growth, were described both *in vitro* and *in vivo*. Clonal events have potential to dominate the cell population and provide selective advantage for accumulation of other abnormalities that could drive neoplastic progression. Indeed clones that have lost p16 and/or p53 function through mutations or LOH, pose an increased risk of progression to malignancy from premalignant conditions such as Barrett's oesophagus (Galipeau et al., 1999; Maley et al., 2006; Maley et al., 2004a; Maley et al., 2004b). In addition, consistent with the data described in this thesis (clonal telomere lengths), clones expanding in multiple 2-cm intervals in the Barrett's segment were detected with 9p LOH only, or 17p LOH only or both 9p and 17p LOH (Galipeau et al., 1999). Similarly, frequent clonal expansion of p53 mutant cells occurs in the epidermis adjacent to basal cell carcinomas (BCCs) during skin carcinogenesis (Urano et al., 1995). This data is consistent with the observation that non-reciprocal translocations in carcinomas and early stage

neoplasia are clonal (Artandi et al., 2000) and could be initiated by telomere dysfunction.

The inspection of telomere length in human tissues comprising patient-matched melanocytic naevi and Barrett's metaplasia revealed extensive telomere erosion in these tissues compared to adjacent normal tissues. Loss of p16 function is very common in melanocytic naevi (Bennett, 2003; Michaloglou et al., 2005) and Barrett's metaplasia (Galipeau et al., 1999; Jenkins et al., 2002). Thus it is possible that the loss of p16 confers an advantage for accumulation of more abnormalities such as the loss of p53 function, extensive telomere erosion and chromosome instability. Indeed telomere-driven genomic instability is associated with transition from benign lesions to malignancy (Chin et al., 2004; Meeker et al., 2004). In addition, p16INK4a has been reported to contribute to the p53-independent response to telomere damage (Jacobs and de Lange, 2004); thus loss of p16 function can allow cells with damaged telomeres to bypass senescence and allow further telomere attrition and instability.

The telomere erosion observed in the skin and Barrett's metaplasia samples could arise from oxidative stress which in skin may be due to chronic UV exposure while in Barrett's metaplasia it may be due to gastro-duodenal acids and salts. Telomeres are prone to UV damage which generates UVB-induced pyrimidine especially at dithymidine sites (Jin and Ikushima, 2004). In addition, oxidative damage due to either metabolism or UV is predominant at guanine sites (Kawanishi and Oikawa, 2004; Oikawa and Kawanishi, 1999). Since the repair of these lesions is less effective at telomeres, their existence at telomeres may result in substantial loss of telomere repeats and thus telomere erosion. Indeed, oxidative stress and/or UVA exposure has been reported to compromise the telomere integrity in cultured fibroblasts (Houben et al., 2008; Kawanishi and Oikawa, 2004; Oikawa et al., 2001; Oikawa and Kawanishi, 1999; von Zglinicki, 2002) and endothelial cells (Kurz et al., 2004). Thus oxidative stress (see section on oxidative damage in Chapter 1 of this thesis) could result in telomere

breakages generating short uncapped telomeres which have potential to fuse and leads to genomic instability through anaphase-bridging-breakage-cycles (Maser and DePinho, 2002). Consistent with telomere breakage rather than telomere erosion, telomere fusion events that contain long arrays of telomere repeats on one side were detected in normal skin samples (chapter 5 of the thesis).

The difference in telomere length between metaplasia, naevi and adjacent tissues could also have been due to replicative history of the cells (Martin-Ruiz et al., 2004). Indeed, it has been demonstrated that the replicative life span of human primary cells in culture is highly heterogeneous such that even within the same clone different cells display different telomere length profiles and each dividing cell population contain a fraction of senescing cells [possibly telomere-induced senescence; (Kill et al., 1994)]. In addition, the rate of telomere erosion varies greatly in cultured fibroblasts and generates large heterogeneity in telomere length (Martin-Ruiz et al., 2004). Moreover, telomere erosion varies in different fibroblasts depending on age of donor, such that reduced telomere loss is reported in foreskin fibroblasts from young donors compared to fibroblasts from older donors (Figueroa et al., 2000; Takubo et al., 2010). Thus it is possible that different cell types within these tissues had different rates of telomere loss. The replicative history can also account for the large heterogeneity in telomere distributions of these tissues. It is also possible that different cell populations could be subjected to end-replication losses as a consequence of cell proliferation, whilst in others shortening at an increased rate is due to other factors such as oxidative stress from the microenvironment as well as stochastic deletion/breakage events within the telomeres. Indeed work in our laboratory (Baird et al., 2006) and others laboratories (Callen et al., 2002; Murnane et al., 1994) have demonstrated that superimposed on end-replication losses, telomeres are subjected to events that generate short telomeres.

7.4 The mutational changes within telomere variant repeat arrays

Telomeric DNA sequences share some properties with simple tandem repeat (STRs) sequences such as length variation between unrelated individuals (Dib et al., 1996; Kong et al., 2002). In humans, the proximal part of the telomere is marked by interspersion of the canonical TTAGGG repeats with variant repeats such as TCAGGG, TGAGGG, TTGGGG etc (Allshire et al., 1989; Baird et al., 1995; Coleman et al., 1999; Letsolo et al., 2009). The role of telomere variant repeats in telomere function is not known, however it is assumed that due to large variability as well as lack of binding to telomere proteins, they do not play an essential role, if any, in telomere function. However, recently it has been demonstrated that some of the variant repeats, notably CTAGGG arrays are bound weakly by TRF1 and 2, and strongly by POT1 (Mendez-Bermudez et al., 2009). In addition, like $(TTAGGG)_2$ (Tsolou et al., 2008), telomere variants, $(TCAGGG)_2$ and $(CTAGGG)_2$ induced DNA damage reponse in fibroblasts (Mendez-Bermudez et al., 2009). Considering these recent findings, it is possible that TVR may play some role in telomere function which is yet to be determined.

