WISKOTT ALDRICH SYNDROME PROTEIN AND IT'S ROLE IN BREAST CANCER

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A dissertation submitted to the School of Medicine, Cardiff University in candidature for the degree of Doctor of Medicine (MD)

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DEDICATION

This work is dedicated to my wife Sunita, as it would not have materialised without her help, prayers and gentle persuasion, and to our beautiful daughter Geve Lolita

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Abbreviations

Aa	Amino acid
Ab	Antibody
ABC	Avidin biotin complex
Ag	Antigen
BCS	Breast Conserving Surgery
bp	Base pair
BSA	Bovine serum albumin
BSS	Balanced Salt Solution
CaCl2	Calcium Chloride
CCR 5	Chemokine receptor 5
CDC42	Cell Division Control Protein 42
cDNA	Complementary Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
DAB	Diaminobenzidine tetrahydrochloride
DCIS	Ductal Carcinoma in Situ
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DFS	Disease free survival,
dNTP	Deoxynucleotide

dTTP	Deoxythymidene triphosphate
dH2O	Distilled water
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECIS	Electric Cell Impedance Substrate
ECM	Extracellular Matrix
E. coli	Escherichia coli
EDTA	Ethylene di-amino tetra-acetic acid
ER	Oestrogen receptor
ERM	Ezrin Radixin Moesin
FCS	Foetal calf serum
FITC	Fluorescine Isothiocyanate
G418	Geneticin
GTP	Guanosine Triphosphate
HGF	Human Growth factor
HER2	Human epidermal growth factor 2
HBSS	Hank's Balanced Salt Solution
HCMF	HEPES buffered salt solution without CaCl2/MgCl2
HRT	Hormone Replacement Therapy

HSE	Heat shock element
HSP90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cell
IGEPAL	Non ionic, non denaturing detergent
Kb	Kilo-base
KDa	Kilo dalton
KCl	Potassium chloride
KH2PO4	Potassium dihydrogen phosphate
LB	Luria Bertani
LFS	Li-Fraumeni Syndrome
m	Metre
M	Molar
ma	Milliamp
MCF-7	Breast Cancer Cell line
MDA-MB-231	Breast Cancer Cell line
mg	Milligram
min.	Minute
ml	Milli litre
mM	Milli molar
MRC-5	Fibroblast cell line
mRNA	Messenger Ribonucleic acid

MTT	Dimethylthiazol diphenyltetrazolium bromide
NaCl	Sodium chloride
NACT	Neo adjuvant chemotherapy
NaHPO4	Sodium phosphate
NaOH	Sodium Hydroxide
Na3 VO4	Sodium Orthovanadate
ng	Nano- gram
NPI	Nottingham Prognostic Index
NRK	Normal Rat kidney
OS	Overall survival
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
pCR	Pathologic complete response
PTHrP	Parathyroid hormone related peptide
QBT	DNA purification buffer
QPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse Transcription

RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel
sec	Second
SH3	SRC Homology 3 domain
SOC	Super Optimal broth with Catabolite repression
Src	protooncogene abbr. for Sarcoma
SYBR	Synergy Brands
TAG	Tubulo-acinar gland
Taq	Thermus aquaticus
TBE	Tris/ Borate/ EDTA electrophoresis buffer
TBS	Tris Buffered Saline
TEMED	Tetramethylethylenediamine
TGF-P	Transforming Growth factor P
TNM	Tumour-Node-Metastasis
TRITC	Tetramethylrhodamine-5-(and 6)-isothiocyanate
TRIS-HCl	Hydroxymethyl aminomethane hydrochloride
tRNA	Transfer Ribonucleic acid
U	Unit
V	Volt
WAS	Wiskott Aldrich Syndrome

WASP	Wiskott Aldrich Syndrome protein
WAVE	Wiskott Aldrich Syndrome protein verprolin homologue
WT	Wild type

SUMMARY

Breast Cancer continues to be the most common form of cancer in women. The ability of tumour cells to spread from primary and metastatic tumours is the primary cause of death in patients with cancer. Thus, it rightly follows that significant research is dedicated to the pathways and mechanisms controlling metastases in order to guide therapeutic approaches.

Wiskott Aldrich Syndrome [WAS] is an X-linked recessive condition with immunodeficiency as the clinical manifestation. It is caused by mutations of the Wiskott Aldrich Syndrome [WAS] gene, which codes for a cytoplasmic protein with multiple functions. Two major complexes that are linked to the NWASP family, namely the ERM family and Rho GTPases are aberrantly expressed in human breast cancer. Additionally, X chromosome inactivation which silences gene expression from one of the two X chromosomes in females, is usually random. Skewed X inactivation has been shown to occur more frequently in breast and ovarian cancer patients. Individuals with WAS are known to have skewed X inactivation. In addition, they are more susceptible to certain forms of malignancy, primarily haematological. This formed the basis of the present study, which sought to elucidate the role of WAS protein in human breast cancer, and to determine if it plays a role as a tumour suppressor. We also attempted to determine its biological role and association with clinical outcome in patients with breast cancer. We examined the correlation of NWASP with human breast cancer *in vitro, in vivo* and in human breast cancer tissue.

Immunohistochemistry studies of frozen sectioned human breast cancer tissues revealed that breast cells stained positively for NWASP and that cancer cells in tumour tissues stained very weakly. Quantitative RT-PCR revealed that breast cancer tissues had significantly lower levels of NWASP compared to normal background breast tissue. Although no significant correlation was found with tumour grade and TNM staging, lower levels of transcript were seen to correlate with clinical outcome following a 10 year follow up. The invasive human breast cancer cell line, MDA-MB-231 was used to over-express NWASP, with over-expression resulting in cells with reduced motility and invasion, increased

adhesion to the basement membrane and more significantly, reduced tumour growth *in vivo*. This has important implications in understanding the mechanism whereby cancer cells become more motile and presents an interesting tool in analysing the progression of human breast cancer.

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CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Epidemiology

Breast cancer is by far the most frequent cancer among women with an estimated 1.38 million new cancer cases diagnosed in 2008 (23% of all cancers) and ranks second overall (10.9% of all cancers). It is now the most common cancer both in developed and developing regions with around 690000 new cases estimated in each region. Incidence rates vary from 19.3 per 100,000 women in Eastern Africa to 89.7 per 100,000 women in Western Europe, and are high (greater than 80 per 100,000) in developed regions of the world (except Japan) and low (less than 40 per 100,000) in most of the developing regions (Figure 1.1). The range of mortality rates is much less (approximately 6-19 per 100,000) because of the more favourable survival of breast cancer in (high-incidence) developed regions. As a result, breast cancer ranks as the fifth cause of death from cancer overall (458 000 deaths), but it is still the most frequent cause of cancer death in women in both developing (269 000 deaths, 12.7% of total) and developed regions.

Breast cancer has been the most common cancer in the United Kingdom [UK] since 1997, despite the fact that it is rare in men (Figure 1.2). It is by far the most common cancer among women in the UK, accounting for 31% of all new cases of cancer in females. In 2010, there were 49,961 new cases of breast cancer in the UK: 49,564 (99%) in women and 397 (less than 1%) in men, giving a female: male ratio of around 125:1.1. The crude incidence rate shows that there are 157 new breast cancer cases for every 100,000 females in the UK, and 1 for every 100,000 males.

The risk is strongly age-related, with more than 80% of cases occurring in women over 50 years of age. The highest incidence is seen in the 50-69 age range [Table 1.4]. Hence, the national screening programme in the United Kingdom targets this age range [recently amended to include women up to 70]. Among cancers in women, Breast cancer is the most commonly diagnosed cancer in women under 35. By age 35 –39, almost 1,500 women are diagnosed annually. There occurs considerable geographic variation, with highest rates in the developed world and lowest rates in Africa and Asia (Ferlay et al., 2012). It has been found that

migrants from low to high risk countries acquire the risk of the host country within two generations (Ziegler et al., 1993, Tominaga, 1985).

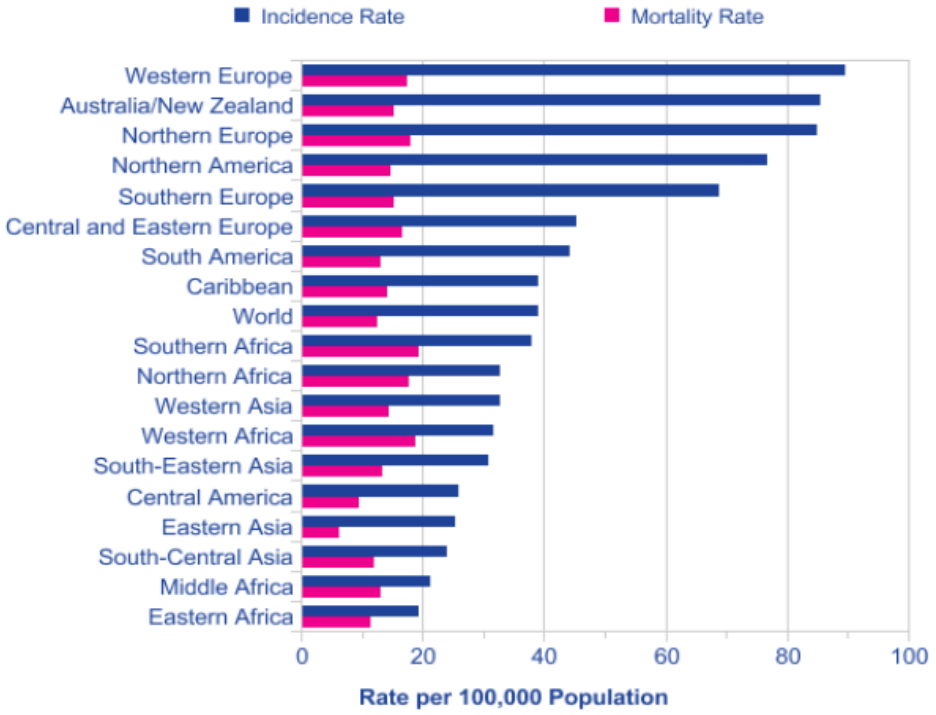


Figure 1. 1 Age standardised incidence and Mortality rates of female breast cancer in selected world regions, 2008. Adapted from (http://www.cancerresearchuk.org)

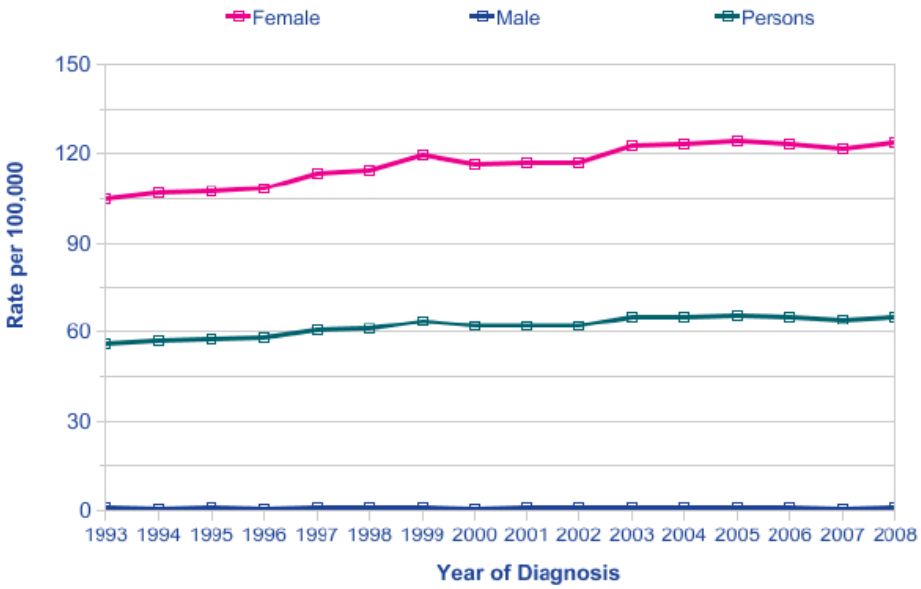


Figure 1. 2 European Age standardised Incidence Rates per 100000 Population by sex, UK 2008. Adapted from (http://www.cancerresearchuk.org)

The incidence of breast cancer is high, and with advancements in management, the overall 5-year survival rates are constantly increasing [now above 75%]. This translates into a large population of women who are alive and have been diagnosed with breast cancer [Figure 1.3]. The most recent estimates suggest this figure is around 172,000 in the UK (Micheli et al., 2002).

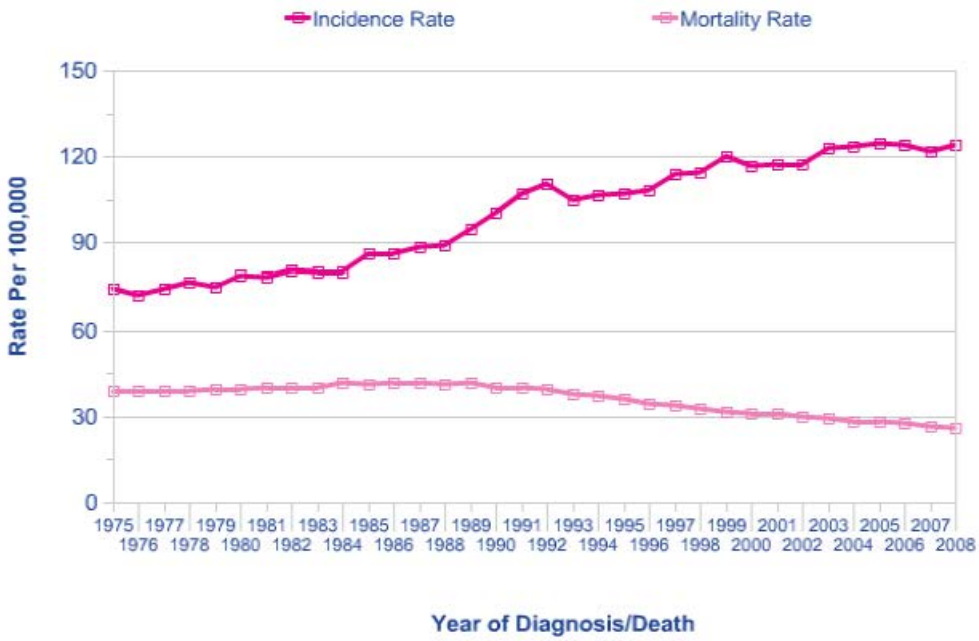


Figure 1. 3 Age standardised incidence and mortality rates of female breast cancer- United Kingdom (1975-2008). Adapted from (http://www.cancerresearchuk.org).

1.2 Aetiology and Risk Factors

Broadly speaking, the risk factors can be divided as those associated with oestrogen exposure [age, lower parity, early menarche, late menopause, Hormone Replacement Therapy or Oral Contraception] or those associated with genetic factors [family history, carriers of BRCA 1 or 2 mutations, or previous breast, endometrial or ovarian cancer]. These have been summarized in Table 1.1.

1.2.1 Age

A diagnosis of breast cancer in the 3rd or 4th decade of a woman's life is associated with a poorer prognosis (Albain et al., 1994). Young women with breast cancer are more likely to have a higher tumour grade and disease positive nodes (de la Rochefordiere et al., 1993). Early onset breast cancers are more likely to be

hormone receptor negative but C-erb-2 positive, and this is associated with more aggressive behaviour (Bertheau et al., 1998, Gusterson et al., 1992). Survival rates were shown to be lower by 10-20% in patients aged 30 or less (de la Rochefordiere et al., 1993, Lee et al., 1992). By the age of 50, around 10,000 women were diagnosed with breast cancer (in the UK in 2010), but 80% of all diagnoses were in the over 50s, and 45% were diagnosed in women aged 65 and over (in the UK between 2008 and 2010). Age-specific incidence rates rise steeply from around age 35-39, level off for women in their 50s, then rise further to age 65-69, drop slightly for women aged 70-74, then increase steadily to reach an overall peak in the 85+ age groups (see Fig 1.4). The estimated risk of developing breast cancer at the different age groups is summarized in Table 1.2.

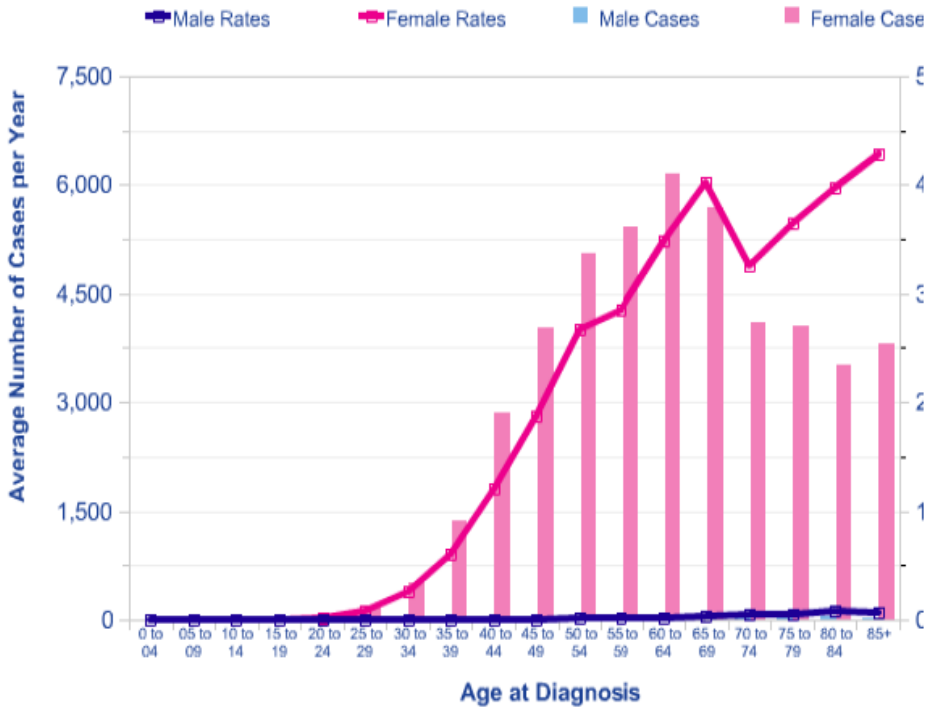


Figure 1.4: Numbers of new cases and age specific incidence rates, by sex, breast cancer, UK. Adapted from (http://www.cancerresearchuk.org)

Nearly half (48%) of female breast cancer cases are diagnosed in the 50-69 age group; currently women in this age group and those aged 70 are invited for screening every three years in the UK through the NHS Breast Screening Programme. In England, a trial is taking place to look at the possible benefits of extending breast screening so that women aged 47 to 50 and 70 to 73 are invited.

1.2.2 Genetic Factors

Although it is estimated that familial breast cancer accounts for 6-19% of cases (Colditz et al., 1993, Slattery et al., 1993), only 5% of breast cancers are hereditary, the remainder occur as a result of spontaneous genetic mutations. Known genetic disorders associated with breast cancer are ataxia telangiectasia, Cowden's disease and Li Fraumeni syndrome (Thompson and Dixon, 1992). Important breakthroughs were the discovery of the tumour suppressor genes-BRCA 1 on chromosome 17q (Miki et al., 1994) and BRCA 2 on chromosome 13q (Wooster et al., 1995). BRCA 1 mutations are spread evenly across the gene (Couch and Weber, 1996), and the gene is involved in regulating the growth of breast epithelial cells (Holt et al., 1996). Women carrying the BRCA 1 gene mutations have an 87% life-time risk of developing breast cancer (Ford et al., 1994), although this has subsequently been estimated at 50% (Struewing et al., 1997). It has been shown that mutations in BRCA1 function as a novel predictor of response to chemotherapy (James et al., 2007). Germ line mutations in this pair of genes, which cause interruption of DNA repair, are responsible for the majority of familial breast cancers (Ingvarsson, 2004).