The pattern of interspersion of telomere variant repeats has been examined by Telomere Variant Repeat mapping by PCR, TVR-PCR (Baird et al., 1995). The analysis of the repeats in human populations revealed that these repeats are extremely variable and display 98% heterozygosities (Baird et al., 2000; Baird et al., 1995; Coleman et al., 1999). In addition, inspection of different telomeric alleles using the specific primers in the telomere-adjacent DNA revealed that the mutational mechanisms underlying the variability in interspersion of the variant repeats was predominately intra-allelic such as replication slippage, and unequal sister chromatid recombination resulting in expansions or contractions of the repeat blocks (Baird et al., 2000; Baird et al., 1995). Replication Slippage is a mutational process in which repetitive sequences expand or contract by addition or subtraction of the simple tandem repeat unit (Viguera et al., 2001). The data described in this thesis was collected by direct sequence analysis of TVR region

from single fusion molecules. This revealed similar mutation pattern to that described for human population studies consisting of contractions and expansions of repeat blocks as well as contractions and expansions of TTAGGG repeat unit [e.g. TTTTAGGG; TTAGGGG; TTA-GG, TT-GGG, T-AGGG, etc] coupled with transversions or transitions changing TTAGGG to another type. E.g. TTAGGG to TTCGGG or TTGGGG or CTAGGG or TCAGGG or TGAGGG etc. Superimposed on simple events, a fraction of complex events that involved insertion of block of repeats with no obvious origin were also detected. It is not known whether these infrequent events arise through inter-allelic recombination or through gene conversion-like processes similar to those known to generate diversity at minisatellites in germ-line (Jeffreys et al., 1994; Monckton et al., 1993). However, unlike minisatellites, there are no known recombination hotspots at telomeres. It is clear from this data, that telomere repeat arrays are unstable structures in somatic cells, if this instability is reiterated in the rest of the telomere repeat array that is composed of homogenous TTAGGG repeats, could lead to loss of telomere function. This mechanism has the potential to create telomere length instability, which has the potential to lead to telomere dysfunction and fusion.

7.5 Conclusion

In conclusion, this thesis has assessed different aspects of telomere dysfunction in human cells and tissues. The knowledge of telomere dynamics *in vitro* has been translated to human tissues *in vivo*. This study has demonstrated that telomere erosion, instability and fusion previously studied in human tissues *in vitro* are physiologically relevant as these events were detected in human tissues. The telomere erosion, instability and fusion were apparent in melanocytic naevi and Barrett's oesophagus. However, from the data described in this thesis it was difficult to determine if telomere dynamics play any role in melanocytic senescence *in vitro* or if telomere dynamics could be used as a prognostic biomarker that will predict those individuals that will progress to melanoma or oesophageal adenocarcinoma. However, our data has provided direct (molecular structure) evidence of telomere-induced chromosome fusions.

7.6 Future work

This study has revealed very interesting findings some of which may be pursued further. For instance, involvement of interstitial loci near sites implicated in malignancies could suggest that telomere instability may be more extensive. To explore this possibility, development of STELA at those ends that we have detected fusion events involving their interstitial loci especially those implicated in certain cancer could indicate whether these telomeres behave differently from other telomeres studied so far. If these telomeres are more unstable their involvement in telomere fusion events could eventually result in terminal deletion creating double stranded breaks that could fuse with dysfunctional telomeres. Alternatively, the analysis would indicate whether the involvement of such interstitial loci is a result of telomere dysfunction at associated telomeres initiating chromosome instability. Whatever, the mechanism that result in these events, it is clear that telomere instability and fusion can be catastrophic. In chapter 4 of this thesis, I have hypothesised that telomere instability and fusion may induce activity of telomerase, if fusion occurs adjacent to genes involved in regulation of telomerase. For example fusion events involving the c-myc locus at 8q24.3 could activate this oncoprotein, which in turn can activate transcription of the hTERT gene (Wang et al., 1998; Wu et al., 1999) on chromosome 5p. Although this locus (8q24.3) is often deleted in human cancers, the increase in copy number of 8q arm has been reported in tumours [reviewed in (Cao et al., 2008)]. Similarly, telomere fusions involving 5p may result in activation of hTERT, probably through localised amplification of the terminally located hTERT locus [5p15.33]. In support of this view, an increase in copy number of the hTERT locus has been reported in human tumours [13%; reviewed in (Cao et al., 2008). I therefore hypothesise that telomere fusion-breakage-bridging cycles involving 5p telomere could result in increased copy number at the hTERT locus and thus increased telomerase activity. Indeed the increase in copy number of the hTERT locus has been correlated with telomerase expression and activity in

neuroblastoma (Zhang et al., 2000) and lung carcinoma cell lines (Saretzki et al., 2002).

Evidence of telomere dysfunction resulting from hTERT deletions comes from the infant conditions called Cri du chat syndrome in which terminal deletion of 5p encompassing hTERT locus is responsible for the characteristic cat-like cry in this condition (Laczmanska et al., 2006). Here, I hypothesise that loss of telomere function may lead to misregulation of telomerase activity and extended proliferation for cells with eroded telomeres and hence accumulation of more abnormalities that may drive neoplastic progression. Thus assays that target these loci may give insights into the mechanistic activation of telomerase, in cells that have bypassed senescence.

In addition, the improved telomere fusion assay appears to be a robust analysis of telomere-related chromosome instability at multiple chromosomes. Thus, the assay could further be extended to detect the fusion events involving short dysfunctional telomeres with fragile sites and/or other repetitive unstable loci within the human genome. In addition, the assay could be expanded to include other ends that are not detected with the current assays.

Analysis of melanocytic naevi has indicated that short telomeres are subjected to fusion. Thus for future investigations, the telomere length and fusion could be investigated in dysplastic naevi and melanoma. This would indicate the role of telomere biology in melanoma development. The role of telomere biology in Barrett's oesophagus progression could be extended by studying telomere dynamics in dysplastic and carcinoma *in situ* tissue samples. In addition, the telomere length dynamics and fusion can be correlated with telomerase expression in different tissue segments derived from Barrett's oesophagus patients. Furthermore, telomere dynamics can also be correlated with genome-wide chromosome instability using comparative genomic hybridisation (CGH) and other biomarkers such as loss of p53, p16, FHIT deletions etc. It would be

interesting to investigate whether the clones that display short telomeres and increased chromosome instability also display loss of p16 and or p53 function. Thus LOH and methylation patterns can be investigated in these genes.

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Appendix

Box 2.1

The frequency of telomere fusions for each experiment was calculated as follows: Number of fragments on the gel detected by either the XpYp or 17p probes, divided by the number of input diploid genome equivalents.