1.2.3 Hormonal Factors

Hormonal risk factors have an important bearing as is suggested by the observation that the longer the breast is exposed to cyclical oestrogens, the greater the risk of developing breast cancer. Late menarche (Peeters et al., 1995), early menopause (Velentgas and Daling, 1994), early full term pregnancy and greater parity (Layde et al., 1989); are all associated with a decrease in the risk of breast cancer development. The use of Hormone Replacement Therapy [HRT] over long periods is associated with an increased risk, but the risk disappears soon after stopping treatment (Vessey, 1997). However, HRT induced cancers appear to have a better prognosis than those in non users with improved survival prospects (Schairer et al., 1999). Obesity (Trentham-Dietz et al., 1997) and excessive alcohol intake (Steinberg et al., 1991, Reichman et al., 1993) have been implicated as associated risk factors. The conversion of adrenal androstenedione to oestrone is related to body weight (Dunn and Bradbury, 1967): in normal subjects, this figure is 1%; in obese individuals, it approaches 10%.

Table 1.1: Risk factors for the development of breast cancer
Adapted from (Kelsey and Berkowitz, 1988)

Risk Factors	High risk	Low risk	Relative risk
Age at 1st full term pregnancy	>30	<20	2-4
Age at menarche	Early	Late	1-1.9
Age at menopause	Late	Early	1-1.9
Family history of premenopausal bilateral breast cancer	Yes	No	>4
Past history of breast cancer	Yes	No	>4
Any 1st degree relative with breast cancer	Yes	No	2-4
Biopsy showing atypical hyperplasia	Yes	No	>4

Table 1.2: Estimated risks for different age group
Adapted from (Kelsey and Berkowitz, 1988)

Age Group	Estimated Risk
Up to age 25	1 in 15,000
Up to age 30	1 in 1,900
Up to age 40	1 in 200
Up to age 50	1 in 50
Up to age 60	1 in 23
Up to age 70	1 in 15
Up to age 80	1 in 11
Up to age 85	1 in 10

1.3 Breast Morphology

1.3.1 Normal Breast

The human mammary gland [breast] is a modified sweat gland, and is essentially composed of 15-20 tubulo-acinar glandular [TAG] units defined by dense connective tissue that separates them. The composition of the connective tissue varies with age, with older women having less adipose tissue. The onset of puberty brings about stromal proliferation with deposition of inter and intra lobular connective tissue, resulting in the expansion of the TAG unit. Each TAG unit is composed of many lobules which in turn comprises a system of terminal alveoli draining into alveolar ducts. Several of these drain into a single lactiferous duct from each TAG unit to the nipple.

The ductal system of the breast is lined by cuboidal epithelial cells flanked by myoepithelial cells, both layered upon a prominent basement membrane. The two epithelial cell components are luminal epithelial and myoepithelial cells, both of which can be purified by immunoaffinity techniques which exploit differences in marker cell surface protein expression (Gomm et al., 1995, Kamalati et al., 1999, Clarke et al., 1994, Slade et al., 1999). Using such protocols, it has been possible to further study and highlights the responses of these two cell types to growth and morphogenic signals, thereby gaining further insight into the functionality of these cells in the normal mammary gland.

The stromal structure of the breast accounts for about 80% of the volume of the breast (Drife, 1986). This component changes remarkably during different phases of the menstrual cycle. The main components of the stroma are adipose tissue, connective tissue (dense and loose) and blood vessels. A prominent cell type in the structure and stroma is the fibroblast. The luminal epithelial cells are highly specialized structures and display structural and functional polarity in their organization (Simons and Fuller, 1985). The apical surface (lumen facing) membrane is bound to the adjacent cell apical membrane by a tight junction preventing molecules from diffusing between adjacent cells (Gumbiner, 1987). In the ducts, they are surrounded by a discontinuous layer of myoepithelial cells. The latter are less marked in the smaller ductules of the lobuli.

In the adult human breast, oestrogen receptors are expressed in approximately 20% of all cells (Russo et al., 1999) and are over expressed in the majority of breast cancers (Petrangeli et al., 1994). The luminal epithelial cells express a variety of keratin proteins, but cytokeratin 18 staining is the most consistent and homogenous, being positive in all luminal epithelial cells and negative in myoepithelial cells (Petersen and van Deurs, 1987). Myoepithelial cells also express cytokeratins, predominantly CK5 and to a lesser extent CK7, 13, 14 and 17 (Moll et al., 1989, Nagle et al., 1986). These also express actins and myosins in keeping with their contractile function.

1.3.2 Breast Cancer

Pathologically breast cancer is classified as non-invasive [carcinoma *in situ*] or invasive. There are different morphological variants. In invasive carcinoma, histological examination shows that myoepithelial cells are absent. The basement membrane is lost and cancer cells express markers for luminal epithelial cells rather than myoepithelial cells (Ronnov-Jessen et al., 1996). Gene expression studies indicate that there may be myoepithelial gene expression signature in a significant number of such tumours (Perou et al., 1999, Perou et al., 2000).

In carcinoma *in situ*, the cancer cells are confined to the ductal epithelium and acini, without having penetrated through the basement membrane [hence non invasive]. Ductal carcinoma *in situ* [DCIS] is visible as microcalcifications on mammograms. This accounts for 20% of all screen detected breast cancers (Pinder, 2001). Based on the degree of nuclear atypia within the cells, this is classified as high, intermediate and low grade DCIS.

DCIS is considered premalignant, and there are conflicting opinions regarding the rate of progression, depending on the variety of DCIS. The natural history of low grade DCIS is that approximately 60 percent of lesions will become invasive at 40 years follow up. The natural history of intermediate grade DCIS is yet unknown. The natural history of high grade DCIS suggests an invasive risk of at least 50 percent at 7 years follow up (Hamilton et al., 2004). Other authors consider DCIS to be a nonobligate precursor to invasive breast cancer, postulating that 47-86 percent of patients with DCIS will not progress to invasive breast cancer even after 30 years of follow up (Fisher and Brown, 1985, Fisher et al., 1986). In one cohort of patients treated with excisional biopsy alone, only 39 percent of patients progressed to ipsilateral breast cancer at 30 years of follow up (Bonadonna et al., 1976). Lobular carcinoma *in situ* [LCIS] is usually found incidentally in biopsy specimens in postmenopausal women. It is less common, often multifocal and occurs bilaterally in 30% of cases. Almost 90% of invasive cancers are ductal in origin; lobular cancers make up the remaining 10% (Forrest and Alexander, 1995). The majority of ductal carcinomas are called no special type [NST] as they do not display any special histological features. Histological variants include

medullary [5%], tubular [3%] and mucoid varieties [2%]. Variants of lobular cancer include cribriform, solid and tubular varieties.

The TNM classification of malignant tumors, initially developed by Pierre Denoix is currently maintained by the Union for International Cancer Control. A common international language for classifying cancer, it is summarized in Table 1.3 below.

Table 1.3: TNM definitions (http://cancernet.nci.nih.gov)

	Primary tumour (T)
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ; DCIS, LCIS, or Paget's disease of the nipple
T1	Tumour 2.0 cm or less in greatest dimension
T1mic	Microinvasion 0.1 cm or less in greatest dimension
T1a	Tumour more than 0.1 but not more than 0.5 cm in greatest dimension
T1b	Tumour more than 0.5 cm but not more than 1.0 cm
T1c	Tumour more than 1.0 cm but not more than 2.0 cm
T2	Tumour more than 2.0 cm but not more than 5.0 cm
T3	Tumour more than 5.0 cm in greatest dimension
T4	Tumour of any size with direct extension to (a) chest wall or (b) skin,
T4a	Extension to chest wall
T4b	Oedema (including peau d'orange) or ulceration of the skin of the breast or satellite skin nodules confined to the same breast
T4c	Both of the above (T4a and T4b) (N):
T4d	Inflammatory carcinoma
	*** Regional lymph nodes (N)**
NX	Regional lymph nodes cannot be assessed (e.g., previously removed)
N0	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral axillary lymph node(s)
N2	Metastasis to ipsilateral axillary lymph node(s) fixed to each other or to other structures
N3	Metastasis to ipsilateral internal mammary lymph node(s)
	Pathologic classification (pN):
pNX	Regional lymph nodes cannot be assessed (not removed for pathologic study or previously removed)
pN0	No regional lymph node metastasis
pN1	Metastasis to movable ipsilateral axillary lymph nodes
pN1b	Metastasis to lymph node(s), any larger than 0.2 cm
pN2	Metastasis to ipsilateral axillary lymph node(s) fixed to each other or to other structures
pN3	Metastasis to ipsilateral internal mammary lymph node(s)
	Distant metastasis (M):
MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis present (includes metastasis to ipsilateral supraclavicular lymph nodes)

1.4 Tumour staging and prognosis

More detailed, and therefore prognostically informative, staging can be determined by including pathological information. Histopathologists specifically include variables such as tumour size, grade, oestrogen and progesterone status, the number of lymph nodes involved and the presence of lympho-vascular invasion. Based on the following three variables, viz., tubule formation, nuclear size/degree of pleomorphism and mitotic count, tumours are assigned a grade from 1-3 (Table 1.4). Each of these variables is further assigned a score from 1-3, and grade is arrived by adding up the final score. Grade 1: 3-5; Grade 2: 6- 7; Grade 3: 8-9. Patients with grade 1 tumours have a better prognosis [85% 10 year survival] as compared to grade 3 patients [less than 45% 10 year survival] (Pinder, 2001).

Table 1.4: AJCC stage groupings (http://cancernet.nci.nih.gov)

Stage 0	Tis, N0, M0
Stage I	T1,* N0, M0 [*T1 includes T1mic]
Stage IIA	T0, N1, M0 T1,* N1,** M0 T2, N0, M0 *T1 includes T1mic **The prognosis of patients with pN1a disease similar to that of patients with pN0 disease.
Stage IIB	T2, N1, M0 T3, N0, M0
Stage IIIA	T0, N2, M0 T1,* N2, M0 T2, N2, M0 T3, N1, M0 T3, N2, M0 *T1 includes T1mic
Stage IIIB	T4, Any N, M0 Any T, N3, M0
Stage IV	Any T, Any N, M1

Table 1.5 Bloom and Richardson Grading System of breast cancer (Cancer, 2010)

Tubule Formation (% of tumour exhibiting gland formation)	**Score**
>75% of tumour	1
10- 75% of tumour	2
<10% of tumour	3
Pleomorphism (Variation in size and shape of nuclei)	
Mild	1
Moderate	2
Marked	3
Mitotic activity	
<6 mitosis/ 10hpf	1
7- 15 mitosis/ 10hpf	2
>15 mitosis/ 10hpf	3
Total Score is derived from adding scores for the 3 parameters. The score varies from 3- 9.	
Grade 1 (well differentiated)	3-5
Grade 2 (moderately differentiated)	6-7
Grade 3 (poorly differentiated)	8-9

It has been universally recognised that the single most important clinico-pathological factor prognostically is the axillary lymph node status (Carter et al., 1989, Fisher et al., 1983). Nodal involvement is reciprocally related to prognosis. In a series of 24,740 patients with breast cancer, the overall incidence of axillary involvement was 46% (Carter et al., 1989). Recent series, albeit smaller, have shown that this figure has fallen towards 25% (Martin et al., 2002). The 10 year survival falls from 60-70% for node negative patients to 20-30% for node positive patients (Dixon and Sainsbury, 1998). Tumour size is probably related to nodal metastasis. The incidence of positive axillary nodes varies from 3-22% for tumours less than a centimetre in size (Carter et al., 1989, Hindie et al., 2011, Hsueh et al., 2000, Veronesi et al., 1998)

As the prognosis is an important issue that concerns patients and physicians alike, and there are a number of variables involved in this determination, an effort to combine these has been made. The Nottingham Prognostic Indicator [NPI] (Galea et al., 1992) has been found to accurately predict the clinical outcome of breast cancer. This is calculated as follows:

NPI = 0.2 X tumour size + histological grade [1-3] + lymph node stage [1-3]*

*** Lymph node stage is arrived at as: stage 1 if the axillary nodes are disease free, stage 2 if 1-3 nodes are involved, and stage 3 if more than 3 lymph nodes are involved.**

An NPI of less than 3.4 is associated with an 80% 15-year survival, NPI of 3.4-5.4 is associated with 42% 15-year survival, and NPI of greater than 5.4 is associated with a 13% 15-year survival. The NPI has limitations in that it does not account for lymphovascular invasion or oestrogen receptor status, which has independent prognostic significance [see below]. Lymphovascular invasion is useful in predicting local recurrence. It is not used in planning adjuvant treatment or determining prognosis.

The role of oestrogen receptor [ER] in breast cancer was elucidated after its isolation (Toft and Gorski, 1966). It became feasible to use an ER assay to predict hormone responsiveness in breast cancer (Brem et al., 1978, Gorski et al., 1968). Improvements in developing monoclonal antibodies and antigen retrieval have vastly facilitated immuno-staining techniques to allow a change in emphasis on ER from a prognostic indicator to a predictive test of a cancer amenable to treatment with anti-oestrogens. Although ER positive tumours have a 5-10% better chance of disease free survival, it is important to note that around 30% of ER positive tumours do not respond to endocrine therapy (Jordan, 1995).

Progesterone receptor [PR] status often correlates with ER status. However, tumours which are both ER and PR have a better prognosis than those which are only ER positive. The newer biological marker being increasingly measured, particularly in younger women with breast cancer, is the Her-2 receptor, also called c-erb-B2 (Mosselman et al., 1996). The *HER2* (human epidermal growth factor receptor 2) gene is part of a family of genes that play roles in regulating cell growth. A fraction of breast cancers, as part of their development, undergo gene amplification. Instead of having two gene copies of the *HER2* gene in a normal cell, there are multiple copies. As a result, there is far more expression of the *HER2* protein on the cell surface, resulting in aberrant cell growth regulation.

Tumours are faster growing, more aggressive and less sensitive to chemotherapy and hormone therapy.

It is universally recognised that breast cancer patients with the same stage of disease can have a markedly different response to treatment and hence to the final outcome. The strongest predictors for metastases (for example, lymph node status and histological grade) fail to classify accurately breast tumours according to their clinical behaviour. Chemotherapy or hormonal therapy reduces the risk of distant metastases by approximately one-third; however, 70–80% of patients receiving this treatment would have survived without it.

Recent advances have focussed around Gene Expression Profiling, initially known as the 70 Gene Amsterdam Signature (Mook et al., 2007). The intention was to develop a gene expression signature that would enable characterisation of those patients with early breast cancer who were either at high risk or at low risk of recurrence. This would then enable more individualized treatment by a process of risk stratification.

To date, the earlier signatures of breast cancer gene expression did not allow for patient-tailored therapy strategies. More recently, DNA microarray analysis has been performed on primary breast tumours of young patients, and applied supervised classification to identify a gene expression signature strongly predictive of a short interval to distant metastases ('poor prognosis' signature) in patients who were lymph node negative (van de Vijver et al., 2002). In addition, researchers established a signature that identifies tumours of *BRCA1* carriers. The poor prognosis signature consists of genes regulating cell cycle, invasion, metastasis and angiogenesis. This gene expression profile will outperform all currently used clinical parameters in predicting disease outcome. These studies and their results provide a strategy to select patients who would benefit from adjuvant therapy.

There are currently 2 commercially available Gene Expression Profiling Kits: MammaPrint (Agendia Inc., Irvine, California) and Oncotype DX (Genomic Health, Redwood, California). MammaPrint was developed on 10 year outcome data from an untreated breast cancer patient population, thereby ensuring the

validity of the results regardless of the ultimate treatment regimen selected (van 't Veer et al., 2002). Additionally, both oestrogen receptor positive and negative patients were included in this evaluation.

Initially, an unbiased genome-wide approach, in which all 25,000 genes in the human genome were evaluated to isolate the 231 most prognostic breast cancer-specific genes. 70 critical genes were shown to best correlate with the likelihood of distant recurrence using two-dimensional cluster analysis followed by a leave-one-out cross validation procedure. These were selected for the Gene Expression profile. These 70 genes affect all steps known to be important for metastasis alluded to in the aforementioned discussion, including cell cycle regulation, angiogenesis, invasion, cell migration and signal transduction. When a Gene expression profile is generated, it enables classification of a tumour as either high or low risk. When this is used in conjunction with other risk factors, it helps identify patients who will benefit from adjuvant therapy. Compared to the standard risk assessment factors described above, MammaPrint significantly reduces the number of patients traditionally classified with a poor prognosis. Simultaneously it helps identify those patients who may be at increased risk of recurrence despite their clinico-pathologic findings. Because gene expression profiling actually analyzes the gene activity of the tumour itself, individual treatments can be tailored to patients. This results in improved patient compliance and reduced adverse effects, the net result being an improvement in patient quality of life outcomes (Buyse et al., 2006).

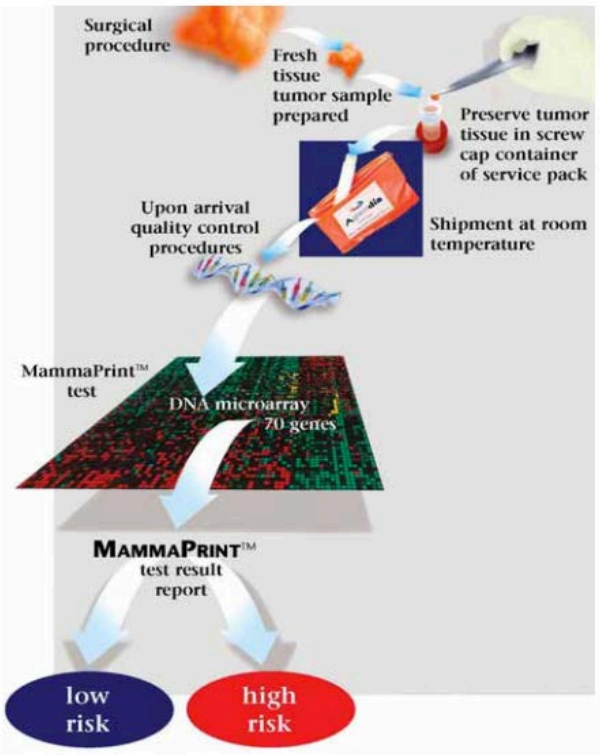


Figure 1. 5 Gene Expression profiling: Adapted from (http://biomedicalcomputationreview.org/)

1.5 Treatment

Treatment overview

The Multidisciplinary Team approach to the management of Breast Cancer has revolutionised the approach to the treatment. The biological spectrum of the disease is incredibly wide, and treatment options and recommendations depend on several factors, including:

- The stage and grade of the tumour
- The tumour's hormone receptor status (ER, PR) and HER2 status
- The patient's age, general health, and preferences
- The patient's menopausal status
- The presence of known mutations in inherited breast cancer genes (*BRCA1* or *BRCA2*)

1.5.1 Surgery

There has been a cascade of growth in the knowledge guiding the care patients with this widely prevalent disease since the observations in the 1800's that surgery could help improve the quality of patients with breast cancer. In its evolution from Halsted to Fisher, surgery continues to be a mainstay of treatment in breast oncology. However, there has always been a difference of opinion with regards to breast cancer being a systemic or local disease at inception. Halsted advocated the Radical Mastectomy in an attempt to gain loco-regional control, reducing his local recurrence rate to 6% (Ghossain and Ghossain, 2009). In recent years, it has been suggested that local therapy is ineffective in reducing breast cancer mortality. This viewpoint has been championed by Fisher, who maintains that "because operable breast cancer is a systemic disease involving a complex spectrum of host-tumor interrelations, local-regional therapy is unlikely to affect survival" (Fisher, 1996). Recently, results from large trials such as National Surgical Adjuvant Breast Project-04 (NSABP-04) and King's/Cambridge trials showed that leaving the axilla untreated in patients having primary breast cancer has no impact on survival (Fisher et al., 1985, Baum et al., 1980). Thus, the axilla does not seem to serve as a nidus for further spread of the cancer, as suggested by Halsted. Additionally, the NSABP-06 randomized patients to lumpectomy, lumpectomy with radiotherapy, or mastectomy and showed that there was an increased risk of local recurrence in the group treated with lumpectomy alone but no difference in survival between the three groups (Fisher et al., 1995). This indicated that the extent of the mastectomy does not influence survival, a conclusion that was inconsistent with the Halstedian view of breast cancer as a locally progressive disease.

In keeping with the history of evolution of breast cancer as a disease, the notion of breast cancer being a systemic disease at presentation is not universally accepted. Indeed, Hellman argues that "persistent disease, locally or regionally, may give rise to distant metastases and, therefore, in contrast to the systemic theory, locoregional therapy is important" (Hellman, 1994). The initial results of the breast cancer screening trials seem to lend weight to the argument of Hellman and others and, at least partially, refute the systemic hypothesis. If breast cancer is indeed systemic at onset, then one can argue that the early diagnosis and timely extirpation of the

primary tumor should have no impact on mortality. Yet, in stark contrast to this, a meta-analysis of eight randomized controlled trials and four case-control studies has shown that breast cancer screening reduces mortality by 30% in women older than the age of 50 (Kerlikowske et al., 1995). Thus, controversy surrounding the impact of local therapy on mortality centers on whether breast cancer is viewed as a systemic or locoregional disease. This debate has been the subject of intense speculation for more than 2000 years, and ongoing research will, no doubt, add weightage to both sides of the argument.

Surgery remains vital in the management of this disease and is vibrantly in evolution. The newer developments have increasingly brought about less invasive procedures and more creative reconstructive techniques. The primary aim of surgery is the removal of tumour and surrounding tissue during an operation. Surgery is also used to examine the nearby axillary lymph nodes. In general terms, the smaller the tumour, the more surgical options a patient has, with the option to perform breast conserving surgery (BCS). Of vital importance in BCS is the margin status, as this has prognostic implications for all patients treated for invasive breast cancer. There exists no consensus on what constitutes a negative margin. As per NICE guideline CG-80, for ductal carcinoma *in situ*, a minimum of 2 mm radial margin of excision is acceptable (http://www.nice.org.uk/CG80, 2009). A recent meta-analysis on margins in early stage breast carcinoma treated with BCS concluded that "increasing the threshold distance for declaring negative margins is weakly associated with reduced odds of local recurrence; however adjustment for covariates (adjuvant therapy) removes the significance of this effect. Adoption of wider margins, relative to narrower widths, for declaring negative margins is unlikely to have a substantial additional benefit for long-term local control in breast conserving therapy" (Houssami et al., 2010).

The types of surgery include the following:

- Wide Local Excision
- Partial or segmental mastectomy or quadrantectomy
- Total Mastectomy
- Modified Radical Mastectomy

- Radical Mastectomy
- Breast Reconstruction

Wide Local Excision

The cancerous area and a surrounding margin of normal tissue is removed. A second incision may be made in order to remove the lymph nodes. This treatment aims to maintain a normal breast appearance when the surgery is over.

After the wide local excision, a course of radiation therapy is usually used to treat the remaining breast tissue. The majority of women who have small, early-stage breast cancers are excellent candidates for this treatment approach.

Partial or Segmental Mastectomy or Quadrentectomy

During a partial or segmental mastectomy or quadrantectomy, more breast tissue is removed than with a wide local excision. As the name suggests, a segment or a quadrant of the breast is removed. The cancerous area and a surrounding margin of normal tissue are removed. Radiation therapy is usually given after surgery.

Simple Mastectomy

With a simple mastectomy, the entire breast is removed, but no lymph nodes are removed. Simple mastectomy is most frequently used to prevent new cancer from developing or when the cancer does not go to the lymph nodes. This procedure is usually performed to treat in-situ or stage IA breast cancers.

Modified Radical Mastectomy

A Modified Radical Mastectomy is a procedure in which the entire breast is removed, including the skin, nipple, areola and most of the axillary lymph nodes, but sparing the pectoralis major. This procedure is usually recommended in patients who have multicentric disease, prior radiation therapy to the breast or chest wall, inflammatory breast cancer, or positive margins after repeat re-excision.

Radical Mastectomy

Also referred to as Halsted's procedure, removes all of the breast tissue including the nipple, areola, lymph nodes and the underlying chest wall muscles. This procedure is rarely performed today as the Modified Radical Mastectomy has proved to be equally effective and less disfiguring. It is now only performed when the cancer has invaded the pectoralis muscle beneath the breast tissue.

Breast Reconstruction

Breast reconstruction is an operation to create a breast shape to match the remaining breast and regain symmetry following mastectomy. In line with guidance issued by NICE in 2002, breast reconstruction should be discussed with all patients who require a mastectomy. This message has been reinforced by the Association of Breast Surgeons and the British Association of Plastic Reconstructive and Aesthetic Surgeons in their Guidelines for best practice, Oncoplastic Breast Reconstruction (www.bapras.org.uk, 2012).

Reconstruction may be performed either at the same time as the initial mastectomy or at a later stage. The decision for immediate reconstruction versus delayed reconstruction will depend on a number of clinical issues (adjuvant treatment plans) as well as patient choice. There are different techniques used to reconstruct a breast which include either implant-only reconstruction or 'autologous' reconstruction using the patient's own tissue or a combination of both these methods.

In a huge undertaking, the United Kingdom National Mastectomy and Breast Reconstuction Audit (NMBRA) prospectively evaluated data between January 2008 and March 2009. It looked primarily at complications and patient reported outcomes from data submitted from more than 200 centres. Nearly 17000 women underwent mastectomy, with 21% undergoing immediate reconstruction, and 11% having delayed reconstruction. The results from the audit- gleaned from questionnaires completed at baseline, 3 and 18 months-found that patients who chose delayed reconstruction were happier with their outcome (Thiruchelvam et al., 2013).

Management of the Axilla

Axillary dissection was first advocated as part of the treatment of invasive breast cancer in the 18th century by Heister (Meyer et al., 1984). This is a surgical procedure that incises the axilla to identify, examine, or remove lymph nodes. Axillary dissection has been the standard technique used in the staging and treatment of the axilla in breast cancer. Axillary lymph node status is a significant prognostic pathologic variable in patients with operable primary breast cancer, and it remains the most powerful predictor of recurrence and survival.

Over the last century, there has been a significant evolution in the management of the axilla in breast cancer patients. Prognostically important information is derived from the status of the axillary nodes. As opposed to Halsted's beliefs, we now know that occult systemic metastases and tumour biology rather than surgical clearance of the axilla determine long-term survival after breast cancer treatment. The individual patient's clinical stage, patient and tumour characteristics, and treatment preferences should determine the management of the axilla.

With the understanding that breast cancer does not always spread sequentially from the breast to successive axillary lymph node levels, to distant sites, emerged an increased emphasis on systemic therapy and a focus towards less radical therapy in the axilla. The National Surgical Adjuvant Breast and Bowel (NSABP) B-04 trial-a prospective, randomised controlled trial- was pivotal in the trend toward individualised management of the axilla in women with breast cancer. The three arms of this study compared (1) radical mastectomy to (2) simple mastectomy with chest wall and axillary nodal radiation to (3) simple mastectomy alone. The ground breaking conclusions were that there was no significant difference in overall or disease-free survival among the 3 treatment arms at any point up to 25 years of follow-up. While the local and regional recurrence rate was highest in those who underwent total mastectomy alone (13 %), the local regional recurrence rate in the radical mastectomy group (9%) was not much lower, and was lowest of all in those who underwent total mastectomy with axillary irradiation (5 %), i.e., no axillary surgery (Fisher, 1977).

The National Surgical Adjuvant Breast Project B-04 trial concluded that axillary dissection has no effect on survival, and this has influenced thinking about the role and benefits of this procedure. However, other studies provided substantial evidence that axillary dissection provides excellent local control of disease in the axilla, with a local recurrence rate of 2% or less (Veronesi et al., 1998, Cabanes et al., 1992) which may lead to improved overall survival (Diab et al., 1998, Haffty et al., 1997).

Axillary dissection should be reserved for patients with proven axillary disease preoperatively or with a positive sentinel node biopsy. Axillary dissection is only therapeutic in patients who are node positive. Therefore, performing axillary dissection in all patients would lead to overtreatment of a large proportion of patients who are node negative. The anatomic disruption caused by axillary dissection may result in lymphoedema, nerve injury, and shoulder dysfunction, which compromise functionality and quality of life. The introduction of sentinel-node biopsy has enabled us to establish the status of the axilla with considerably less morbidity for patients when compared with complete axillary dissection.

Sentinel Lymph Node Biopsy (SLNB)

In 1992, Morton -working with cutaneous melanoma- described the technique of sentinel node localisation, based on the notion that the first lymph node draining the primary tumour reflects the status of the regional lymph node basin (Morton et al., 1992). In 1994, Giuliano et al. reported on the feasibility of lymphatic mapping and sentinel node biopsy (SNB). In this study with no prior technique established, SNB had a positive predictive value of 96 % and false negative rate of 11.9 % (Giuliano et al., 1994). Further studies further confirmed the applicability of SNB using a variety of techniques: subareolar (Kern, 1999), peritumoral (Klimberg et al., 1999), or intradermal injection (Povoski et al., 2006) using blue dye alone, blue dye in combination with technetium- 99 m labelled sulfur colloid, or radiolabelled colloid alone. Among inexperienced surgeons and others, the highest success rates with SNB are achieved when both blue dye and technetium-99 m labelled sulfur colloid are used together (Rodier et al., 2007). Irrespective of the technique used, between 1 and 3 lymph nodes are usually removed during SNB. The Axillary Mapping Against Nodal Axillary Clearance (ALMANAC)

study found that 99.6 % of lymph node metastases were identified within the first 4 sentinel nodes removed (Goyal et al., 2005).

The Sentinel Lymph node (SLN) technique is based on the observation that tumour cells metastasize to one or a few lymph nodes before involving other lymph nodes. In patients with clinically node negative breast cancer, SLNB identifies patients without axillary node involvement, thereby obviating the need for more extensive surgery (Mabry and Giuliano, 2007). A systematic review, performed by the American Society of Clinical Oncology expert guidelines panel, included 69 eligible trials of SLND in early stage breast cancer, representing 8059 patients (Lyman et al., 2005). The study concluded that SNB is an appropriate initial alternative to routine staging Axillary Lymph Node Dissection (ALND) for patients with early-stage breast cancer with clinically negative axillary nodes. Completion ALND remains the standard treatment for patients with axillary metastases identified on SNB. Appropriately identified patients with negative results of SNB, when done under the direction of an experienced surgeon, need not have completion ALND.

Traditionally, patients with involved nodes have been offered further therapy, either by subsequent complete axillary dissection or axillary radiotherapy. There is an increased awareness that there exists a cohort of patients whose risk of having residual lymph node metastases is so small that they can be spared axillary clearance or completion axillary lymph node dissection. In light of this, a number of algorithms have been developed, and these continue to be refined in light of new evidence. Significant among these is the Z0011 study (Giuliano et al., 2011) which has challenged the need for axillary dissection in patients with one or two positive sentinel lymph nodes. This study -on patients who had breast-conserving surgery and on sentinel node biopsy had one or two positive nodes- randomised them to either axillary dissection or no subsequent axillary surgery. All patients had whole-breast radiotherapy and 58% in both arms had adjuvant chemotherapy, 47% in both arms had hormonal adjuvant therapy. Local recurrence and overall survival at a median of 6.3 years were not significantly different.

Similar findings were reported in the IBC SG23-01 trial (Galimberti et al., 2013). This multi-centered randomised controlled trial looked to see if patients with

micro-metastatic disease (less than or equal to 2mm) might be over treated if subjected to axillary dissection. The 5 year disease free survival – which was the primary endpoint - was 87.8% in the group without axillary dissection, and 84.4% in the group with axillary dissection. This suggests that axillary dissection, and its attendant morbidity, could be avoided in patients with early breast cancer and limited sentinel lymph node involvement.

This lack of benefit of axillary clearance in such patients raises questions about the routine use of intraoperative node assessment. After Z11 and SG23-01, new algorithms need to be developed to ensure that there is consistency of axillary node management between units in patients with disease that would have made them eligible for entry into Z11. The available current evidence cannot support routine axillary dissection for all patients with positive axillary nodes.

1.5.2 Radiation Therapy

Post-operative radiotherapy following breast conserving surgery or mastectomy has been shown to reduce the risk of loco regional recurrence. Randomised Clinical trials in Canada and the Netherlands have also shown that post mastectomy radiotherapy has a beneficial effect on survival (Ragaz et al., 1997, Overgaard, 1999).

Traditionally, standard schedules of curative radiotherapy for a wide range of cancers deliver multiple small daily doses, called “fractions”. These are gentler on healthy tissues than they are on the cancer. However, if cancer is to be eradicated a high total dose has to be delivered. This way of treating cancer certainly works for squamous carcinomas of the head and neck, lung and cervix.

The development of a nationwide initiative called the Standardisation of Breast Radiotherapy (START) Trial-following on from the results of the Royal Marsden Hospital/Gloucestershire Oncology Centre Trial (RMH/GOC) - came about to adequately assess efficacy as well as safety of the hypothesis that a lower total dose in fewer, larger fractions would be just as safe and effective in women with breast cancer.

The Standardisation of Breast Radiotherapy (START) Trials led by Professors John Yarnold and Judith Bliss was the largest evaluation of radiotherapy

fractionation ever undertaken in women being treated for early breast cancer (Bentzen et al., 2008). In START-A, a regimen of 50 Gy in 25 fractions over 5 weeks was compared with 41·6 Gy or 39 Gy in 13 fractions over 5 weeks. The use of two dose levels of the 13-fraction schedules made it possible to directly estimate the fractionation sensitivity of breast cancer and late- responding normal tissues. The START A trial was significant because it challenged the traditional view of radiotherapy treatment for early breast cancer. Meta–analysis of the data generated from START A in conjunction with data from the RMH/GOC trial suggests that continuing to use small fractions of ≤ 2.0 Gy spares the cancer as much as the healthy tissue, which is of no patient benefit. Meanwhile, START B randomised patients with early breast cancer to receive either 50 Gy in 25 fractions of 2.0 Gy over five weeks or 40 Gy in 15 fractions of 2.67 Gy over three weeks. The researchers concluded that, after surgery for early breast cancer, a radiotherapy schedule delivering 40 Gy in 15 fractions over three weeks seems to offer local-regional tumour control and rates of late normal tissue effects at least as good as the accepted international standard of 50 Gy in 25 fractions over five weeks.

Since the START results were published in March 2008, this 3 week schedule has largely replaced the 5 week schedule. Ten years on the principal results of the START trials have now been reported and published recently (Haviland et al., 2013). This long-term follow-up confirms that appropriately dosed hypo fractionated radiotherapy is safe and effective for patients with early breast cancer. The results support the continued use of 40 Gy in 15 fractions, which has already been adopted by most UK centres as the standard of care for women requiring adjuvant radiotherapy for invasive early breast cancer. Similar results were mirrored other studies; it has been shown that ten years after treatment, accelerated hypo fractionated whole breast irradiation was not inferior to standard radiation treatment in women who had undergone breast conserving surgery for invasive breast cancer with clear surgical margins and negative axillary nodes (Whelan et al., 2010).

1.5.3 Chemotherapy

The use of cytotoxic drugs in the treatment of breast cancer has exponentially expanded since the first studies detailing the use of nitrogen mustard and folates as anticancer drugs. Evidence to support the use of adjuvant chemotherapy comes from the Early Breast Cancer Trialists Collaborative group (EBCTCG). The EBCTCG meta-analysis in 2011 suggested that there was a significant improvement in risk of recurrence, reduction of breast cancer mortality and reduction in overall mortality using either the cyclophosphamide-methotrexate-5 fluorouracil (CMF) or anthracycline based regimens (Peto et al., 2012).

It is important to note that there is no single standard adjuvant chemotherapy regimen in the treatment of breast cancer. This is because breast cancer is a phenotypically diverse and heterogeneous disease, composed of various biologic subtypes which have distinct responses to therapy. The regimen selected will take into account various tumour specifics such as tumour histology, expression of oestrogen and progesterone receptors, tumour size and nodal status.

Neo adjuvant-or preoperative- therapy refers to the systemic treatment of breast cancer prior to definitive surgical therapy. Whereas the goal of all systemic therapy with non-metastatic invasive breast cancer is to reduce the risk of distant recurrence, the primary objective of neoadjuvant therapy is to improve surgical outcomes in patients where a primary surgical approach is not technically feasible or for patients with operable breast cancer who desire breast conservative surgery who would otherwise need a mastectomy, or where a partial mastectomy would leave them with a poor cosmetic result (Kaufmann et al., 2006, Gralow et al., 2008). It is of critical importance that patients receiving neoadjuvant systemic therapy are followed up by clinical examination at regular intervals during treatment to ensure that there is no disease progression.

Evidence in support of neo adjuvant chemotherapy (NACT) came initially from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-18 trial. The two main findings in NSABP B-18 however were (1) no difference in overall survival and disease-free survival between pre- and postoperative chemotherapy; (2) patients achieving a pathologic complete response (pCR) had a superior

disease free survival (DFS) and overall survival (OS) compared to patients not achieving a pCR. Additionally, there was a trend in favour of neoadjuvant chemotherapy compared with adjuvant therapy for OS and DFS in women younger than 50 years (Rastogi et al., 2008). A meta-analysis by Mauri and colleagues of nine randomised studies comparing adjuvant with neoadjuvant therapy found no difference with regard to death, disease progression, or distant disease recurrence (Mauri et al., 2005).

Compared to the classical adjuvant treatment, NACT offers several advantages. It provides an opportunity to monitor response during treatment and allows modification or discontinuation of treatment in case of non-responsiveness. Even if an advantage by changing therapy has not yet been proven, it helps avoid the toxicity of an ineffective treatment. Unsurprisingly, the demonstration of treatment efficacy motivates patients to continue therapy despite toxicities. The rate of breast conservation can be increased, and in case of breast conserving therapy, the extent of surgery can be reduced. Importantly, primarily inoperable tumours can be downsized allowing a curative intervention. The residual cancer burden (RCB) is a powerful prognostic marker, sometimes changing the initial prognostic profile. From an ongoing research perspective, in neoadjuvant trials predictive markers, tumour biology, mechanisms of resistance, and new treatment approaches can be investigated more rapidly and with fewer patients than in adjuvant studies.

Currently, interest is increasing in a new design of trial: the so-called window-of-opportunity trial. In this case, a short course of targeted therapy is administered prior to surgical resection or prior to standard therapy. The endpoint of such a trial is not necessarily response rates, but changes of biological markers, for example, for proliferation or apoptosis. The purpose of Window trials could be to prove the expected mechanism of action, to identify tumour resistance and sensitivity, or to establish a “biologically effective” dose of the investigated targeted agent. A hypothesis-generating trial like this was done with metformin in operable breast cancer, where metformin was given twice daily for a median of 18 days prior to surgery. The proliferation biomarker Ki-67 staining in invasive

tumour tissue decreased significantly and TUNEL staining increased (Niraula et al., 2012).

The POETIC (Perioperative Endocrine Therapy for Individualising Care) trial is based on the hypothesis championed by Fisher that endocrine therapy given before primary therapy for breast cancer might improve outcomes. This is the primary end point of the study. In this trial, postmenopausal patients with ER positive tumours are randomised to a non-steroidal aromatase inhibitor (either letrozole or anastrozole) or to no treatment for two weeks before and for two weeks after excision. The overexpression of human epidermal growth factor receptor type 2 (HER2, also called ErbB-2, was first described more than two decades ago (Schechter et al., 1984) and occurs in 20 to 30% of invasive breast carcinomas. Broadly speaking, patients with breast-cancer cells that overexpress HER2 have decreased overall survival, and may respond differently to a variety of chemotherapeutic and hormonal agents (Albanell et al., 1996, Ellis et al., 2001). This underlies the principle behind strategies targeting HER2, recognising its importance in breast cancer. The humanized monoclonal antibody trastuzumab (Herceptin, Genentech) is one such medication.