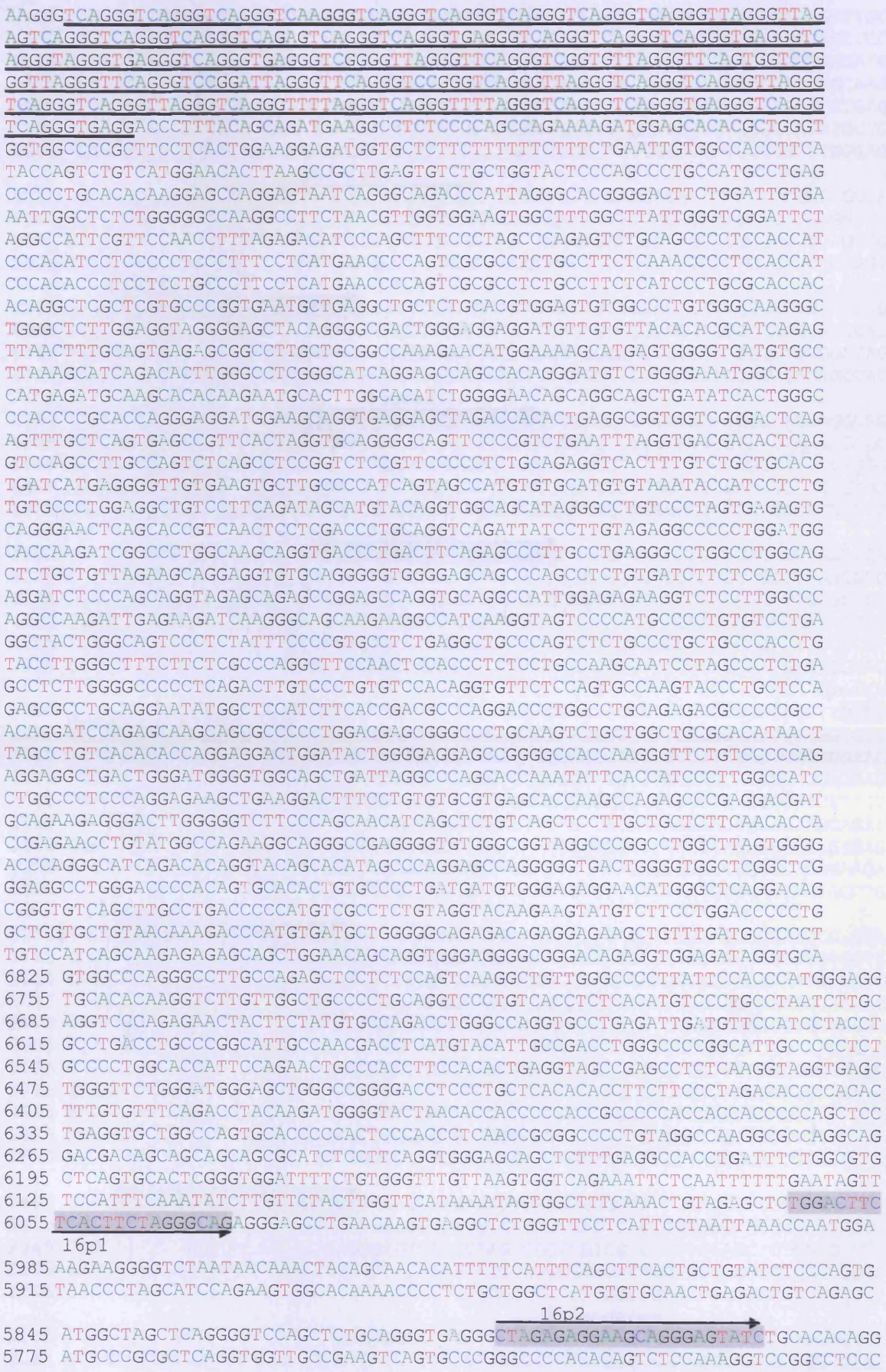
[Number of input diploid genome equivalents=input DNA per reaction (picograms) x number of reactions (typically 6-18, rarely 36-38)/6 picograms (diploid genome equivalents)]. Then the average fusion frequency was estimated from the average of all frequencies per experiment (based on 3-5 experiments depending on the sample concentration).

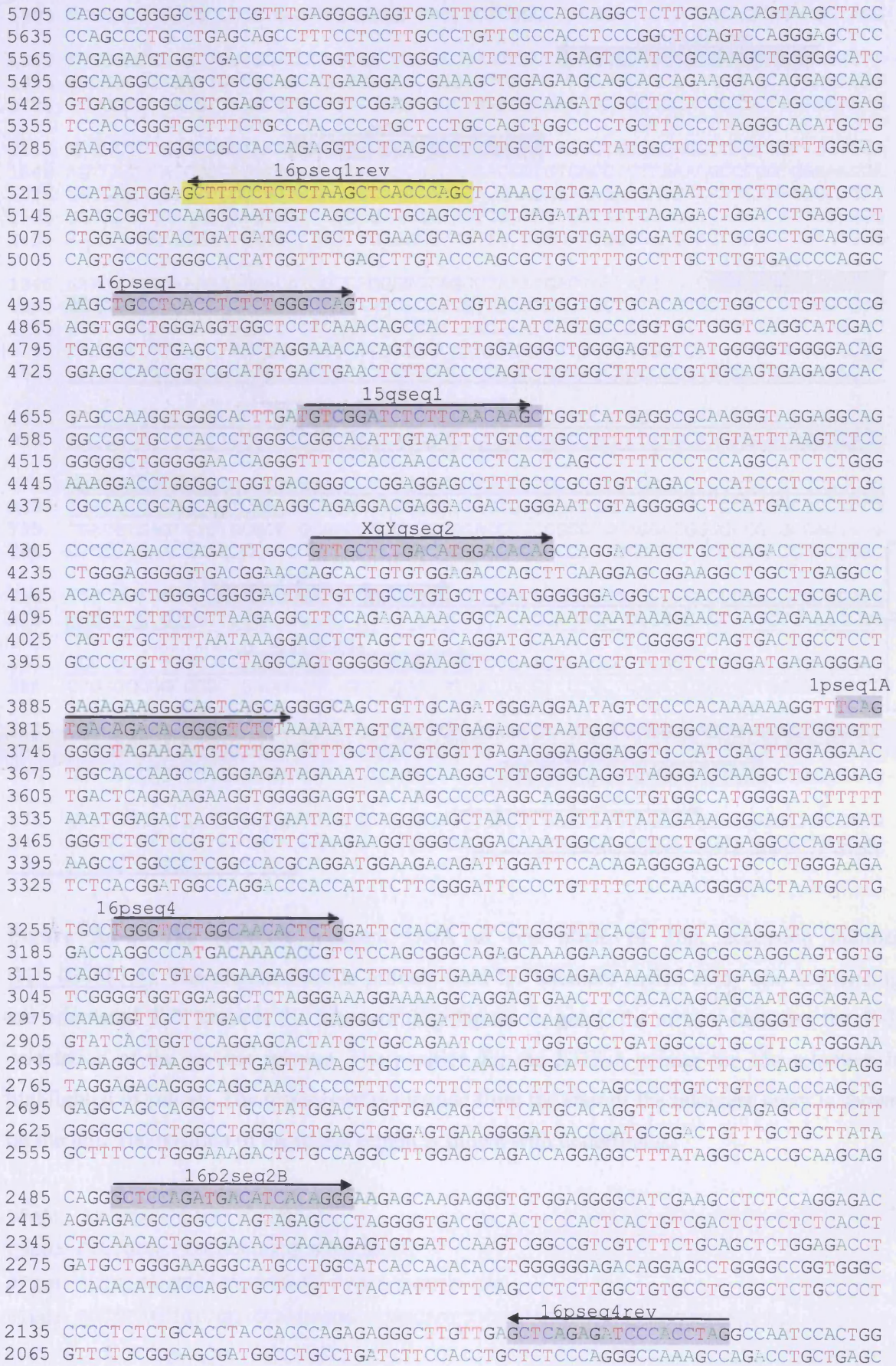
Table 3A-1B. Illustrates the experiments carried out for determining the fusion frequency in MRC5 with XpYp, 17p, 21q and/or 16p primers