Trastuzumab binds to the extracellular juxta-membrane domain of HER2 and inhibits the proliferation and survival of HER2-dependent tumours. An intracellular tyrosine kinase domain exists for HER2. Phosphorylation of the tyrosine kinase domain by means of homodimerization or heterodimerization induces both cell proliferation and survival signalling. Cleavage of the extracellular domain of HER2 leaves a membrane-bound phosphorylated p95, which can activate signal-transduction pathways. Binding of trastuzumab to a juxta-membrane domain of HER2 reduces shedding of the extracellular domain, thereby reducing p95. Trastuzumab may reduce HER2 signalling by physically inhibiting either homodimerization or heterodimerization. It is postulated that trastuzumab may recruit Fc-competent immune effector cells and the other components of antibody-dependent cell-mediated cytotoxicity, leading to tumour-cell death. Additionally, mechanisms such as receptor down-regulation through endocytosis have been proposed (Hudis, 2007).

The study by Slamon et al was a pivotal randomized clinical trial that showed the activity of trastuzumab in combination with chemotherapy with previously untreated, HER2-positive, metastatic breast cancer (Slamon et al., 2001). Patients received first-line chemotherapy either alone or in combination with the antibody. The primary end point of this study was time to disease progression, which increased from 4.6 months among patients who received chemotherapy alone to 7.4 months among those who received trastuzumab in addition to chemotherapy. The study also demonstrated an improved overall survival. Subsequently the results from the M77001 group- a randomized trial of docetaxel alone or with trastuzumab had similar results (Marty et al., 2005).

Trastuzumab should be considered for the management of all metastatic breast cancers with HER2 overexpression. Patients with moderate- to high-risk, rapidly progressive cancer characterized by a negative hormone-receptor status, extensive visceral metastases, and a short disease-free interval (typically less than 2 years) are candidates for immediate treatment with chemotherapy and should receive the appropriate agent (or agents) with trastuzumab. The chemotherapy regimen of choice should be predicated on the patient's previous adjuvant therapy and coexisting conditions. Trastuzumab does not appear to diminish the quality of life in patients who are already receiving concurrent chemotherapy, although prospective quality-of-life testing is not available. It is not clear whether antibody therapy should precede, follow, or be added to hormone therapy for the subgroup of patients with HER2-positive and hormone-receptor–positive disease. Since trastuzumab monotherapy appears to be effective for the treatment of metastatic breast cancer, its use as a single agent for newly discovered metastatic disease can be considered (Piccart, 2001). This strategy would delay the initiation of chemotherapy with its attendant side effects, possibly resulting in a better quality of life.

Adjuvant Therapy for Early-Stage Breast Cancer A high rate of pCR has been demonstrated in patients with HER2 positive breast cancer treated with NACT, particularly tumours that are hormone-receptor negative, thus reflecting the aggressive nature of these tumours (Esserman et al., 2012). Significantly, a pCR after chemotherapy followed by HER2 directed therapy is associated with

recurrence and survival advantages. A recent meta-analysis confirmed an increased event free survival in HER 2 positive patients who achieved a pCR with NACT who concurrently received chemotherapy (Cortazar et al., 2012). The TECHNO (Taxol, Epirubicin, Cyclophosphamide, Herceptin Neoadjuvant) trial confirmed a pCR of 39 percent, and showed an improvement in both event free and overall survival in patients who demonstrated a pCR (Untch et al., 2011).

1.5.4 Endocrine therapy

Breast tissue possesses high affinity protein receptors for oestrogen and progesterone and these play key roles in both normal breast cancer development and in breast cancer progression. These receptor proteins become activated when they are occupied by their specific hormone ligand. Activation of oestrogen receptor leads to the induction of numerous cellular genes, including those that may encode critical enzymes and secrete peptide growth factors.

Surgically induced menopause in the form of bilateral oophorectomy became the first effective means to control advanced breast cancer. Over the last two decades, endocrine organ ablation has been replaced by hormonal therapy-either antioestrogen therapy or by the use of aromatase inhibitors. Breast cancers are said to be hormone sensitive if they are either Oestrogen receptor positive (ER+), or progesterone receptor positive (PR+). This is assessed by measuring the ER and PR receptor status of the tumour using immunohistochemistry. In general, if greater than 10% of tumour cells stain positive for the relevant nuclear receptor, the assay is reported as positive, and a response to hormonal therapy is likely. The majority of human breast cancer contain hormonal receptor either oestrogen or progesterone, or both. Approximately 75% of primary breast cancers express ER, and over half of this also expresses PR (McGuire, 1978). The presence of oestrogen receptor predicts the clinical response to all types of endocrine therapies including the hormonal therapy. Treatment aimed at inhibiting the action of ER represents a highly successful example of targeted therapy for clinical breast cancer (Campos and Winer, 2003).

ER positive tumours have a 5-10% better chance of survival than ER negative tumours. Patients with ER positive tumours respond well to anti-oestrogen

treatment with better clinical outcome. Patients with both ER and PR positive have slightly better prognosis than those with ER positive but PR negative tumours. The introduction of Tamoxifen and aromatase inhibitors has simplified hormonal manipulation for the treatment of breast cancers.

Tamoxifen is an oestrogen receptor modulator and acts as a weak agonist/ antagonist. It acts a competitive antagonist of oestrogen activity in breast tissue, but not in the other oestrogen sensitive tissues. Tamoxifen reduces the risk of local recurrence by 25% and mortality by 17 percent in ER/PR positive breast cancer patients. Aromatase inhibitors are agents that block the conversion of androstenedione to oestrone by inhibiting the aromatase enzyme. There is emerging evidence that aromatase inhibitors are at least as effective as tamoxifen in reducing the risk of recurrence in breast cancer with less adverse effects.

The Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial, a double-blind randomised trial, compared the aromatase inhibitor anastrozole alone with tamoxifen alone, or the combination, as adjuvant therapy with localised breast cancer (Baum et al., 2002). Initial analyses of the ATAC trial at 33 and 47 months of median follow-up showed that anastrozole significantly prolonged disease-free survival and time-to-recurrence, and reduced the incidence of contralateral breast cancer, compared with tamoxifen. The combination treatment arm was closed because of low efficacy demonstrated at the above analyses. The conclusion from the trial was that anastrozole should be the preferred initial treatment for postmenopausal women with localised hormone-receptor-positive breast cancer.

More recently, the long-term outcomes in the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial that compares anastrozole with tamoxifen were published (Forbes et al., 2008). At a median follow-up of 100 months, disease free survival, time to recurrence, time to distant recurrence, and the incidence of contralateral breast cancer were improved significantly in the hormone-receptor-positive populations. Fracture rates were higher in patients receiving anastrozole than in those receiving tamoxifen during active treatment, but were not different after treatment was completed. No significant difference in risk of cardiovascular morbidity or mortality between anastrozole and tamoxifen treatment groups was noted. The study establishes clearly the long-term efficacy of anastrozole

compared with tamoxifen as initial adjuvant treatment for postmenopausal women with hormone-sensitive, early breast cancer. Additionally, it shows a safety profile and provides statistically significant evidence of a larger carryover effect after 5 years of adjuvant treatment with anastrozole compared with tamoxifen.

1.6 Tumour Metastasis

1.6.1 The biology of tumour metastasis

Metastasis refers to the transfer of disease from one part of the body to another. In cancer, metastasis is the migration of cancer cells from the original tumour site through the blood and lymph vessels to produce cancers in other tissues. Metastasis is also the term used for a secondary cancer growing at a distant body site.

Metastasis is responsible for a large proportion of deaths related to cancer. With the growth of the primary tumour, cells begin to dissociate and travel around the body to settle at different sites. They then begin to replicate to form “secondaries”. The process may then repeat itself, and this is referred to as the “metastatic cascade”. There are various steps involved in this process (Jiang and Mansel, 2000). For dissemination to occur, there must be a reduction in the adhesiveness between the cells and also between the cells and the extra cellular matrix. Subsequent migration of the cells through the matrix and vessel walls allows for dissemination via either the haematogenous or lymphatic routes. This migration is dependent upon stable adherence and traction produced by adhesion molecules and their ligands. Arrest in distant sites may be facilitated by the adherence of tumour cells to lymphoid cells/platelets resulting in larger aggregates composed of many cells, thereby increasing the likelihood of entrapment in capillaries (Gasic et al., 1973). Survival and growth of the metastatic deposit will only occur if there is a suitable environment [nutrients and extracellular matrix substrate].

Cell adhesion is mediated by several families of cell-surface expressed molecules including the cadherins (Stappert and Kemler, 1993), immunoglobulin super family (Simmons, 1999), selectins (Tedder et al., 1993) and the integrins (Hynes,

1992). Metastasis is also dependant on the development of new blood vessels [angiogenesis] and new lymphatics [lymphangiogenesis]. When a primary tumour first appears, proliferation of cancer cells may be balanced by apoptosis [programmed cell death] so that the tumour may remain static for years (Hanahan and Folkman, 1996). This gives rise to another concept of micrometastatic disease; i.e., an initial high rate of apoptosis in the metastatic deposits may help keep them clinically undetectable for years, until the rate of apoptosis is exceeded by the rate of proliferation. This phenomenon of tumour dormancy may depend on the rate-limiting role of neo-vascularisation (Holmgren, 1996).

In breast cancer, the initial period of tumour dormancy is usually long, and tumours are usually in the *in situ* stage when neo-vascularisation occurs (Brem et al., 1978). Clinical and pathological observations have for long suggested that the most common pathway for initial dissemination is via the lymphatics, with patterns of spread via afferent vessels following natural routes of lymph drainage (Cotran et al., 1999).

1.6.2 Breast Cancer Metastasis

Metastatic disease in breast cancer is common. At the time of initial diagnosis, 7% of women with breast cancer will have metastases (Mansel et al., 2000). Even in women with no evident metastases who undergo potentially curative surgery, 20-30% will develop widespread metastases (Wingo et al., 1995); this figure rises to 50-60% in those with positive axillary nodes. This has led some to argue that breast cancer is a systemic disease at the time of presentation (Fisher, 1977).

The rate of tumour growth decreases as the size of the tumour increases (DeWys, 1972). This is because large tumours have inhibiting factors, which result in a larger number of cells being in a dormant state. This applies to both primaries and secondaries. An extension of this theory is that the rate of growth of the metastases may increase when the primary is removed. This would make metastatic foci particularly vulnerable to chemotherapy after surgery for the primary. An alternative theory is that of micrometastatic dormancy, whereby the rate of growth decreases after the initial growth spurt, only to be followed by a delayed increase in the rate of growth (Demicheli et al., 1997). The trigger for

this sudden activity has not been established yet. Recently, it has been shown that angiogenesis may act as one such trigger and that VEGF and VEGF-1 may be key to this event (Kaplan et al., 2005).The VEGF serum level may be one of the important indexes for monitoring forecast of therapy efficiency and disease prognosis. The late occurrence of local recurrence and distant metastases lends credence to this theory.

W.S. Halsted was the first to put forward his theory of the natural history of breast cancer. He suggested that the disease remains localised at its site of origin up-to the point when lymphatic invasion takes place and then it becomes loco-regional. After a further indeterminate interval, vascular invasion and dissemination occur, resulting in metastatic disease. This concept was the basis for Halsted's radical mastectomy, as the chance of cure was thought to be better with the amount of tissue excised. Fisher's hypothesis proposed that breast cancer is a systemic disease by the time of clinical presentation (Fisher, 1977). Controversially, it was proposed that a delay in diagnosis of breast cancer does not reduce survival (Fisher, 1988). The first tumour cell multiplies exponentially with time: 1-2-4-816-32 and so forth. For a tumour to reach a size of $1cm^3$, it will consist of 10^9 cells. According to the Gompertzian model, tumour growth over time follows a sigmoid curve (Demicheli et al., 1997). As the tumour grows, the doubling time increases and the growth fraction decrease. The maximum growth rate is at approximately one third of maximal tumour volume, and slows to a plateau near lethal tumour volume. When plotted as a growth curve, there exists an upper horizontal asymptote, which for human malignant tumours, is the upper limit that the cancer cannot exceed because the tumour burden has become lethal to the host. This limit is conceptualized as the "lethal burden". A 1cm or $1cm^3$ tumour has already undergone 30 doublings as compared to the lethal tumour burden of 40 doublings (Khonji et al., 2000). Hence, it is argued that dissemination is likely to have already occurred. The failure of loco regional surgery to prevent metastasis is further evidence in support of this theory. Contrary to this, data from screened trials indicate a 30% decrease in mortality (Tabar et al., 1985). This suggests that surgery in such patients with impalpable cancers may remove the primary before any viable metastases have occurred. Continuing studies and

analysis of data from prospective screening should shed further light on this ongoing debate.

Breast cancer shows a predilection to metastasise to bone, liver and lungs. Other sites are the brain, adrenal gland, ovaries, peritoneal cavity and thyroid. The bones are the most common site for metastases. The bony deposits cause an increase in osteoclastic activity-this is reflected as bony destruction and hypercalcaemia in 10-15% of patients (Body, 1995). One theory postulated for this is the inherent capacity of the cancer cells themselves to cause bony destruction (Eilon and Mundy, 1978). Another theory suggests that the tumour cells produce a local peptide such as parathyroid hormone related peptide [PTHrP] (Vargas et al., 1992). In its turn, PTHrP is regulated by one of the growth factors TGF-P [Transforming Growth Factor P] (Yin and Stull, 1999). Liver is the second most common site to be affected by breast cancer metastases, and its involvement usually carries a poor prognosis. Local growth factors have been implicated in liver involvement; these are believed to induce preferential growth of these tumour cells. Insulin-like growth factor has been shown to be present in liver. This itself is a growth and motility factor for breast cancer (Nicolson, 1993).

1.6.3 Tumour Suppressor Genes

A tumour suppressor gene or antioncogene is a gene that protects a cell from one step on the path to cancer. When this gene is mutated to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes.

Cell growth is regulated by tumour suppressor genes which are normally present within the cell (Cuschieri et al., 2002). They exert control by slowing down the cell cycle and encode protein products that normally exert a negative control on cellular growth and division, or are integrally involved in apoptosis. Damage to these genes may result in unregulated cell growth or failure to direct DNA damaged cells to a programmed death. Thereby, a loss, inactivation or malfunction of tumour suppressor genes may result in uncontrolled cell proliferation. The net result is a dysregulated proliferation of cloned cells with nuclear damage, possibly resulting in neoplastic growth (Cuschieri et al., 2002).

Unlike oncogenes, tumour suppressor genes generally follow the 'two hit hypothesis,' which implies that both alleles that code for a particular gene must be affected before an effect is manifested. This is due to the fact that if only one allele for the gene is damaged, the second can still produce the correct protein. In other words, tumour suppressors are usually recessive as opposed to oncogenes which are generally dominant. The two hit hypothesis was first proposed for cases of retinoblastoma (Knudson, 1971). It was observed that the age of onset of retinoblastoma followed 2nd-order kinetics, implying that two independent genetic events were necessary. He recognized that this was consistent with a recessive mutation involving a single gene, but requiring biallelic mutation. Oncogene mutations, in contrast, generally involve a single allele because they are gain of function mutations. There are notable exceptions to the 'two hit' rule for tumour suppressors, such as certain mutations in the p53 gene product. p53 mutations can function as a 'dominant negative', meaning that a mutated p53 protein can prevent the function of normal protein from the un-mutated allele. Other tumour suppressor genes which are exceptions to the 'two-hit' rule are those which exhibit haploinsufficiency. An example of this is the p27Kip1 cell cycle inhibitor, in which mutation of a single allele causes increased carcinogen susceptibility (Fero et al., 1998).

Tumour suppressor genes, or more precisely, the proteins for which they code, either have a dampening or repressive effect on the regulation of the cell cycle or promote apoptosis, and sometimes do both. The functions of tumour suppressor proteins fall into several categories including the following:

1. Repression of genes that are essential for the *continuing* of the cell cycle. If these genes are not expressed, the cell cycle will not continue, effectively inhibiting cell division (Sherr, 1994).
2. Coupling the cell cycle to DNA damage. As long as there is damaged DNA in the cell, it should not divide. If the damage can be repaired, the cell cycle can continue.
3. If the damage cannot be repaired, the cell should initiate apoptosis (programmed cell death) to remove the threat it poses for the greater good of the organism.

4. Some proteins involved in cell adhesion prevent tumour cells from dispersing, block loss of contact inhibition, and inhibit metastasis. These proteins are known as metastasis suppressors (Hirohashi and Kanai, 2003, Yoshida et al., 2000).

The first tumour suppressor protein discovered was the Retinoblastoma protein (pRb) in human retinoblastoma. The retinoblastoma gene-which codes the pRb-can be considered a model for a class of recessive human cancer genes that have a "suppressor" or "regulatory" function. The loss or inactivation of both alleles of this gene appears to be a primary mechanism in the development of retinoblastoma (Murphree and Benedict, 1984). However, recent evidence has also postulated pRb as a tumour survival factor (Goodrich, 2006). Another important tumour suppressor is the p53 tumour suppressor protein encoded by the TP53 gene. p53 is a nuclear phosphoprotein transcription factor that controls the expression of proteins involved in the cell cycle. Mutations in p53 are found in a large percentage of human cancers (Morris and Wood, 2000). Homozygous loss of p53 is found in 70% of colon cancers, 30-50% of breast cancers and 50% of lung cancers. Mutated p53 is also involved in the pathophysiology of leukaemias, lymphomas, sarcomas and neurogenic tumours. Abnormalities of the p53 gene can be inherited in Li-Fraumeni syndrome (LFS), which increases the risk of developing various types of cancers. Cells with p53 mutations are more resistant to the effects of radiation and chemotherapy since producing lethal abnormalities in DNA is a critical step in their mode of action (Morris and Wood, 2000).

Abnormalities in *BCL2* and *Bax* also occur in some tumour types and cause many of the same effects as p53 mutations. The restoration of wild type p53 expression in cells with defective p53 is sufficient to cause growth arrest or even apoptosis. There is mounting evidence that expression of wild type p53 in established tumours can induce apoptosis *in vivo* as well. This has been accomplished using retroviral, adenoviral and non viral gene delivery vectors expressing wild-type p53. The induction of apoptosis by certain chemotherapeutic agents may require the presence of wild type p53. Transfection of the wild-type p53 gene into a variety of human tumor cells was shown in the late 1980s and early 1990s to induce apoptosis and growth inhibition. In lymphoma models, when this approach

was used, the activity of p53 that induced antitumor activity was seen to be apoptosis (Ventura et al., 2007) whereas, in a liver tumor model, the p53 activity induced a senescent phenotype. Dramatically, this was shown to then induce an intense macrophage response that cleared the tumor (Xue et al., 2007).

In 1996, Jack Roth was credited with being the first to attempt p53 gene therapy in man. He used direct injection of a retroviral vector expressing human p53 under the control of an actin promoter to treat non-small cell lung carcinoma (Roth et al., 1996). Later studies identified adenovirus vectors expressing human full length wild-type p53 as suitable for large scale production. In vitro studies showed that such viruses could infect and inhibit the growth of many different human tumor cells. Remarkably such viruses did not seem to induce apoptosis or senescence in normal tissues or cells. The virus effectively results in a burst of p53 production in the infected cell and remarkably normal cells can recover from this process. In many tumor cells, however, an irreversible induction of apoptosis takes place Thus p53 gene therapy has an excellent safety profile.

Unlike retrovirus based systems that have proved to be oncogenic in man, the adenovirus delivery system has the advantage that it does not result in integration of the vector DNA into the host cell. Using this initial approach has enabled many thousands of patients in clinical trials primarily in China and the United States to receive p53-based gene therapies. In 2003, the use of adenovirus gene therapy for the treatment of head and neck cancer in combination with radiation was approved in China and the product “Gendicine” has been marketed since then. An analysis of 2500 patients treated by Gendicine has been published by Shi and Zheng (2009).

1.7 Wiskott Aldrich Syndrome and Wiskott Aldrich Syndrome Protein

The Wiskott Aldrich Syndrome [WAS], also known as Wiskott Aldrich Huntley syndrome and as Werlhof’s disease bears the name of the two authors who are credited with first describing it-Wiskott in Germany in 1937 and Aldrich in the United States in 1954 (Aldrich et al., 1954). In its original descriptions, it was

characterised by an illness manifesting as eczema, thrombocytopenia, susceptibility to infection, and bloody diarrhoea, with death usually occurring before ten years of age. Subsequently, the main causes for death were found to be infection and bleeding, and also due to the development of malignancies such as lymphoreticular tumours, leukaemia and reticuloendothelial system malignancies (Bensen et al., 1996, Perry et al., 1980, Sullivan et al., 1994). Currently, it is characterised by the triad of recurrent bacterial infections of the sinuses and lungs, eczema that appears atopic in nature, and a bleeding tendency due to thrombocytopenia and platelet dysfunction. Less than a third has the full triad, but 90% present with the features of thrombocytopenia. Around 5% have only infection and 20% only haematological problems.

Two major complexes that are linked to the NWASP family, namely the ERM family and Rho GTPases are aberrantly expressed in human breast cancer (Jiang et al, 2003, Martin et al, 2003). Additionally, X chromosome inactivation which silences gene expression from one of the two X chromosomes in females is usually random. This skewed (nonrandom) pattern of inactivation has been observed in a wide range of neoplastic tissues (Busque and Gilliland, 1998) and can be considered a consequence of the monoclonal origin of the neoplasia. Nonrandom X-chromosome inactivation is frequent in the somatic tissue of females with invasive ovarian cancer and BRCA1 mutations (Buller et al., 1999). Individuals with WAS are known to have skewed X inactivation. In addition, they are more susceptible to certain forms of malignancy, primarily haematological. This formed the basis of the present study, which sought to elucidate the role of WAS protein in human breast cancer, and to determine if it plays a role as a tumour suppressor

The incidence is about 1 in 250 000 male births or 1 in 500,000 births overall. Being X linked and potentially lethal, it would be expected to be almost invariably in males, but females have been reported in the literature. Females usually have no family history and so are presumable spontaneous mutations. In some cases, females have been shown to have non-random inactivation of the X chromosome bearing the functional WAS allele. The WAS gene is located on the Xp 11.22-23 region of the X chromosome and is linked as an X linked recessive condition.

However, recently, an apparently autosomal dominant form has been described, but this is in 3 generations of a single family. A male child of a female carrier has a 50% chance of being affected, a female child has a 50% chance of being a carrier (Schwaber and Rosen, 1990).

The discovery of the causative gene has revealed a spectrum of clinical types demonstrating a strong genotype/phenotype correlation. Four clinical phenotypes are seen: classic WAS, X-linked thrombocytopenia, intermittent thrombocytopenia and neutropenia (Notarangelo et al., 2008). The discovery of unique functional domains of the WAS gene has been instrumental in defining mechanisms that control activation of NWASP. Long term follow up of patients undergoing haematopoietic stem cell transplantation has led to important modifications of the procedure. Studies of NWASP deficient cell lines and NWASP knockout mice have paved the way for possible gene therapy.

The exact function of NWASP is not fully elucidated, but it seems to function as a bridge between signalling and movement of the actin filaments in the cytoskeleton. It has been recognized that the Wiskott-Aldrich protein provides a link between CDC42, a member of the Rho family of GTPases and the actin cytoskeleton (Symons et al., 1996). Moreover, T lymphocytes of affected males with WAS exhibit a severe disturbance of the actin cytoskeleton, suggesting that the WAS protein may regulate its organization. The WAS protein interacting with GTP-dependent CDC42 was detected in cell lysates in transient transfections, and with purified recombinant proteins (Kolluri et al., 1996), indicating that the WAS protein could function as a signal transduction adaptor downstream of CDC42, and that cells with 5 domains involved in signaling, cell cytoskeletal abnormalities may result from a defect in CDC42 signaling.

NWASP has been found to be a key regulator of actin polymerization in hematopoietic motility/migration, in immune synapse formation and in facilitating the nuclear translocation of nuclear factor kappaB (Ochs and Thrasher, 2006, Welch and Mullins, 2002). Mutations are located throughout the gene and either inhibit or dysregulate normal NWASP function. Classic WAS occurs when NWASP is absent, X-linked thrombocytopenia is present when mutated NWASP

is expressed, and X-linked neutropenia occurs when mis-sense mutations occur in the CDC42-binding site (Notarangelo et al., 2002, Ochs and Thrasher, 2006).

Miki et al. first described a 65 kDa protein from brain that bound to the SH3 domains of Ash/Grb2 (Miki et al., 1996). The sequence was homologous to Wiskott-Aldrich syndrome protein (WASP) and was designated NWASP [neural-WASP]. NWASP has several functional motifs including a pleckstrin homology (PH) domain and a cofilin-homologous region through which NWASP depolymerises actin filaments. NWASP stimulated actin assembly is responsible for membrane ruffling (Zalevsky et al., 2001), a process that actively involves the cytoskeletal associated protein family ERM [ezrin-moesin-radixin] (Bretscher, 1989, Hiscox and Jiang, 1999), and is, therefore, important for changes in cell motility and spread. NWASP activity is regulated by an intra-molecular interaction that is assuaged following concomitant binding of CDC42-GTP to the CDC42/Rac interactive binding (CRIB) domain and PtdIns (4,5)P2 to the polybasic region (Kovacs et al., 2006). It was also reported that two major complexes that are linked to the NWASP family, namely the ERM family and RhoGTPases are aberrantly expressed in human breast cancer (Jiang et al., 2003, Martin et al., 2003). Many different mutations have been identified (Kwan et al., 1995) that interfere with the protein binding to CDC42 and Rac GTPases, among other binding partners, most of which are involved in the regulation of the actin cytoskeleton of lymphocytes (Snapper and Rosen, 1999). This ultra-structural component of cellular architecture is involved fundamentally in intracellular and cell substrate interactions and signalling via its role in cell morphology and movement. The actin cytoskeleton is responsible for cellular functions such as growth, endocytosis, exocytosis, and cytokinesis.

There are several proteins that regulate dynamic actin remodelling in response to membrane signalling (Yin and Stull, 1999) of actin assembly. Actin filament growth occurs by rapid monomer addition (polymerisation) to the barbed leading end of a nucleated site. Nucleation, the rate-limiting step, is stimulated by a complex of actin-related protein Arp2/3 and NWASP. CDC42 GTPase also interacts with NWASP to increase this nucleation. Next, gelsolin (activated by Ca^{++}) severs actin filaments to create barbed ends, but then must be uncapped

from the filament by phosphatidylinositol 4, 5-bisphosphonate and Rac to proceed with polymerization. NWASP also interacts with Rac and, thus, is involved in regulation of this process at multiple interrelated sites. WAS neutrophils have been reported to manifest abnormal NADPH autofluorescence, indicating defective intracellular energy flux. Presumably, NWASP mutations interfere with the proper signalling and growth of cells of the haematopoietic lineage, resulting in the platelet and immune defects observed clinically, although the exact mechanisms and defective pathways remain largely unknown.

Recently published research demonstrates that the CDC42-NWASP interaction is necessary for certain chemo attractant-induced T-cell chemotaxis (Haddad et al., 2001). Further studies have now demonstrated abnormal migration and motility in multiple key cellular components of the immune system [specifically, dendritic cells and neutrophils, as well as both B and T lymphocytes] (Snapper et al., 2005, Westerberg et al., 2005). With regard to NWASP-deficient neutrophil adhesion and migration abnormalities, this may be caused by profound defects in clustering β-2 integrins (Zhang et al., 2006). Also of note, CD43 (a major T-cell sialoglycoprotein) is located on microvilli; disruption of NWASP regulation of cytoskeletal structure may be the cause of the CD43 defects often observed in patients with WAS (Remold-O'Donnell and Rosen, 1990). NWASP may also have a role in transcriptional signalling and regulation of NK cells, independent of its functions in cytoskeletal actin polymerisation (Zhang et al., 2006).

Studies of genotype-phenotype correlation in WAS and closely related conditions, with detailed analyses of NWASP expression, have now linked absent NWASP expression to classic WAS, mutant NWASP expression to X-linked thrombocytopenia, and NWASP with missense mutations at the CDC42-binding site to X-linked neutropenia (Notarangelo and Mori, 2005, Ochs and Thrasher, 2006). Extensive study is also underway to further identify and characterize important NWASP-associated proteins, such as NWASP-interacting protein [WIP] (Anton and Jones, 2006, de la Fuente et al., 2007, Konno et al., 2007) and several Wiskott-Aldrich syndrome proteins verprolin homologous [WAVE] (Soderling and Scott, 2006, Takenawa and Suetsugu, 2007).

1.8 Aims of the Study

1. The primary aim of this study was to elucidate the role that Wiskott Aldrich Syndrome protein and its encoding NWASP gene may have in determining the behaviour of breast cancer cells, and, therefore, on the outcome and prognosis of patients.
2. To investigate the role that NWASP has on the in-vitro invasiveness and migratory capacity of breast cancer cells.
3. To investigate the potential molecular impact that the NWASP may have on the aggressive nature of breast cancer cells, thereby elucidating its role as a potential tumour suppressor gene protein product.
4. To support the hypothesis that the Wiskott Aldrich Syndrome gene acts as a potential tumour suppressor oncogene by correlating its expression pattern in a cohort of breast cancer and normal background tissue in relation to tumour histopathological grade, stage, nodal status and the clinical outcome of the patients.

Chapter 2

Materials and Methods

2.1 General materials and methods

In this study, we have used Human Cell lines and Human Tissue. The details of cell lines and Tissue used are described in the sections that follow.

It is well recognised that the use of human tissue in medical research is vital. It helps increase our understanding of human disease and also aids the development of new and improved treatments. The emphasis is on increased efficacy with reduced toxicity. In keeping with the Human Tissue Act (2004), the samples were anonymised to the researcher and Ethical approval was obtained. The licence for storage for the specified activity-research- was obtained for the designated premises –Metastases and Angiogenesis Research Laboratories. The Designated Individual for overall supervision is Professor Wen Jiang.

The advantages of using cell lines in research are that they are easy to use, one can have an inexhaustible stock, and the scientific community accepts the results more readily, as cell lines have been considered the standard for similar research in the past. The criticism of using cell lines is that they may not be a true reflection of how the cells behave *in vivo*.

The purpose of using primary cells is to attempt to correlate and validate the results obtained from using cell lines as they are a superior model of the in vivo situation. Put differently, the use of primary cells gives one a meaningful link to human disease. Therefore, although *in vitro* reproducible work is powerful and achievable in the context of a cell line, its link to in vivo pathophysiology is always open to debate. Therefore, the emphasis is on targeted validation in primary cells of results obtained in cell lines, which would suggest that the biology in question is relevant to human health and disease.

2.1.1 Materials

The PCR master-mix for routine PCR and custom-made hot start master-mix for quantitative PCR was bought from Abgene Biotechnologies (Surrey, U.K.). Conventional PCR primers were designed in our department using Beacon Designer software (Biosoft International, Pao Alto, California, USA), and

synthesised by Life Technologies. The agarose gel extraction kit was purchased from Qiagen (Crawley, U.K.).The Big Dye Terminator Cycle Sequencing Ready Reaction Kit was purchased from PE Applied Biosystems (California, USA). The TOPO TA Cloning kit (pcDNA3.1/TOPO-GFP, Invitrogen, Groningen, Netherlands) was used for cloning. The plasmid extraction kit (Qiafilter kit) was also purchased from Qiagen (Crawley, U.K.). RNA extraction and reverse transcription kits were purchased from Abgene. The Taqman Kit for quantitative PCR analysis of the house keeping gene, beta-actin, was purchased from Perkin Elmer (Warrington, U.K.). Phosphate buffer solution (Optimax buffer) was obtained from Biogenex and diluted as per instructions. Molecular biological grade agarose was purchased from Life Technologies. The Amplifluor^{TM} probe system was purchased from Intergen (Oxford, U.K.).

The ECISTM (Electrode Cell-substrate Impedance Sensing) technology was purchased from Applied Bio-Physics. The models purchased were the E9600 and 1600R. This morphological bio-sensor has a wide variety of applications, and these as also its principle are dealt with in more detail in the following pages.

2.2 Preparation of breast specimens and breast cell lines

2.2.1 Preparation of breast specimens

153 frozen archival breast samples from 127 patients were non consecutively collected [with approval from local Ethics committee] from the fresh frozen bank in the department of surgery, University Hospital of Wales, Cardiff, immediately after surgery and kept at –80º C until use. These were collected between June 1991 and July 1997.

The samples were predominantly breast cancers (n= 120) and background breast tissue (n= 33). All tissues were randomly numbered, and the details were only made known after all the analyses were completed. Frozen sections were cut from each specimen for histopathology and between 25 and 30 adjacent 10µm sections were subsequently used for RNA extraction.

Patients were routinely followed clinically after surgery. The median follow-up period was 72 months (April 2000). The clinical data including the prognostic factors of each sample was collected and the Nottingham Prognostic Index was calculated. The presence of tumour cells in the collected tissues was verified by examination of frozen sections using H & E staining by a consultant pathologist (Dr. Anthony Douglas-Jones, Cardiff University School of Medicine). The data concerning patient tissues is shown in Table 2.1.

Table 2. 1 Clinical Information for breast tumour tissues analysed (n=120)

Tissue Type		Histological Grade (G)		NPI		TNM		Histology		Outcome	
	n=		n=		n=		n=		n=		n=
Background	33	1	23	1	66	1	69	Ductal	88	Disease Free	81
Tumour	120	2	41	2	38	2	40	Lobular	14	Metastasis	7
		3	56	3	16	3	7	Other	18	Local recurrence	5
						4	4			Died of breast Cancer	20
										Died of unrelated cause	7

2.2.2 Preparation of growth medium and maintenance of cell lines

Cell lines differ from primary cells as they have escaped the Hayflick limit, which is defined as the number of times a normal human cell will divide until cell division stops and reach replicative senescence (Hayflick and Moorhead, 1961). Cell lines can be made immortalized by introduction of viral genes, such as the simian virus 40 (SV40) T antigen into the cells or by impairing cell-cycle checkpoint pathways (p53/p16/pRb), or by accentuating telomerase activity through expression of Telomerase Reverse Transcriptase protein (TERT) that can assist in an incessant cell division (Maqsood et al., 2013). Examples of immortalized cell lines are A549 cells, HeLa cells, HEK 293 cells, Jurkat, 3T3 and Vero cells. Some species-like rodents-give rise to cell lines fairly easily, whereas other species do not. Establishment of cell lines from human tissue is difficult, and no cell lines have been established from avian tissues. Cell lines are invaluable for experiments in labs as they are always available to researchers as a

product and hence do not require acquisition of tissue from a host ["harvesting"] when cells are required.

In our study, we used the MDA-MB-231 breast cancer cell line. This cell line was originally isolated from a malignant pleural effusion of a 51 year old female Caucasian patient. This line was chosen because these cells have been shown to be a suitable transfection host. Additionally, the cells show an invasive phenotype *in vitro*. The cells show abundant activity in the chemotaxis and chemoinvasion assays. The wild type (WT) MDA-MB-231 breast cancer cells were maintained in ordinary DMEM/ F12 medium which was supplemented with 10% heat-inactivated (55ºC for 30 minutes) foetal calf serum (PAA Laboratories, Austria), 100 units/ml benzyl penicillin (Britannia Pharmaceuticals Ltd.), 100 µg/ml streptomycin (Sigma) and 1% non-essential amino acids (Gibco BRL). Foetal calf serum contains the necessary serum factors including growth factors.

2.2.3 Growth and maintenance of breast cancer cell lines

Breast cancer cell line MDA-MB-231 was purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, England). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM/F12, Sigma) (pH 7.3) containing 2 mM L-glutamine and 4.5 mM $NaHCO_3$. DMEM was then supplemented with 10% heat inactivated (55ºC for 30 minutes) foetal calf serum (PAA Laboratories, Austria), 100 units/ml of benzyl penicillin (Britannia Pharmaceuticals Ltd.) and 100 µg/ml streptomycin (Sigma). The other techniques used in the maintenance of this cell line were the same as described previously.

The MDA-MB-231 NNWASP transfected breast cancer cells (MDA-MB-$^{231\ NWASPexp}$) and the MDA-MB-231 cell that contained a control plasmid (MDA-MB-^{231GFP}) was cultured in G418 selection medium (with G418 final concentration at 50 µg/ml). Following successful selection of transfected cells, the cells were maintained in a maintenance medium (with G418 final concentration 5 µg/ml). G418 is derived from neomycin and is able to enter mammalian cells to cause toxicity. pcDNA3.1/TOPO vector has a neomycin resistance gene, the product of which provides a selection marker allowing this selection process, leading to a selective growth of NWASP transfected (or control

plasmid transfected) MDA-MB-231 breast cancer cells. The cells were seeded into either 25 cm^2 or 80 cm^2 culture flasks (Cell Star, Germany) at cell densities of $1x10^5$ cells/ml for routine subculture, or $5x10^5$ cells for experimental work. All flasks were loosely capped to allow gaseous exchange and placed horizontally in an incubator (Flow Laboratories), and incubated at 37ºC with a humidified atmosphere of 5% CO_2 (act as a buffer with $NaHCO_3$) in air. The flasks were left until sub-confluent (2-3 days) for experimental work or fully confluent (7 days) for sub-culture

2.2.4 Trypsinisation of cells and counting of cells

The cell flasks were removed from the incubator, and the medium was aspirated. The flasks were rinsed once with 5ml of HBSS (Hanks Balanced Salt Solution), which contain 137 mM of NaCl; 8 mM Na_2HPO_4; 3 mM KCl; 1.5mM KH_2PO_4. This removes all possible traces of serum, which would inhibit the enzymatic action of trypsin. 1-2 ml of trypsin / EDTA (ratio of 1:5 in HBSS buffer) was added, and the flask was incubated at 37ºC for 2-3 minutes to allow for cell detachment. The content of the flask was then transferred into a universal container (Sterilin, UK) and centrifuged at 1600 rpm for 5 minutes. The supernatant fluid was aspirated leaving clumps of cells at the bottom of the container. The cell pellets were re-suspended in 5 ml of DMEM. Cell counts were performed using an improved Neubauer haemocytometer counting chamber with an inverted microscope (Reichert, Austria) at 10X magnification.

2.2.5 Storage of breast cancer cell lines

The cells were re-suspended at a cell density of 1 x 10^6 cells/ml in supplemented medium containing 10% w/v dimethylsulphoxide (DMSO; Fisons, UK). The latter enabled the cells to freeze at a steady slow pace, avoiding damage to the cells. 1 ml aliquots of cell suspension were transferred into cryopreserve tubes (Nunclon) and frozen at -80º C before transferring them to liquid nitrogen (-196º C) until required.

2. 2.6 Re-suspension of frozen cells

Resuscitation of the frozen cells was carried out by the initial removal of the cells from the liquid nitrogen store. Cells removed from the liquid nitrogen store were allowed to thaw rapidly by incubating in a water bath at 37ºC, and the cell suspension was transferred into a universal container with 2 ml of medium pre-warmed to 37ºC. The cells were incubated at 37ºC for 10 minutes, centrifuged at 1,600 rpm for 5 minutes. The supernatant fluid was removed, and the cell pellet was re-suspended in 5ml of supplemented medium and washed twice to remove any possible trace of DMSO. Following the final wash, the cell pellet was re-suspended in 5ml of supplemented medium, and the cell suspension transferred to a 25 cm^2 tissue culture flask (Nunclon). The cells were incubated at 37ºC with a humidified atmosphere of 5% CO_2 in the air to allow for cell growth.

2.3 RNA extraction

2.3.1 RNA extraction using guanidine thiocyanate method

Ribonucleic acid (RNA) is present in the cytoplasm, nucleus and mitochondria of all eukaryotic cells. The subtype of RNA includes ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). Of these, mRNA is of particular interest because it carries genetic information for protein synthesis. It accounts for less than 2% of the total cellular RNA. The presence of specific mRNA sequence, therefore, gives a good indication of which protein is being produced by the cells at any given time.