				XpYp:17p:21q (3 primers)				XpYp:17p:21q:16p (four primers)			
				Amplified bands		Fusion Frequency		Amplified bands		Fusion Frequency	
	Number of Reactions per experiment	Concentration per reaction (ng)	Input genome equivalents	XpYp	17p	XpYp	17p	XpYp	17p	XpYp	17p
MRC5	18	300	900000	0	3	0	3.33×10^{-6}	0	5	0	5.56×10^{-6}
	6	300	300000	0	2	0	6.67×10^{-6}	1	1	3.33×10^{-6}	3.33×10^{-6}
	6	300	300000	0	0	0	0	2	2	6.67×10^{-6}	6.67×10^{-6}
		Total frequency				0	1.00×10^{-5}			1.00×10^{-5}	1.56×10^{-5}
Average Fusion frequency						0	3.33×10^{-6}			3.33×10^{-6}	5.19×10^{-6}


LTR/mer
21Q
GROUP
Line L1
LTR
Sine/Alu
LTR
Satellite
21Q1
21Q2
10q2
21q3
Subtel2
Start of telomere

Figure 3A-1: Telomere adjacent DNA of 21q telomere. The primers are indicated with grey highlighting and underlining. Different repeat regions are indicated. The numbering of the nucleotide is based on accession number (AP001761).


16p1
16p2


16pseq1rev
16pseq1
15qseq1
XqYqseq2
1pseq1A
16pseq4
16p2seq2B
16pseq4rev

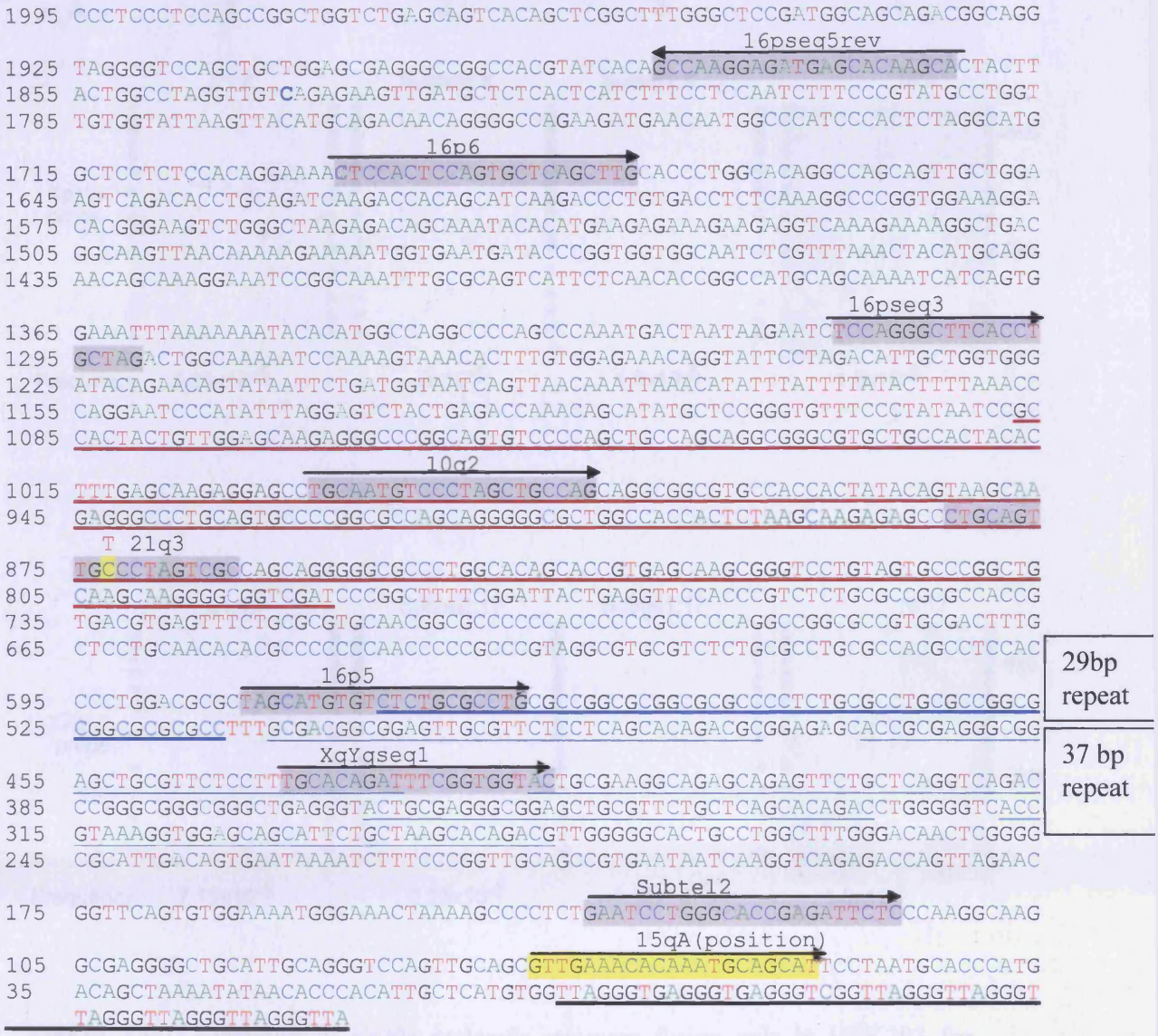


Figure 3A-2. The telomere adjacent DNA of one allele of 16p, accession number (NT_037887.4). The oligonucleotide primers used for telomere fusion assay and sequencing are orientated 5′-3′ towards the telomere (highlighted in grey), the arrows indicates the 5′-3′ orientation of the reverse primers. The position for the STELA primer for 15q telomere is highlighted in yellow. The distance of nucleotide from the start of the telomere array is shown on the left. The position of the repeat region is shown with underlining.