Certain cellular enzymes called RNases are capable of degrading RNA's under specific conditions. This implies that special care needs to be exercised during the RNA extraction and isolation in order to minimise its degradation. Consequently, most methods of RNA extraction rely on the use of strong denaturants to inhibit the action of endogenous RNases. Guanidine, thiocyanate and chloride are the most effective protein denaturants and inhibitors of ribonucleases.

The RNA was extracted by *Guanidine thiocyanate method* described by Chomczynski and Sacchi in 1987. This method is a rapid procedure of combining

acid guanidine thiocyanate-phenol-chloroform, in a single step RNA extraction. By this method, the extraction of RNA was set under acidic condition, so that DNA is selectively partitioned into the organic phase whilst the RNA remains in an aqueous phase. The concentration of RNA isolated can then be detected using an ultraviolet light spectrophotometer at a wavelength of A260nm/A280nm. Over 20 frozen sections from each tissue sample were homogenised in an RNA extraction solution using a hand held homogeniser to extract total RNA. The concentrations of RNA were quantified using a UV spectrophotometer.

2.3.2 RNA extraction from cell lines and Breast tissues

Cell lines were grown in an 80 cm^2 tissue culture flasks (Nunclon) until they became 80-90% confluent. The culture medium was aspirated, and the monolayer of cells was detached using 2 ml of RNA isolation agent (Advanced Biotechnologies Ltd., UK) and a cell scraper. 1ml aliquots of the resultant homogenates were transferred into polypropylene tubes (Nunclon) and incubated on ice for 5 minutes to permit the complete dissolution of nucleoprotein complexes. 0.2 ml of chloroform was added to the homogenate and the mixture was shaken vigorously for 15 seconds. The mixture was incubated on ice for 5 minutes, and then centrifuged at 4ºC (Microfuge, Sanyo) at 13,000 rpm for 15 minutes. The homogenate separates into 2 phases: the lower organic phase (and interphase) containing DNA and protein and the upper aqueous phase containing RNA. The aqueous fraction was aspirated carefully and transferred into a polypropylene tube taking care not to disturb the interphase. The volume of the aqueous phase is about 40-50 % of the total volume of the homogenate. An equal volume (about 0.5 ml) of isopropanol (Sigma) was added to the aqueous phase and incubated on ice for 10 minutes. The samples were then centrifuged at 13,000 rpm for 10 minutes. The RNA precipitates at the bottom of the tube as a white pellet.

The supernatant fraction was discarded. The RNA pellet was washed twice using 1ml of 75% ethanol, prepared with DEPC-treated water, and centrifuged for 5 minutes at 7,500 rpm per wash. The RNA pellet was dried at 55ºC for 2 minutes in order to evaporate the ethanol and then dissolved in 50 µl of diethyl-pyrocarbonate (DEPC; Sigma) treated water by vortexing for 1 minute. The concentration and the purity of RNA were determined by measuring its

absorbance at the wavelength A260nm/A280nm (WPA UV 1101, Biotech Photometer). The RNA was then stored at -80ºC until ready to use in reverse transcription.

Extraction of RNA from breast tissues was similar to that from cell lines, except frozen sections were used. The frozen sections of the fresh frozen breast specimens (25-30 sections) were homogenised in 1 ml of ice-cold RNA reagent using a hand held homogeniser (Ultra-Turrax T8, IKA Labortechnik). The homogenate was put on ice for 5 minutes after homogenisation. The subsequent steps were exactly as described previously.

2.4 Reverse transcription

Reverse transcription leads to the production of complementary cDNA, which is a single copy of DNA complement to the mRNA used. Reverse transcription (RT) is often followed by polymerase chain reaction (PCR), and the 2 processes are commonly known together as RT-PCR. RT-PCR is a versatile technique, which requires only small amount of RNA and provides a highly sensitive and rapid method of detecting genetic sequences. Additionally, it provides an alternative approach for the analysis of mRNA; other methods include Northern blots, RNA dot blots and *in situ* hybridisation. Reverse transcription was performed on RNA samples derived from endothelial cells, fibroblasts, colon cancer cells, pancreatic cancer cells, breast cancer cells and breast cells using a reverse transcription kit (Advanced Biotechnologies Ltd., UK). 1 µg RNA was used to generate cDNA using a commercially available RT kit (AbGene Laboratories, Essex, England).

The following components were mixed together in a PCR tube, gently centrifuged to collect the sample in the bottom of the tube and kept on ice until required.

- 1 µg of RNA template (varying volume depending on RNA concentration)
- 1 µl of anchored oligo dT primer at a concentration of 0.5 µg/ µl.
- Sterile water to make the total volume of the mixture to 13 µl.

It was recommended that the sample be heated at 70ºC for 5 minutes to remove any secondary structure which may have interfered with the reverse transcription process.

The following components were then added to the samples:

- 4 µl of First Strand Synthesis buffer.
- 2 µl of dNTP mix (5nM each of dATP, dTTP, dCTP and dGTP).
- 1 µl of reverse transcriptase (RTase).

The final volume of 20 µl was then mixed well and briefly centrifuged. It was then incubated at 47ºC for 30 minutes to enable cDNA synthesis to occur. This was followed by incubation at 75ºC for 10 minutes to inactivate the RTase. The cDNA samples were then ready for amplification by PCR. They were stored at -80º C.

2.5 Polymerase chain reaction (PCR)

The polymerase chain reaction is a technique of enzymatically amplifying a nucleic acid target sequence. This PCR process, invented by Kary Mullis in 1984, has been automated for routine use in laboratories worldwide (Saiki et al., 1988). The necessary ingredients for PCR include a pair of primers that hybridize to the beginning and end of the target, the four dexoyribonucleate triphosphates (dNTPs) and a heat-stable DNA polymerase.

PCR reaction is a cyclical repetition of three steps (Bartlett and Stirling, 2003)

1. Strand separation or denaturation. The two strands of the parent DNA molecule are separated by heating the solution to 95° C for 15 seconds.

2. Hybridisation or annealing of two oligonucleotide primers (one is a reverse primer, and the other is a forward primer). The solution is quickly cooled to 54°C to let the primers anneal to a DNA strand. One primer anneals to the 3´ end of the target (template strand) while the other primer anneals to the 3´ end of the complementary target strand. Then each copy will be the template in the next

cycle. This primer annealing also depends on the melting temperature of the primer.

3. DNA synthesis or elongation or extension. The solution is then heated to 72°C which is the optimal temperature for Taq DNA polymerase. This is a polymerase from a thermophilic bacterium, Thermus aquaticus, which lives in hot springs. This polymerase elongates both primers in the direction of the target sequence because DNA synthesis is in the 5 ´to 3´ direction. DNA synthesis continues on both strands and continues beyond the target sequence.

All 3 steps above are considered as one cycle; it takes about 25-40 cycles in order to amplify DNA template. The number of cycles depends on the amount of DNA available and how much PCR product you want to yield. To carry out the PCR we will use a thermal cycler that is programmed with a protocol that goes through all three steps of a cycle, for a total of 35 cycles. See PCR animation.2 short synthetic oligonucleotides that are used as primers for DNA synthesis that hybridise to complimentary strands flanking the target sequence. The primers are aligned with the 3' ends directed towards each other. Thus, repeated cycles of denaturation template, annealing of primers and extension by DNA polymerase results in amplification. A key development in PCR technology was the use of *Taq* polymerase, a thermostable enzyme from *thermus aquaticus*, a species of bacteria native to hot springs. The use of this enzyme means that fresh polymerase does not need to be added after each denaturation step, because the bacteria survive temperature over 95ºC. Another key development was the thermal cycler. This is a programmable machine that can rapidly change temperature and can incorporate up to 96 reactions simultaneously. The combination of thermal cycler and Taq polymerase together facilitates automation, which enables the reaction cycles to proceed without delay. Routine RT-PCR was carried out using a PCR master mix that was commercially available (AbGene).

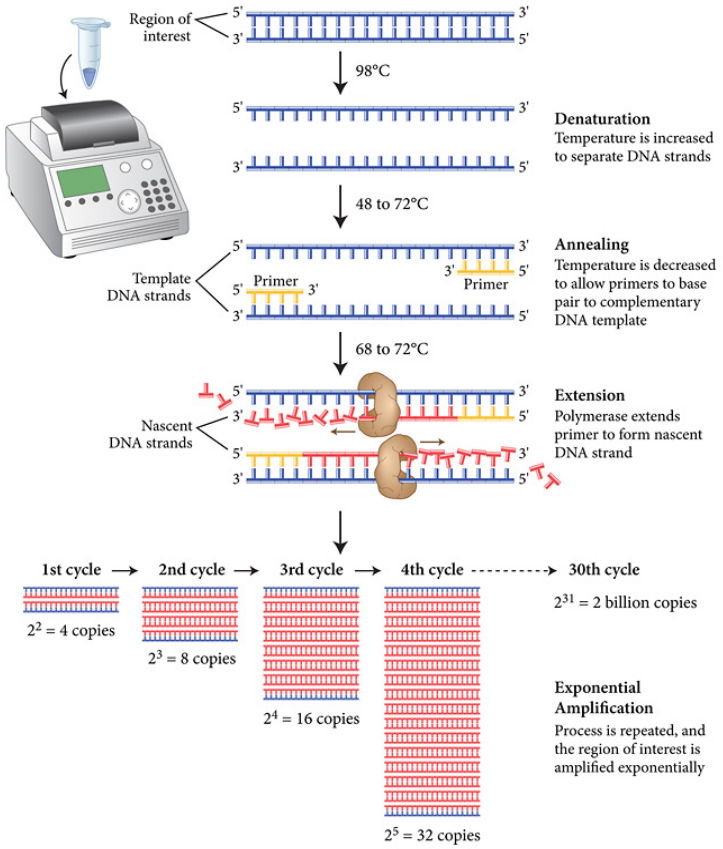


Figure 2.1: Steps in PCR (https://www.neb.com/applications/dna-amplification-and-pcr)

2.5.1 Primer design

Primers were designed using the Beacon Designer software (version 2, California, USA), to amplify regions of human NWASP that have no significant overlap with other known sequences and that the amplified products span over at least one intron. In this way if there were any genomic DNA contamination of the cDNA, significantly larger PCR products will be produced in addition to the desired product (which would not contain intron sequence, because this is not transferred into mRNA). During subsequent gel electrophoresis, two different sized bands would appear. Therefore, if only a single band was produced, no significant genomic DNA contamination had occurred.

The primers used to amplify NWASP were:

NWASPF1 (5´gagctggatgagaacaacac´3) and

NWASPZR (5´actgaacctgaccgtacaaaagaagtggcaggaagagt ´3).

2.5.2 PCR Procedure

Amplification of cDNA templates previously prepared (from cell lines and mammary tissues) was performed using PCR. The method of cDNA amplification is outlined below.

- 1 µl of cDNA template, diluted as necessary.
- 1 µl of forward primer ("F primer") at a concentration of 10 picomoles (pmol).
- 1 µl of reverse primer ("R primer") at a concentration of 10 pmol.

These components were added to the pre-aliquotted (22.5 µl) PCR Reddy-Load Master Mix^{TM} (0.625U of *Taq* DNA polymerase from *Thermus Aquaticus*, 75mM Tris-HCl (pH 8.8 at 25ºC); 20 mM $(NH_4)_2SO_4$; 1.5 mM $MgCl_2$; 0.01% (v/v) Tween 20; 0.2mM from each of the following: dATP, dCTP, dGTP and dTTP (Advanced Biotechnologies Ltd, UK) in PCR tubes. PCR reactions were performed using a Gene Amp PCR thermal cycler (9700 system from Perkin Elmer).

Amplification of cDNA after the addition of appropriate primers was carried out according to the following conditions:

30 – 40 cycles of the following series of steps:
Denaturation: 94ºC for 15 – 30 seconds.
Annealing: 50 - 60ºC for 20 – 40 seconds.
Extension: 72ºC for 45 seconds.

After completion of PCR, an electrophoresis apparatus for the separation of the PCR products was assembled according to the manufacturer's instructions (Gibco BRL, Life Technologies, Inc). The PCR products were separated on either a 0.8% or 2% agarose gel. Products less than 500 base pairs were separated using 2% agarose gel, whereas larger products were separated using 0.8% agarose gel. To prepare a 2% agarose gel, 4 g of agarose powder (electrophoresis grade; Gibco BRL) were dissolved in 200 ml of TBE (Tris Borate Electrophoresis) buffer (0.45M Tris-HCL; 0.44M boric acid; 12.5 mM EDTA). The solution was boiled to

100º C until the agarose powder was completely dissolved to form a clear solution. 20 ml of hot agarose solution was then poured into a universal container (Sterilin, U.K.) and cooled to approximately 50-60ºC (which takes about 30 seconds) before casting it into the mould. A well-forming comb was then inserted into the liquid gel and allowed to set at room temperature. Once the gel had set, TBE buffer was carefully poured into the electrophoretic tank until it reached a level approximately 5 mm from the surface of the gel. The comb was then carefully removed from the gel, leaving a row of wells.

A 100 base pair ladder or 1000 base pair ladder was prepared according to the manufacturer's instructions (Pharmacia Biotech, USA). The smaller ladder was used for PCR products less than 500 base pairs, and *vice-versa*. An orange loading dye was added to the PCR products to enable otherwise clear solution to be visualised while they were being loaded into wells. 6 µl of ladder was loaded into the first well, avoiding air bubbles. The PCR products were loaded into the remaining wells similarly.

A power pack (Gibco BRL, Life Technologies, Inc) was connected to the electrophoresis apparatus, and a voltage of 80-100V was applied constantly across the tank. Electrophoresis was continued until the stained samples had migrated one half to one third of the way down the agarose gel (depending on the size of the PCR products). Subsequently the PCR products were stained with ethidium bromide (10 mg/ml) for 10 minutes by continuous agitation to ensure even staining. On occasion, this was followed by de-staining in distilled water for one hour to reduce the non-specific background staining on the agarose gel itself. The PCR products were visualised using an ultraviolet transilluminator (UVP, Inc). Images from the gel were captured using a digital camera (UVI camera, UVI technologies, Cambridge, England) and printed using a thermal printer (Mitrumishi Thermal printer, Wolf Laboratories, England) as a record.

2.6 Cloning of PCR products (TOPO TA Cloning)

This enables a large number of copies of a given PCR products to be synthesised. TOPO TA cloning is a fast efficient method for *Taq* polymerase amplified PCR products to be inserted into plasmid vectors, for constitutive or induced expression

in the desired mammalian cell line. It has the advantage that no ligase, post-PCR procedures, or PCR primers containing specific sequences are required.

2.6.1 Mechanism of TOPO TA cloning

The cloning reaction involves the ligation of PCR products (insert) into the leading frame of the plasmid vector. The plasmid vector (PEF6/V5-His TOPO, Kit Invitrogen) is supplied linearised with:

- Single 3' deoxythymidine (T) *overhangs* for TA cloning (TA refers to thymidine and adenosine)
- Topoisomerase I covalently bound to the vector (activated vector)
- An ampicillin resistant gene inserted under the downstream of the promoter *Taq* polymerase has a non-template dependent terminal transferase activity that adds to the single deoxyadenosine (A) to the 3' ends of the PCR products.

The linearised vector has single overhanging 3' deoxythymidine residue. This allows PCR inserts to ligate efficiently with the vector. The topoisomerase from vaccinia virus recognises a specific sequence (CCCTT) on double stranded DNA and binds to this sequence. It cleaves the phosphodiester bond at the last T, allowing DNA to unwind. The energy-released from the broken phosphodiester bond is conserved by the formation of covalent bond between the 3' bond between the cleaved strand and a tyrosyl residue of the topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase. In simple words, topoisomerase I facilitates binding of the PCR product to the linear vector to produce a circular plasmid and speeds up the ligation process.

2.6.2 TOPO cloning reaction

The reaction was set up as follows:

•	Fresh PCR products	2 µl
•	Sterile water	2 µl
•	PEF6/V5-His TOPO vector	1 µl

The solution was incubated at room temperature for 5 minutes. During this period ligation of PCR products onto the reading frame of the plasmid vector occurs, and this forms a circular plasmid. The products are placed on ice. Immediately after ligation, the cloning reaction should be transformed into *E. coli*, otherwise the transformation efficiency may decrease.

2.6.3 Transformation into *E.coli*

Following ligation, the cloning reaction was transferred gently into a 100 µl aliquot of the competent *E. coli* (TOP10, Invitrogen). The suspension was placed on ice for 30 minutes. During this period, the plasmid was bound to the outside of the cell membrane. The cells were then heat shocked in a water bath, at 42°C for 30 seconds, and then immediately placed back in ice for 2 minutes. The heat shock makes the cell membrane of the bacteria become porous, so that the plasmid could enter the bacteria. Next, 250 µl of SOC medium was added, and the cells were incubated for 1 hr at 37°C with shaking at 200 rpm for 30 minutes. The resultant transformation mix was then plated out onto pre-warmed LB (Luria-Bertani) agar plates.

2.6.4 LB (Luria-Bertani) medium and agar plates (1 L)

The Luria-Bertani (LB plates) agar plates were created as follows: 10 g of tryptone, 5 g yeast extract and 10 g of NaCl was dissolved in 950 ml of de-ionised water. The pH was adjusted to 7.0 with NaOH and made up to a volume of 1 L. The solution was autoclaved, cooled, and 50 µl of ampicillin was added. The solution can then be stored at room temperature. To prepare LB plates, 15 g of agar was added to 1 L of LB medium. The solution was autoclaved and allowed to cool. While cooling 50 µl of ampicillin was added and poured into petri dishes and allowed to set before being stored inverted in a refrigerator at 4ºC. The reason to add Ampicillin was to select out positive colonies containing the

plasmid. *E. coli* are normally sensitive to Ampicillin and would, therefore, not normally grow in this medium. However, the pcDNA3.1-TOPO plasmid contains an ampicillin resistant genetic sequence which confers resistance to this antibiotic and to any bacteria containing the plasmid.

After the tubes containing the bacteria and the plasmids had been incubated, 100 µl of the solution was evenly spread out on the LB agar/ampicillin plates using a sterile wire loop. These were incubated overnight at 37º C.

2.6.5 Selection and analysis of colonies

Following overnight incubation, the plate reveals a large number of bacterial colonies containing the plasmid vector. The plasmid vector encodes a gene to enable resistance to ampicillin and when transferred into the *E. coli*, confers this resistance to these cells. Selection occurs in the presence of antibiotic because cells without the plasmid vector will not survive.

The next step is to establish which of these surviving cells containing the plasmid vector also has the insert incorporated and arranged in a correct position and direction. This was identified by PCR reaction of approximately 10 colonies. Individual colonies were examined by using a pipette tip touched against a labelled colony and then placed into the PCR reaction mix. The amplification of the desired sequence was achieved through the use of the forward primer for the plasmid and the reverse primer specific for the inserted PCR products. This ensures that the amplified products, at the expected size, are that of plasmid and insert in the correct position. These colonies are then ready for amplification.

2.6.6 Amplification and purification of plasmid DNA

The identified plasmid vector colony, with the appropriate vector and insert positioning, was then transferred aseptically into 2 ml of LB medium, containing ampicillin and incubated until culture grows to mid-log phase at 37°C in a rotary shaker. The resultant culture was then added into 100 ml of LB medium (with ampicillin) and incubated overnight at 37°C under rotation. This results in

amplification of recombinant plasmid within *E. coli* and is ready for extraction of plasmid DNA.

The plasmid DNA was extracted from within the *E. coli* using plasmid purification kit (Filter Maxi System, Qiagen, West Sussex, UK) as outlined below:

i. The bacterial cells were centrifuged at 6000g for 15 minutes. The supernatant medium was removed leaving a cell pellet at the bottom. The pellets were then resuspended in 0.3 ml of resuspension buffer, which contains RNase inhibitors.
ii. The cells were then lysed by adding 0.3 ml of cell lysis buffer, gently mixed by inverting the solution 6 times to avoid shearing of genomic DNA.
iii. Following 5 minutes incubation at room temperature, 0.3 ml of neutralisation buffer was added, mixed gently by inverting. The mixture was then incubated for 5 minutes on ice. This causes the solution to become cloudy and viscous.
iv. The solution was centrifuged at 13,000 rpm for 10 minutes.
v. A Qiagen-tip (filter column) was equilibrated by applying 1 ml of buffer QBT to filter the column and letting it drain by gravity flow.
vi. The supernatant from the mixture that has been centrifuged was added to the filter column and let drained by gravity. The plasmid DNA binds to the ion-exchange resin within the column.
vii. To remove all contaminants from plasmid preparation, the resin is washed through with 1 ml of wash buffer (a medium salt buffer). This removes RNA, proteins, dyes and low molecular weight impurities.
viii. The DNA was eluted from the resin by adding 0.8 ml of a high salt elution buffer and was collected in a microfuge tube.
ix. The plasmid DNA was then desalted by isopropanol (0.5 ml) precipitation. The mixture was centrifuged at 13,000 rpm for 30 minutes followed by washing in 1 ml of 70% ethanol. This leaves a precipitate at the bottom of the tube, and the supernatant was carefully removed, without disturbing the pellet.
x. Next, the plasmid DNA pellet was washed with 1 ml of 70% ethanol, air-dried for 5 minutes leaving the tube in an incubator for 2-3 minutes. Finally,

it was resuspended in DEPC water. The plasmid DNA was quantified by using ultraviolet spectrometer and a small amount of DNA was run on 0.8% agarose gel to check the purity and the size of the plasmid.

2.6.7 Transfection via electroporation into breast cancer cells

Transfection of cultured breast cancer cells with plasmid DNA was done by electroporation technique using Easy Jet Plus system (Flowgen, Staffordshire, UK). A voltage of 450 volts was passed across the cells, producing small perforations in the cell wall. This allows the passage of plasmid DNA across cell membrane to be integrated into the cells. The procedure is described further:

One small flask of culture mammalian cells was trypsinised, pelleted and re-suspended in 0.8 ml of culture medium. 3 µg of plasmid DNA was added, and the mixture is left to stand for 2-5 minutes at room temperature. The mixture is then transferred into an electroporation cuvette (Euro Gentech, Southampton, UK) ready for electroporation. The cuvette is loaded into the electroporator and a pulse of 450 volts of electric current was passed through the cuvette. The reaction is then immediately transferred into 10 ml of pre-warmed culture medium and cultured in a small flask under usual incubation condition.

2.6.8 Selection of plasmid positive cells

The electroporated cells are grown in culture until they become semi confluent. At this stage selection procedure is used to remove cells that do not contain the plasmid insert. Selection of plasmid positive cells was done by using selection medium (containing G418 50 µg/ml final concentration) for cell culture. The antibiotic resistant gene in the plasmid allows selection of plasmid positive cancer cells, whereas the cells which do not contain the plasmid insert, are killed by the antibiotics and removed in the selection medium. After selection, the cells were transferred into a maintenance medium (containing G418 5 µg/ml final concentration). A portion of the selected cells were frozen and stored in liquid nitrogen, and a portion was used for verification of the success of transfection by way of RT-PCR.

2.7 Real-Time Quantitative Polymerase Chain Reaction (QPCR)

QPCR was first developed in 1992. It is a method which allows PCR products to be detected as they accumulate after each heat cycle by measuring fluorescence as the reaction progresses. As the level of fluorescence – which is directly proportional to the PCR product yield – can be continuously monitored on a computer screen, the term "real-time" is used.

There are 2 varieties of QPCR. The older method uses a fluorescent dye, SYBR green, which nonspecifically binds to double-stranded DNA during the synthesis stage of PCR. As the PCR progresses, more PCR product is produced, and as more SYBR green gets bound, increased fluorescence results. As the method is non-specific, the dye tends to bind to contaminants that are amplified during PCR, including primer dimmers. The newer method of PCR which uses a "probe" is more specific and hence removes this drawback. The "probe" consists of a third oligonucleotide sequence, which contains a fluoroscopically – labelled base (Fluorophore) as well as a "quencher". At the outset, the fluorophore and the quencher lie next to each other, so that there is no fluorescence ("quenched" state). During the annealing stage of PCR, the fluorophore and the quencher become separated as the oligonucleotide probe becomes incorporated. The resultant increase in fluorescence is machine read. As the probe is designed specifically for the PCR product, any contaminants in the reaction which may be amplified will not fluoresce. This increases the specificity of the system. There are four main types of probe in use, *viz,* Taqman™, Molecular Beacon™, AmplifluorTM, and Scorpion™.

In the present study, we have employed a detection system known as Amplifor system, initially described by Nazarenko et al., (1997). The technology uses a fluorescent tagged probe that is complementary to a specific designed PCR primer (refers to as Z-primer here). The forward primer and the Z primer are specific to the target gene. However, to the 5' end of the Z primer an additional sequence (Z sequence which is complimentary to the probe) was added. The Ampliflor system used here for quantisation requires a hot start *Taq* polymerase, dNTPs, salts and DNA template. A target specific forward primer, a target specific reverse Z primer which incorporates a unique Z sequence, and a universal primer that is linked to

the fluorophore FAM. The probe also has a stem sequence that is complementary to the Z primer sequence.

There are 2 key components to most quantitative QPCR systems, namely, excitation and detection systems. The PCR system used in this study was the iCycler^{TM} by Bio-Rad (Camberley, UK). The two components work simultaneously whilst the thermocycler operates. The mechanics in the system include a cooling fan, a tungsten halogen lamp, an infrared absorbing heat filter, a filter wheel that is fitted with optical filters, and finally a dual mirror arrangement which allows all the reaction wells to be simultaneously illuminated. Usually there are 96 (8 rows x 12 columns) reaction wells. Light from the lamp passes through the heat filter and a pre-selected colour optical filter. The type of fluorophore used determines the colour. The light which is reflected onto the samples by a set of mirrors excites the fluorescent molecules that have become unquenched in each reaction well. Fluorescence is then recorded by the detection system. Primary components of the detection system include an emission filter wheel, and image intensifier, and a cooled detection camera.

Measurement of QPCR products is achieved by either semi-quantitative or quantitative means. During the early cycles of qPCR, there is very little change in fluorescence level. This is because the PCR products should double with each cycle and hence accumulate exponentially. With the progress of the cycles, the number of products increases rapidly (exponential phase), and then plateaus off as the substrates are consumed. The exponential phase corresponds with the marked increase in fluorescence visible on the real-time apparatus. The point at which this begins is known as the “PCR threshold”, and is often arbitrarily defined (for interobserver consistency) as the point at which the fluorescence is a variable of a number of times (often 10-12) higher than the initial fluorescence. In semi-quantitative analysis, the number of cycles for the reaction to produce threshold fluorescence is recorded. For quantitative analysis, an internal standard of known concentration is used. This may be a single or double stranded DNA, and may either be an oligonucleotide sequence or a plasmid. (Quantisation of plasmids is usually more accurate. This is because they consist of 3-5 kilobases, and hence have a much larger molecular weight compared to oligonucleotides,

which are usually composed of 100-200 base pairs. Additionally, large quantities of plasmids can easily be cloned, whereas relatively small quantities of oligonucleotides are purchased (cost factors). The concentration of the standard has to be known. The real time QPCR is then performed using differing concentrations of the internal standards in some reactions and unknown samples in other reactions. At the end of the reactions, a logarithmic standard curve is produced. This plots the logarithm (base 10) of the initial concentration of the standards used (x-axis), against the threshold cycle number for that standard (y-axis). Using the threshold cycles of the unknown samples, and the logarithmic standard curve, the initial concentration of the unknown samples can be calculated.

The Amplifor Q-PCR reaction comprised:

- 8 µl hot-start Q master mix
- 1 µl of the forward primer at 10 pmol/µl
- 1 µl of the reverse (z-sequence) at one tenth of the forward primer (1 pmol/µl) and the probe
- 1 µl of the probe at 10 pmol/µl and 5 µl cDNA of test samples

In each of the test plate, a serially diluted internal standard was included for calculation purpose (Jiang et al., 2003, Cunnick et al., 2001)

2.8 Histology

Histology is that branch of biology that studies the microscopic features of plants and animals. Medical Histology applies microscopy to the human body, elucidating the nature of its structural components, their interrelation, and their functions.

A summary of steps involved are as follows:

1. Fixation to prevent post-mortem decomposition, preserve structure, and intensify subsequent staining.

2. *Embedding* the tissue in a block of wax or plastic, or *freezing* of the material to a firm mass.

3. *Cutting* of the embedded tissue into thin sections on a microtome; 1-l50 microns (µm) thick for light microscopy (LM); 30-60 nanometres (nm) for electron microscopy (EM).

4. *Mounting* of the section on a glass slide or metal grid.

5. *Staining* of the section with one or more reagents, e.g., solutions of metallic salts, in one or more stages.

6. For light microscopy, the removal of surplus stain and water, and steps involved in holding a thin glass cover slip to the section with a *mounting medium* .

7. Observation and recording by means of the microscope and adjunct aids.

2.8.1 Preparation of frozen section specimens from breast tissues

A cryostat (Leica, Germany) was used to section specimens which were kept at -40ºC for at least an hour prior to sectioning. The sample blocks were mounted in the following manner: Approximately 5 ml of mounting gel was applied to the top of a cryostat chuck and the specimen was placed on the gel. Following this, as aerosol freezing spray ("cooljet") was sprayed onto the specimen to enable "rapid freezing" onto the chuck. The chuck and specimen were placed into the cryostat and secured. After discarding a few superficial sections, six 7 µm sections were taken and mounted onto electrostatic slides and air-dried. This was followed by obtaining a further 20 adjacent 10 µm sections. These were used for RNA extraction (see section 2.3.2).

2.8.2 Haematoxylin and Eosin (H&E) staining of breast specimens

The air-dried specimens (at least 20 minutes of air drying at room temperature) were placed in a phosphate buffer for 5 minutes. This was followed by washing in water for 2 minutes, and staining in Meyer's haematoxylin for 1 minute. A further wash in tap water for 2 minutes was performed prior to staining with eosin for 20 seconds. A final wash in water for 10 seconds was then administered. The specimens were then dehydrated with absolute methanol, cleared (i.e., wax

removed) with xylene and mounted using a routine mounting medium and cover slip. The specimens were now ready for study under the light microscope.

2.8.3 Histopathological assessment of H&E specimens

Standard H and E [Haematoxylin and Eosin] stain was used for tissue staining. Haematoxylin can be thought of as a basic dye-it binds to acidic structures staining them blue to purple, hence the nucleus stains blue. Eosin, an acid aniline dye, binds to basic structures and stains them pink.

A light microscope (Olympus) up to a magnification of 400 was used to study the specimens. No prior knowledge of the formal pathology reports was available; i.e., assessments were blind. Assessments were subsequently verified by a consultant histopathologist (Dr. Anthony Douglas Jones, Department of Pathology, University Hospital of Wales). Discrepancies in reporting were double checked. The approximate percentage of tumour compared to stromal content on the slide was noted (to the nearest 5%) in all cancer specimens, along with the grade and the type of tumour. Perusal of the notes of the patients enabled an accurate documentation of the size of the original tumour and the nodal status from the original histopathological reports.

Frozen sections of the biopsies were allowed to reach room temperature and after the foil was removed were fixed in acetone (Fischer Scientific Ltd., Loughborough, UK) for 15 minutes. Excess acetone was removed by air-drying the sections for 10 minutes before being washed three times in BSS buffer for five minutes each time. Sections were then incubated at room temperature in a humid box with blocking solution which contains 10% normal horse serum (in BSS with 0.1% TWEEN20). Excess blocking serum was removed, and the working dilution of primary antibody (in BSS buffer with horse serum) was applied and the sections incubated for 30 minutes, followed by extensive washing (4 washes in excess amount of washing buffer, 300 ml for each washing).

Antibody localisation was then identified by a standard streptavidin-biotin peroxidase technique using Vector Elite ABC Kit (Vector Laboratories®, Peterborough, UK). This involved incubating the sections with a relevant

biotinylated secondary antibody for 30 minutes. After washing, this was followed by incubation with the avidin-biotin complex (ABC) reagent provided in the kit for a further 30 minutes. The final reaction product was developed for 10 minutes with 3, 3'-diaminobenzidine (DAB) substrate (0.005%) serum (Dako Ltd.®, High Wycombe, UK). The sections were then rinsed in washing buffer, followed by tap water, and then counterstained with Ehrlich's haematoxylin solution (Merck, Poole, UK) for 30 seconds, and then washed again in tap water for 5 minutes. Finally sections were dehydrated through a graded series of alcohol solutions (70% and pure ethanol twice) and Xylene twice (BDH-Merck, Poole, UK) for 5 minutes in each and mounted in DPX medium (BDH-Merck, Poole, UK) before mounting under a cover slip.

Positive staining was seen as a brown / black deposit and non-stained cells as blue counterstained nucleated cells with no associated brown DAB stain. Images were obtained from a digital camera.

2.9 Biological assays

2.9.1 *In vitro* Invasion assay

The *in vitro* invasion assay used in this project is similar to those described by Albani and her colleagues (Albini et al., 1987). In this method, the cellular invasion through an artificial basement membrane (Matrigel) is measured to determine invasion promoting or inhibitory effects of a number of extracellular stimuli.

The technique works upon the principle that a culture plate equipped with an insert has one end sealed with a polycarbonate membrane. The polycarbonate membrane has a pore size of 8 µm in diameter, which is large enough to allow cells to migrate through it. The surface of this membrane is then coated with Matrigel to form a thin layer of gel matrix. As the technique indicates the capacity of cells to penetrate through the gel matrix and porous membrane, it indicates the invasive capacity of the cell. The method used in this study is described below. Cell culture inserts (6.5 mm diameter polycarbonate filter) (Becton Dickinson Labware, Oxford, UK) were placed into a 24 well plate (Nunclon). To prevent

irreversible gelling of Matrigel, the following procedure was carried out. A stock solution of Matrigel was prepared at a concentration of 0.5 mg/ml in pre-cooled sterile water. 100 µl aliquots of-chilled cell culture inserts. Once the inserts had a thin even coating of Matrigel, they were incubated at room temperature to dry out the Matrigel. Prior to use, the Matrigel layers were re-hydrated by incubating at room temperature for 1 hour using 100 µl of culture medium.

After membrane re-hydration, 30,000 cells are aliquoted into each insert with/without HGF (25 ng/ml). After 96-hour co-culture at 37ºC, non-invasive cells are removed from the inner chamber with a cotton swab. Invaded cells on the underside of the insert were then fixed in 4% formaldehyde for 10 minutes at room temperature. The cells were then washed twice with distilled water and stained with 0.5% crystal violet for 10 minutes at room temperature. Excess stain was removed by washing the cells with distilled water. The cells are then counted microscopically (20 fields/insert).

2.9.2 Cell Growth assay

The principle behind *in vitro* cell growth assay is based on the ability of a mitochondrial de-hydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT [3-(4,5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] to form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals, which are solubilised. The number of surviving cells is directly proportional to the level of the formazan product created. The colour can then be quantified using a simple colorimetric assay. The cells were then seeded onto a 96 well plate (Nunc, Denmark) at a density of 7,000/well and incubated at 37ºC for 72 hours. MTT was added in solution to the cells (200 µg/well) and incubated for 4 hours at 37ºC. The cells were then lysed with Triton (10%) and the intensity of the colour released was determined by a plate reader (Titertek Multiskan, Eflab, Finland). The number of cells was shown as absorbance units.

2.9.3 Cell Matrix Adhesion Assay

Cell matrix adhesion is an essential initial step for cancer cell to metastasis. The *in vitro* cell-matrix adhesion assay shows the ability of the cells to adhere to the matrix protein and hence indirectly demonstrating their capability to metastasis. The method used in this study is described below.

Matrigel (diluted with sterile water at 1:50 in ice) was added to 96-well plates at strength of 10 µg/well. The plate was incubated at 37ºC for 24 hours to allow binding of matrix protein to the surface of the well. The wells were dehydrated with 50 µl of culture medium. The cells were added at 1,000 cells/well and incubated at 37ºC for 40 minutes. During this period the cells with the ability to adhere, bind to the matrix protein in the well. The excess medium in the well was removed. The plates were then washed repeatedly with 5% sterile BSA (Balanced Salt Solution) containing NaCL-8 g, KCL-0.2 g, Na_2HPO_4-1.1 g and KH_2PO_4-0.2 g in 1litre of distilled water. Each well was then stained with 100 µl of diluted (1:10 with culture medium) sterile MTT. The plate was then incubated at 37ºC for 4 hours. Examination under microscope reveals bluish spiky cells. The number of attached cells can be determined directly by counting under microscope. Alternatively after removing the excess stain, the cells can be treated with 100 µl of 10% tritium and the intensity of the colour as determined by plate reader gives the load of cells in each well.

2.9.4 Cytodex-2-bead motility assay

Cells are pre-coated onto cytodex-2 carrier beads (Sigma) for 2 hours in complete medium. After the medium is aspirated, and the cells washed (X2 in complete medium), they are aliquoted into wells of a 96-well plate in triplicate (300 µl/well). HGF (25 ng/ml) is added, and the cells are incubated over-night. The beads are washed off in medium, and the cells that have migrated onto the floor of the well are fixed in 4% formaldehyde and stained with crystal violet. The cells are then counted microscopically.

2.10 Immunocytochemistry

The two methods employed in our study for the staining of cellular adhesion proteins were immunocytochemistry and immunofluorescence. Both techniques enabled visualisation of sub-cellular location of an antigen with the use of labelled antibodies which were facilitated with either light or fluorescent microscopy. A condensed but succinct description of the techniques is described in the sections below.

2.10.1 Immunocytochemical staining

The primary reagent used in this study for immunocytochemical staining was 3, 3'-diaminobenzidine (DAB; Sigma). DAB is a substrate for peroxidase and in the presence of hydrogen peroxidase (H_2O_2), a brown coloured precipitate is formed. This indicates the presence of a specific protein when used in conjunction with specific antibodies.

Cell lines were grown in 16 well chamber slides (LAB-TEK) until confluent and subsequently treated according to the conditions outlined in the relevant experimental chapters (chapters 3 and 4). The cells were fixed with 4% formaldehyde for 5-10 minutes after aspirating the culture medium from the wells. HBSS buffer (137 mM NaCl; 8 mM NA_2HPO_4; 3 mM KCL; 1.5 mM KH_2PO_4) was used to wash the cells twice. The cells were then permeabilised with 0.1% Triton X-100 (Sigma) detergent for 5 minutes. Cells were again rinsed twice with HBSS buffer, and non-specific protein binding sites were blocked with 10% milk solution (5 gm of semi-skimmed milk powder dissolved in 50 ml of TBS buffer (20 mM Tri-HCl; 150 mM NaCl [adjusted to pH 7.5], containing 50 µl [0.1%] of Tween 20 detergent) for 40 minutes at room temperature on a rotating wheel.

Cells were again rinsed in HBSS buffer, and 100 µl aliquots of primary antibody (details of antibody concentrations used are in relevant chapters 3 and 4) prepared in TBS buffer with 3% milk (1.5 g of semi-skilled milk powder dissolved in 50 ml of TBS buffer) were added to each well. Cells were incubated with the primary antibody for 1 hour at room temperature on a rotating wheel, to ensure

an even coating of the antibody. Cells were washed twice with 3% milk solution and 100 µl aliquots of secondary conjugated antibody, prepared in the same manner as the primary antibody, were added to each well (relevant concentrations in appropriate chapters). Cells were incubated with secondary antibody for 1 hour at room temperature on a rotating wheel, to ensure an even coating of the antibody. Cells were then washed twice initially with 3% milk solution followed by two washes with HBSS buffer. 200 µl aliquots of DAB substrate (25mM Tri-HCl buffer [pH 7.4]; 0.03% H_2O_2; 200 µg/ml of DAB) was added to each well and these were incubated in the dark at 37 ºC for 5 minutes (allowing a brown precipitate to be formed between the DAB and peroxidase). Cells were washed with distilled water, allowed to air dry, and the slide was mounted with styrolite mounting medium (BDH). DAB stained cells were used using a light microscope.