15qA: ATCGGAACGCAAATGCAGCAG

29bp: CTCTGCGCCTGCGCCGGCGCGGCGCGCCC

37bp: ACTGCGAAGGCAGAGCAGAGTTCTGCTCAGGTCAGAC

61bp: GCCACTACTGTTGGAGCAAGAGGGCCCGGCAGTGTCCCCAGCTGCCAGCAGGCGGGCGTGC

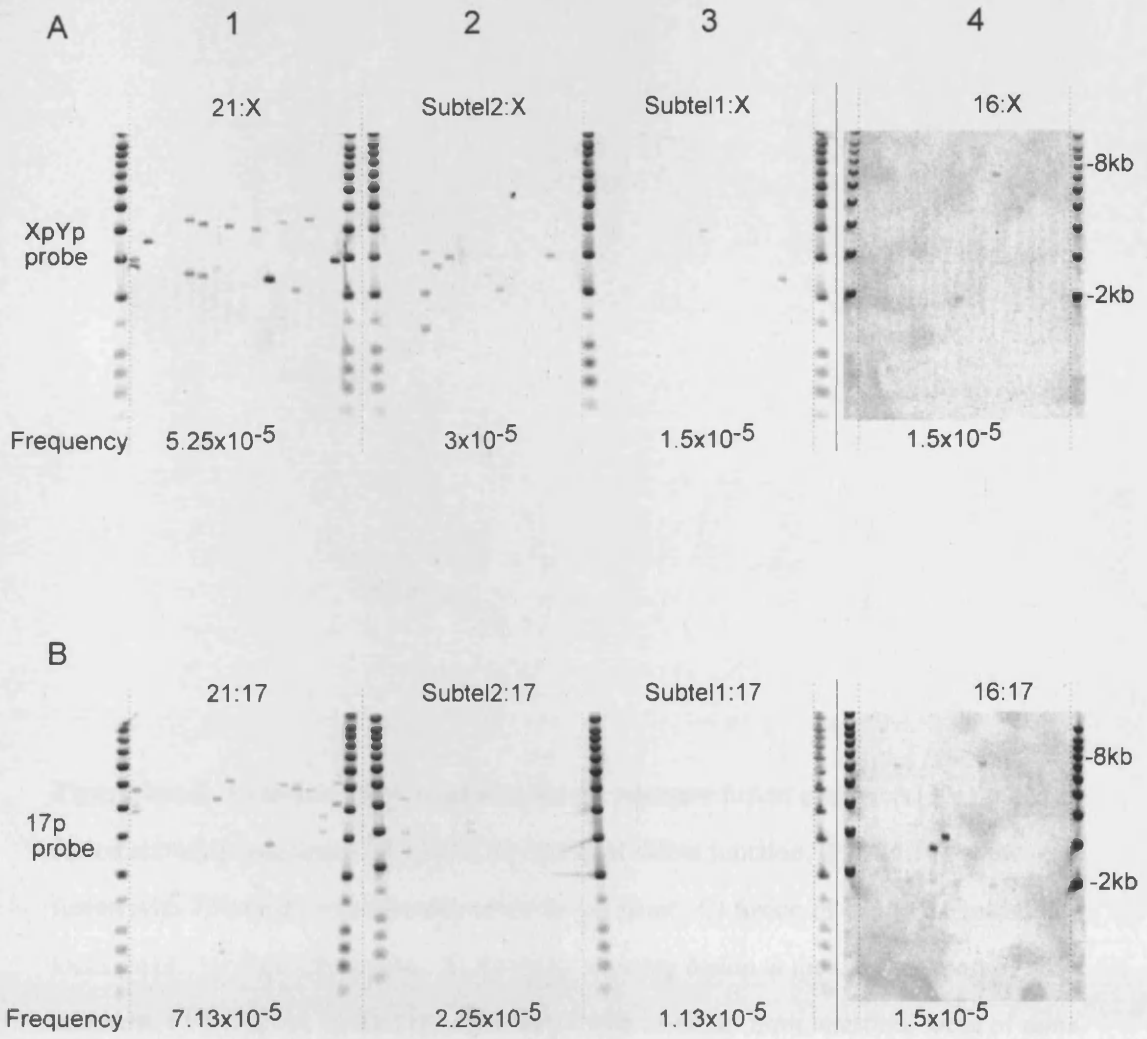
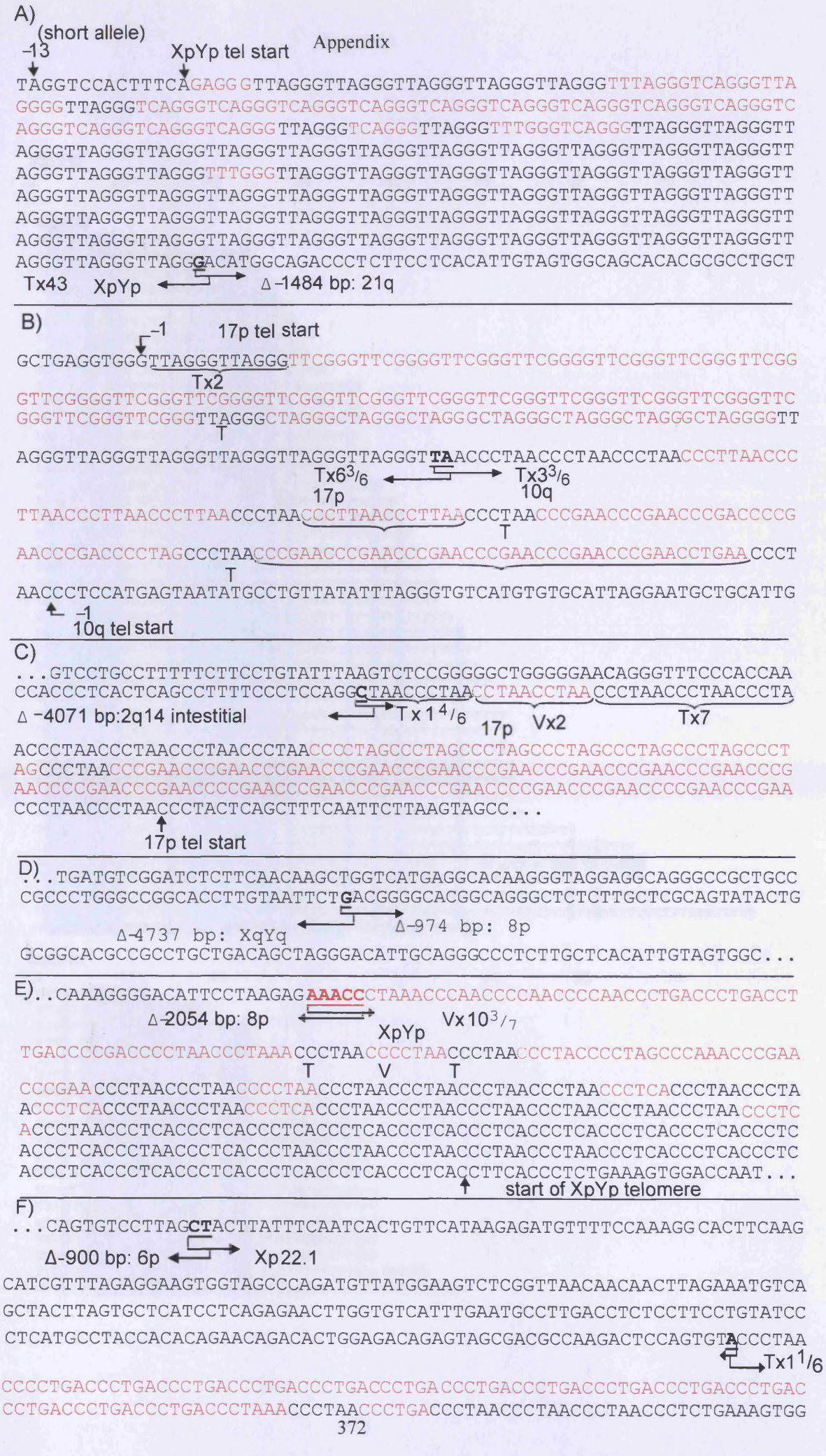
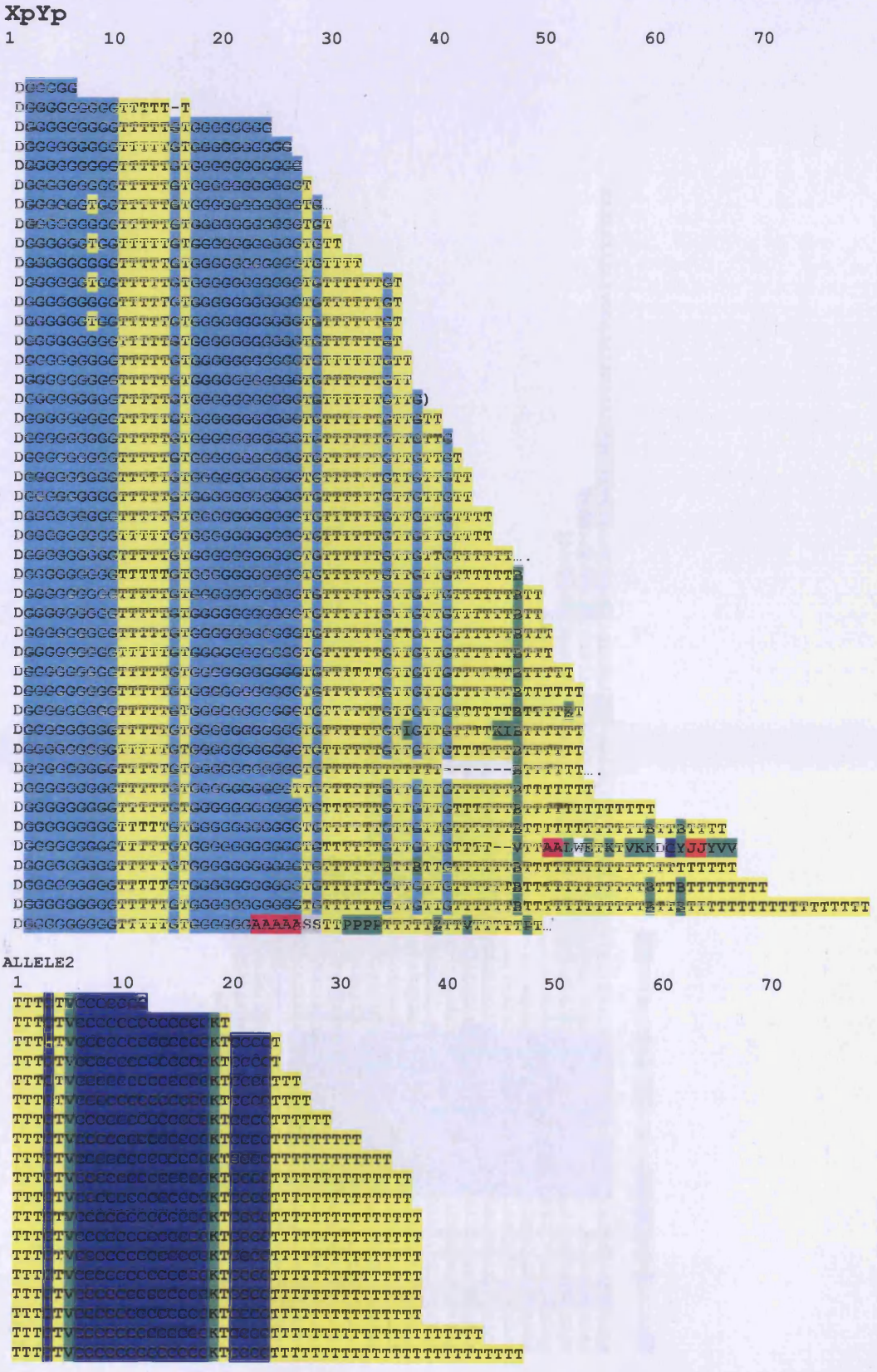
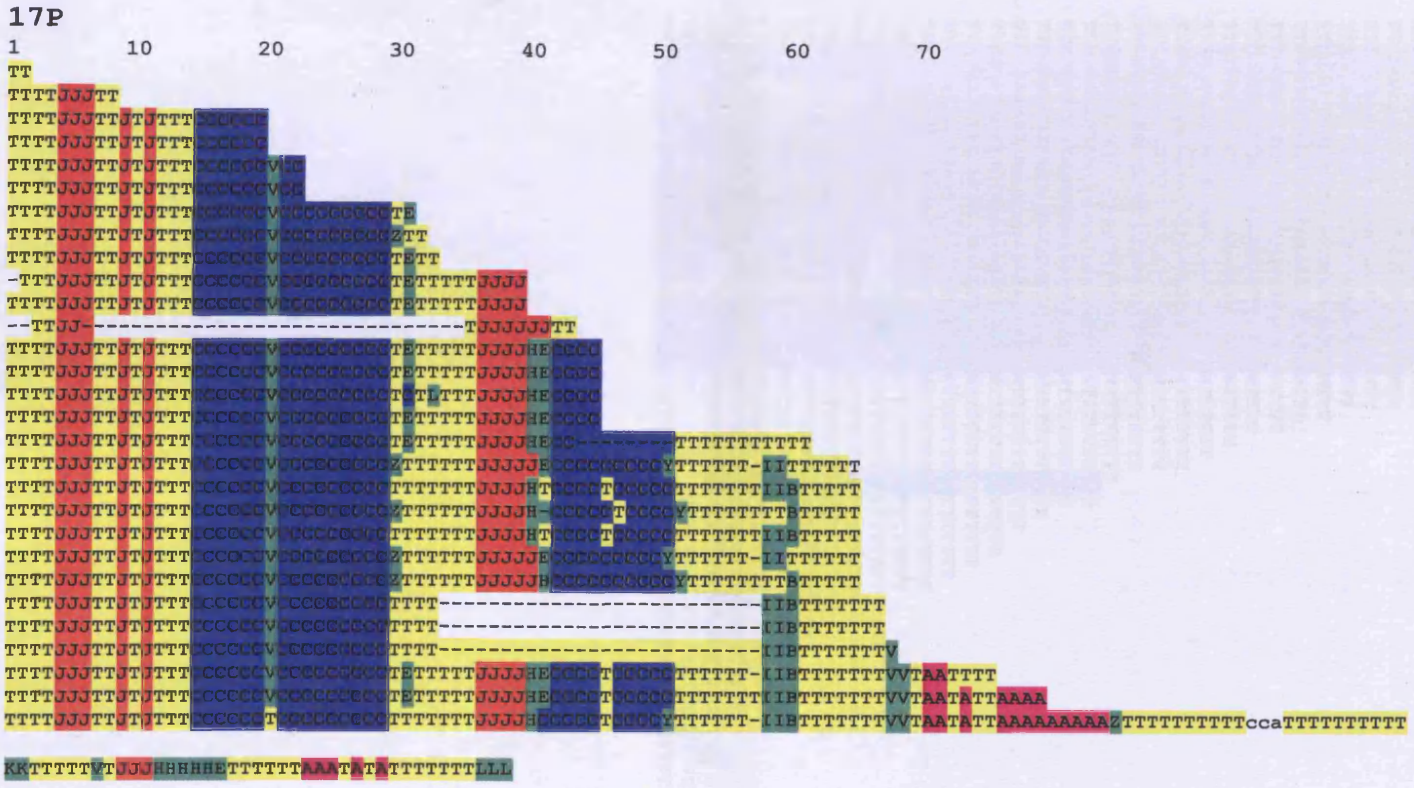


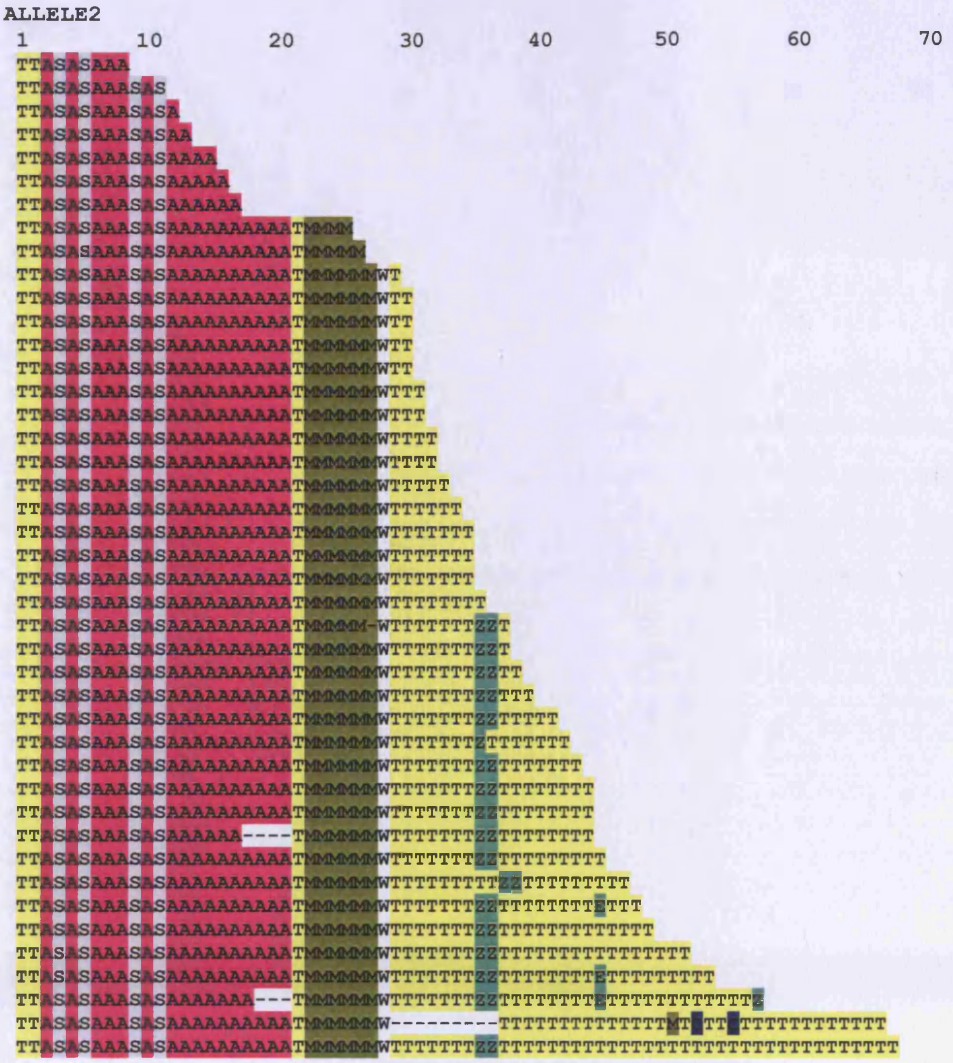
Figure 4A-1. Examples of single molecule telomere fusion gels in HEK293 for detecting fusion events between XpYp (A) or 17p (B) telomere and telomeres of 21q (panels 1, 2) and 16p (panels 3, 4) families. Each reaction contains 10ng of DNA, fusion products were detected with XpYp, and 17p probes respectively. The oligo-nucleotide primers used for each assay are shown above each gel, 21 represents 21q1, 16 represents 16p1. The frequency at which fusion was detected is indicated below each gel. Molecular weight markers are indicated on the right.

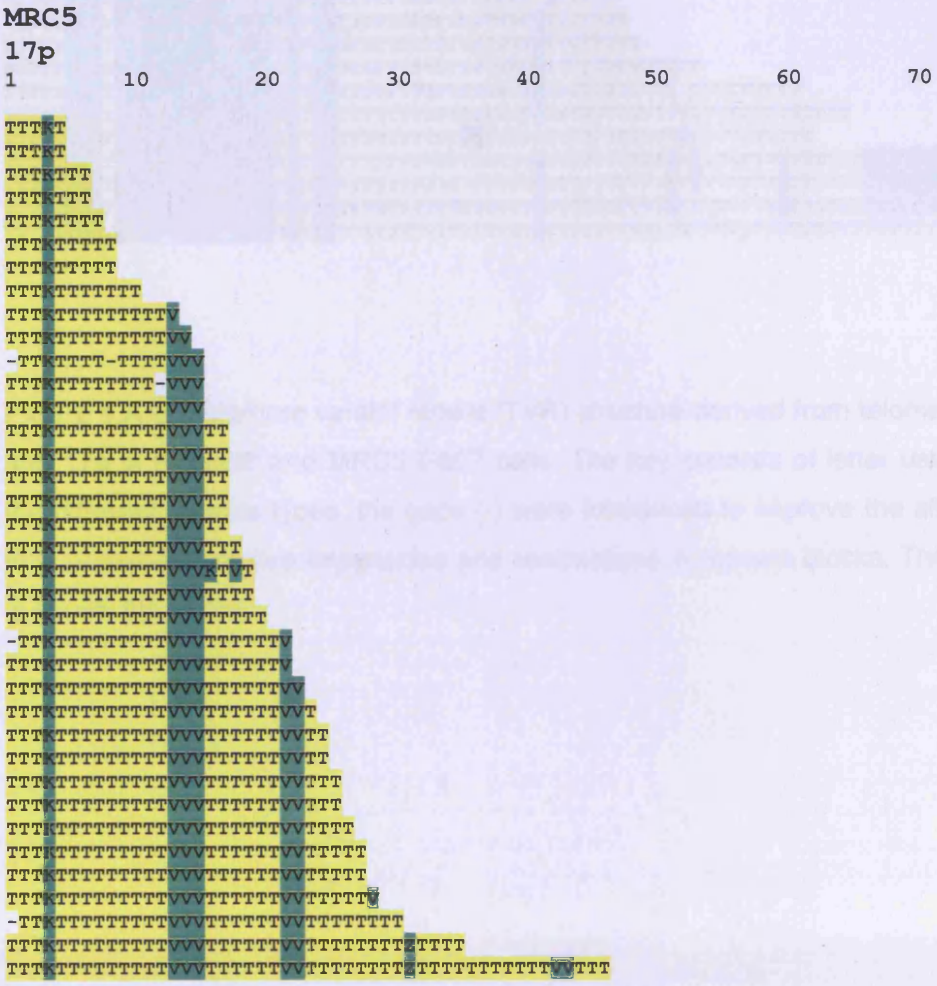
Figure 4.A-2. Extensive DNA sequences for the telomere fusion events. A) XpYp:21q fusion showing long arrays of TTAGGG repeats at fusion junction. B) 17p:10q showing fusion with TTAGGG on either side of the fusion point. C) fusion of 17p to the intestitial locus 2q14. D) XqYq:8p fusion. E) XpYp:8p showing fusion at the proximal part of the telomere. F) 6p:XpYp, an example of an event with insertion from intestitial locus of same telomere (Xp22.1) in reverse orientation.

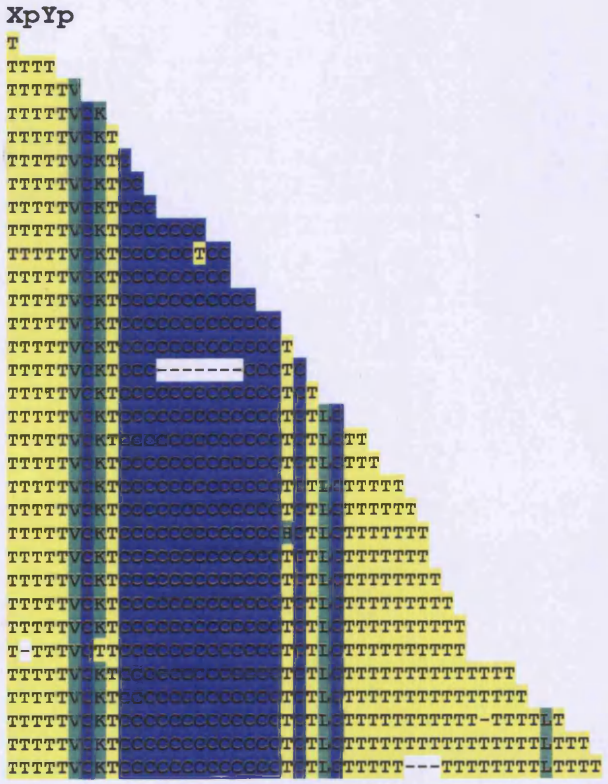

A)
(short allele)
-13
XpYp tel start
Tx43
XpYp
Δ-1484 bp: 21q
B)
-1
17p tel start
Tx2
Tx6 3/6
17p
Tx3 3/6
10q
-1
10q tel start
C)
Δ -4071 bp:2q14 intestitial
Tx1 4/6
17p
Vx2
Tx7
17p tel start
D)
Δ-4737 bp: XqYq
Δ-974 bp: 8p
E)
Δ-2054 bp: 8p
XpYp
Vx10 3/7
start of XpYp telomere
F)
Δ-900 bp: 6p
Xp22.1
Tx1 1/6


XpYp
1
10
20
30
40
50
60
70
ALLELE2
1
10
20
30
40
50
60
70


17P
1
10
20
30
40
50
60
70


ALLELE2
1
10
20
30
40
50
60
70


MRC5
17p
1
10
20
30
40
50
60
70


XpYp

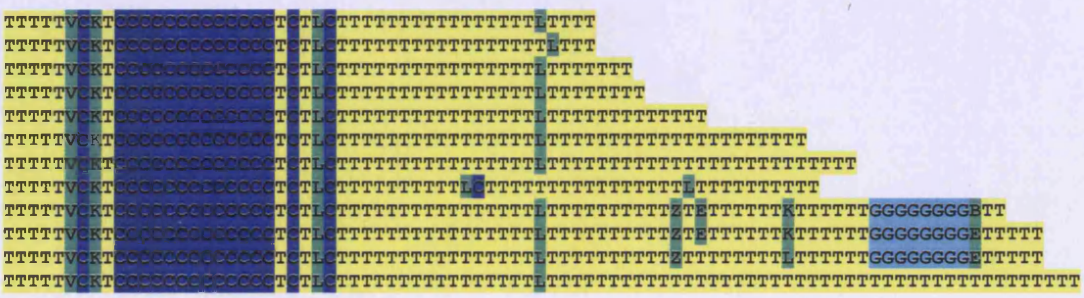


Figure 4.A-3. Telomere variant repeat (TVR) structure derived from telomeres of XpYp and 17p in HEK293 and MRC5 E6E7 cells. The key consists of letter used to denote the different repeats types, the gaps (-) were introduced to improve the alignment and thus represent putative expansions and contractions in repeats blocks. The duplication is shown by arrows.