2.11 Immunofluorescence staining

Cell lines were grown in 16 well chamber slides (LAB-TEK) until confluent and treated according to the conditions outlined in the relevant experimental chapters (chapters 3 and 4). The procedure for immunofluorescence staining is as described in the previous section. However, in this protocol, the cells were probed with fluorescent conjugated secondary antibodies. The antibodies are tagged with either fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (TRITC) conjugates. Mounting is performed using FluorSave™ (Calbiochem-Novabiochem Ltd., U.K) mounting reagent. Subsequent viewing is performed using a fluorescent microscope.

2.12 Immunoprecipitation

Immunoprecipitation provides a useful method for the detection and quantification of specific target antigens within cellular lysates. In addition, it is highly sensitive, detecting protein to the tune of around 100 pg. Immunoprecipitation was invaluable in unravelling events during intracellular phosphorylation following extra-cellular stimulation.

The first step in this process involves cell lysis. This is followed by the addition of a specific antibody directed against the target protein present within the cell lysate

sample. The resultant antigen-antibody complexes are harvested by the addition of staphylococcal protein A, which is covalently attached to sepharose or agarose. These immune complexes are precipitated by centrifugation, separated by SDS-PAGE and analysed by immuno-probing.

2.12.1 Preparation of protein for immuno-precipitation

Following adjustment of the protein concentration, appropriate primary antibodies were added to each sample at the appropriate concentrations, in order to precipitate out the desired protein for subsequent detection by immunoprobing (section 2.12.6). The samples were incubated at 4°C for an hour, and this was followed by the addition of A/G protein agarose beads at the appropriate concentrations. Further incubation at 4°C for an hour was followed by micro centrifugation (Biofuge 13, Heraeus Sepatech) at 13000 rpm for 15 minutes. This removes cellular debris and unbound protein complexes form the supernatant faction. The agarose pellets (with antigen-antibody now bound to the bead surface) were washed twice using 200-300 µl of a lysis buffer with the addition of the following ingredients: 10 mM Na_3VO_4, 10 U/ml apotinin, 1 mM phenylmethylsulfonyl fluoride, 1.5% Triton X-100, 0.1% IGEPAL CA-630 and 4 mM CaCl2. The pellets were suspended in 40-60 µl of sample buffer (10% glycerol; 5% 2-mercaptoethanol; 3% SDS; 80 mM Tris-HCl [pH 6.8]; 0.012% bromophenol blue, and denatured under reducing conditions by boiling at 100°C for 5 minutes. The samples were then stored at -20°C until ready to use for SDS-PAGE.

2.12.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to determine the expression of cell adhesion molecules at the protein level in the cancer cell lines used in this study, and was based on the method used by Laemmli (1970). The technique works on the basis that when a protein sample is heated in the presence of a reducing agent such as 2-Mercaptoethanol (2-ME; Sigma) SDS, the protein becomes denatured with subsequent protein coating from SDS. This coating gives the protein a high negative charge which is proportionate to the length of the polypeptide chain.

The samples are then loaded onto a polyacrylamide gel, and a high voltage is applied; this causes the protein components to migrate in the direction of the positively charged anode. The ionic detergent SDS binds to proteins in proportion to their molecular weights and this binding is independent of their sequence. Furthermore, the resultant protein-SDS complexes are then separated on the basis of their molecular weights, and show different mobilities due to sieving properties of the polyacrylamide gel matrix. The size of the separated proteins can then be determined by comparing the gel mobilities of a particular band, with that of a known protein standard. Alternatively, a plot of molecular weight from known protein standards can be plotted against their mobility, in order to determine the size of unknown protein bands. Following electrophoresis, proteins are electro blotted onto nitrocellulose membranes for subsequent probing with relevant antibodies.

2.12.3 Preparation of polyacrylamide gels for SDS-PAGE

Electrophoresis using a polyacrylamide gel is the most commonly used method employed for the separation of proteins from cell and tissue lysate samples. The electrophoretic procedures are relatively rapid and sensitive enough for the detection of protein size. Acrylamide gels (30% acrylamide mix, crosslinker ratio 29:1; Bio-Rad) are formed by polymerising acrylamide with a cross linking agent (methylene-bis-acrylamide) in the presence of a catalyst/chain initiator mixture, producing a cross linked matrix with a particular pore size. The most commonly used initiator is the persulfate ion (s208-) obtained in the form of ammonium persulfate (Sigma), and the catalyst used most frequently is TEMED (N,N,N,N-tetramethylethylenediamine; Sigma). Varying the concentration of the 2 components allows control at which rate the gels set. The relative proportions of the acrylamide monomer and the cross linking agent determine the porosity of the gel.

Gels are usually referred to in terms of the total percentage of acrylamide, monomer and bis present. Most protein separations are carried out using gel concentrations of between 5 and 15%. The appropriate choice of acrylamide concentration used in this study was based on the separation range required for the protein. Therefore, a low percentage gel (large pore size) would be used if one

were dealing with proteins of a high molecular weight, and conversely, a high percentage gel (small pore size) would be used if one were to separate proteins of low molecular weights. The table below outlines the reagents used and concentrations employed in preparing the acrylamide gels used in this study.

Table 2.2 Preparation of SDS-PAGE Gel

Resolving gel composition (10mls)	Strengths (mls)	Strengths (mls)
	10%	8%
Distilled Water	4.0	4.6
30%Acrylamide/ 0.8% Bisacrylamide	3.3	2.7
1.5M Tris (pH = 8.8)	2.5	2.5
10% SDS	0.1	0.1
10% APS	0.1	0.1
TEMED	0.004	0.006

Table 2. 3 SDS-PAGE resolving and stacking gel components. The volumes shown are for 10ml (resolving gels) and 2 ml (stacking gels) respectively.

Stacking gel composition (2mls)	Strengths (mls)
	5%
Distilled Water	1.4
30% Acrylamide/ 0.8% Bisacrylamide	0.33
1.0M Tris (pH = 6.8)	0.25
10% SDS	0.02
10% APS	0.02
TEMED	0.002

SDS PAGE was carried out by assembling the gel (Mini Protean II gel system) apparatus according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond CA, USA).

The appropriate concentration of resolving gel was chosen and prepared as described in the table above. A 1 ml transfer pipette was used to add the resolving gel mixture to the gel system by carefully running the gel solution down one side between the glass plates. The solution was added until it reached a position of 0.3 cm from the cut-away edge of the gel plate. To ensure that the gel set with a smooth surface, a 0.1% solution of SDS was run down the side between the glass plates until a layer of about 2 mm formed on top of the gel solution (the great difference in densities between the SDS solution and the gel resulted in the SDS solution resting on the surface of the gel mixture without causing any untoward mixing of the two solutions). This was allowed to set for 30 minutes. When this was achieved, a very clear change in the refractive index was observed between the polymerised gel and the overlaying SDS solution. The excess SDS solution was absorbed using a filter paper and the gel rinsed once with distilled water. The stacking gel was then prepared as outlined in the previous table. Its purpose in SDS PAGE is detailed below.

2.12.4 The use of stacking gels in SDS-PAGE

The use of SDS gels enables protein samples to be directly applied onto the top of the resolving gel in which separation of the protein is to occur. However, in these cases, the sharpness of the protein bands produced in the gel is limited by the size (or its volume) of the sample applied to the gel. This problem may be overcome by polymerising a short stacking gel on the top of the resolving gel. The stacking gel allows samples to be electrophoretically concentrated into a thin band during the initial stages of electrophoresis. Furthermore, it allows proteins to stack resulting in sharper protein bands forming in the resolving gel. This modification to SDS-PAGE allows relatively large sample volumes to be applied to the gel without any loss of resolution.

The underlying principle of sample concentration within the stacking gel is based on isotachophoresis of the protein sample. The band sharpening effect relies on

variable electrophoretic mobility of the constituents. Thus, the negatively charged glycinate ions (in the reservoir buffer) have a lower electrophoretic mobility than the protein-SDS complexes, which in turn have a lower mobility than the Cl^- ions present in both the loading buffer and the stacking gel. When the current is turned on, all the ionic species have to migrate at the same speed in order to maintain the electric circuit. The glycinate ions can only move at the same speed as the Cl^- ions if they are in a region of higher field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The net result is that the three species of interest adjust their concentrations so that (Cl^-) > (protein-SDS) > (glycinate). There is only a small quantity of protein-SDS complexes, and hence they concentrate into a very tight band between the Cl^- and glycinate boundaries. Once the glycinate ions reach the resolving gel, they become fully ionised and their mobility increases within the higher pH environment. Therefore, the interface between the glycinate and the Cl^- ions leaves behind the protein-SDS complexes which are then left to electrophorese at their own rates.

In the present study, the stacking gel was added to the gel cassette in the same manner as the resolving gel until it reached the cut-away edge of the gel plate. A well forming comb was gently inserted between the glass plates until it reached within 1mm from the top of the resolving gel, and then left to set at room temperature. The refractive changes around the comb indicate that the gel had set. The comb was carefully removed from the stacking gel, and the gel cassette was assembled in the electrophoresis tank as per the manufacturer's instructions (Bio-Rad Laboratories, Richmond, California, USA).

The central reservoir was then filled with the running buffer (25mM Tris-HCl; 3.5 mM SDS; 192 mM glycine) so that it flooded over and filled the wells. The bottom of the electrophoresis tank was filled with about 300 ml of running buffer. The previously prepared protein samples were loaded into the wells (10-15µl per well). This was done by placing the loading gel tip through the buffer and locating it just above the bottom of the well. The sample was slowly delivered into the well until it was full. The protein samples were pre-mixed with a dense loading buffer (10% glycerol; 5% 2-mercaptoethanol; 3% SDS; 80 mM tris-HCl

[pH 6.8]; 0.012 bromophenol blue) which ensured that the sample settled at the bottom of the well. This procedure was continued until all the samples had been loaded onto the gel. The protein standard or high molecular weight markers were prepared according to the manufacturer's instructions (detection range 29-205 kDa) and loaded (10µl/well) in the same manner as the protein samples. The power pack was connected to the apparatus, and the gel was run at a constant current of 15 mA per gel. Electrophoresis was continued until the samples had reached the bottom of the gel (as indicated by the bromophenol blue present in the loading buffer).

2.12.5 Western Blotting of proteins onto nitrocellulose membranes

This technique, also called Electroblotting, involves the immobilisation of protein antigens which have been separated by SDS-PAGE. Proteins are transferred onto an inert membrane support (eg., nitrocellulose or nylon). Once attached, the protein of interest may be detected by a specific monoclonal or polyclonal antibody against it. Following SDS-PAGE, the gel was removed from the gel cassette and rinsed in transfer buffer (25 mM Tris-HCl; 192 mM glycine; 20% [v/v] methanol) to facilitate the removal of electrophoresis buffer, salts and detergents. The gel was then left to equilibrate in transfer buffer for 10-20 minutes. Simultaneously, nitrocellulose membrane (Amersham, International plc) and filter paper were cut to the dimensions of the gel (9x6 cm) and soaked in transfer buffer for 10-20 minutes. This ensures proper binding of the protein to the membrane. Electroblotting of the proteins was carried out using the following procedure:

1. Two pieces of wetted filter paper were placed on the bottom electrode (cathode), and on top of this was placed one piece of pre-wetted nitrocellulose membrane.
2. The gel was carefully transferred on top of the nitrocellulose membrane, and this was then covered by two further pieces of wetted filter paper, forming a sandwich
3. The surface of this sandwich was carefully smoothed out using a transfer pipette as a rolling pin. This prevents the formation of air bubbles which may interfere with protein transfer.

4. The second electrode (anode) was applied to the top of this sandwich, and a current was applied.
5. The proteins were then transferred for 30-40 minutes (depending on their size) using the following settings: 5 Volts, 500 mA and 8 Watts.

Following electroblotting, the nitrocellulose membranes were removed and stained in Ponceau S (40 ml of 2% w/v Ponceau S concentrate containing 30% w/v trichloroacetic acid and 30% w/v sulphosalicyclic acid (Sigma) was diluted with 360 ml of distilled water to provide a 1:10 working solution) for one minute at room temperature. The staining of proteins on nitrocellulose membranes using Ponceau S has two functions: firstly, it verifies that the proteins have been transferred to the membranes, and secondly, it aids in the visualisation of the molecular markers. Additionally, Ponceau S stain is a reversible protein stain, and does not interfere with subsequent immuno-probing steps. Excess stain was then removed by rinsing in distilled water, and the position of the molecular weight markers were indicated on the membranes using a pencil.

2.13 Electrode Cell-substrate Impedance Sensing (ECIS)

The ECIS core technology is based on a technique of measuring the change in impedance of a small electrode to AC current flow. The heart of the measurement is a specialized slide that has 8 individual wells for cell culturing. The base of the device has an array of gold film electrodes that connect the ECIS electronics to each of the 8 wells [Figures 2.1 - 2.5]

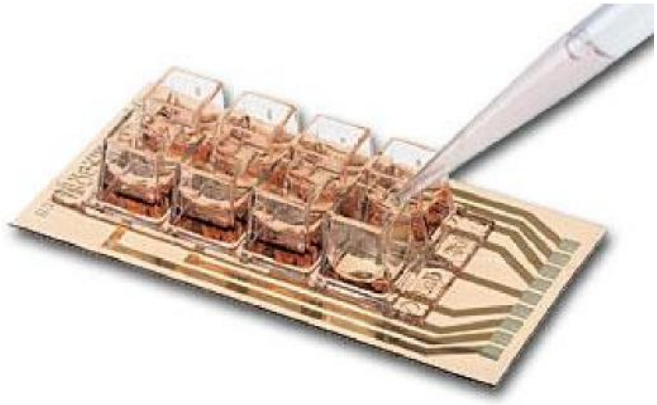


Figure 2. 2 8-Well Electrode Array. Adapted from (www.biophysics.com)

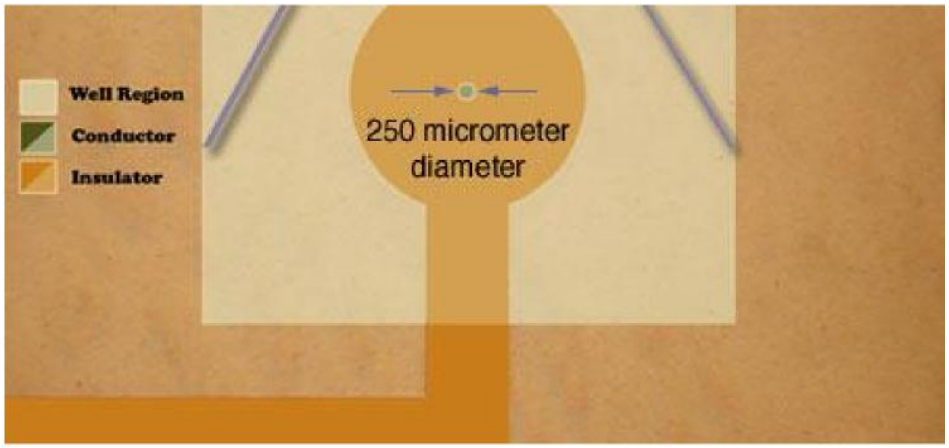


Figure 2. 3 Array well detail. Adapted from (www.biophysics.com)

Array with the wells removed showing more clearly the patterns of gold and insulating films. The current flows between a 250 µm diameter electrode and a larger counter electrode using normal culture medium as the electrolyte.

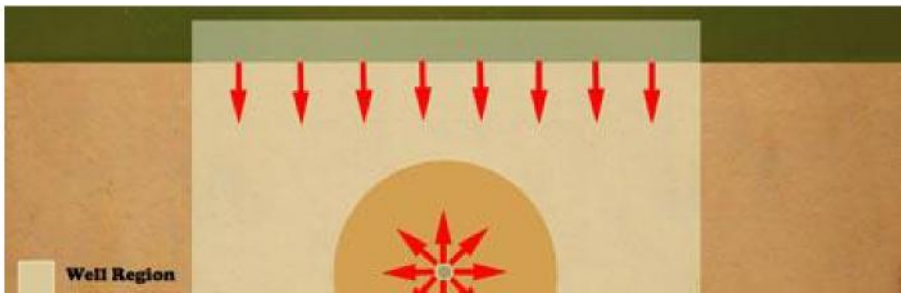


Figure 2. 4 Schematic representation of current flow. Adapted from (www.biophysics.com)

Electrode top view indicating AC current flow between the small active electrode and the counter electrode. Without cells, the current flows unrestrained from the surface of the electrodes.

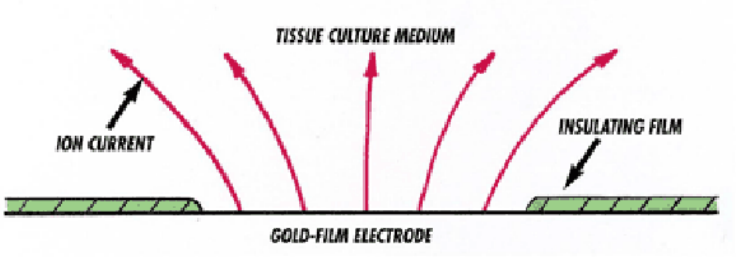


Figure 2. 5 Electrode cross section. Adapted from (www.biophysics.com)

With cells attached and spread upon this region, the current must now flow in the spaces under and between the cells, as the cell membranes are essentially insulators.

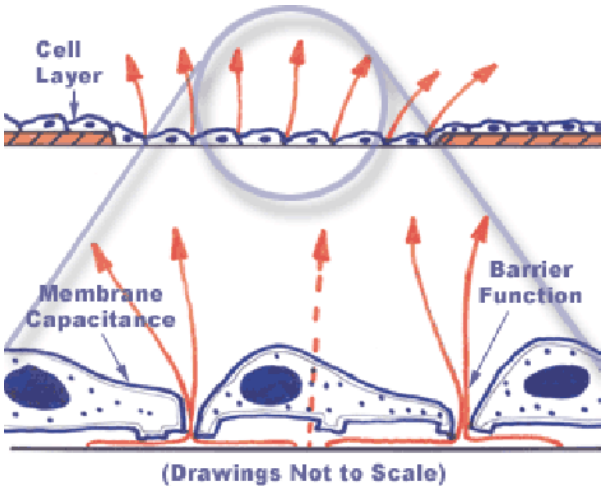


Figure 2.6 Micrograph of the gold electrode. Adapted from (www.biophysics.com)

This is a micrograph of the 250 µm diameter gold electrode. The shaded-hatched area outside the circular electrode is an insulating film that defines the electrode perimeter. Both regions are excellent substrates for cell culture and essentially mimic the surfaces of normal tissue culture ware. At the start of the measurement, the electrode has no cells attached to it and the resistance is about 2000 ohms [Figure 2.6]. Upon inoculation, cells anchor and spread on the base of the well including the active 250 µm electrode. With the presence of the cells, their insulating plasma membranes constrain the electrical current and force it to flow in regions beneath and between the cells. This convoluted current path causes large changes in the measured impedance. Although this is taking place at both the small electrode as well as at the counter electrode, the impedance of the small electrode is several hundred times larger, and so the contribution of the large counter electrode is a fraction of a percent and can be ignored. In addition to the overall increase in the impedance, small fluctuations can be easily observed, because the live cells continuously alter their morphology and hence the impedance. With the confluent cell layer in place, the resistance now has reached nearly 15,000 ohms. It is important to note that the AC current used in making these measurements

(approximately 1 microampere) and the resulting voltage drops across the cells (a few millivolts) has no detectable effects upon them; the measurement is non-invasive.

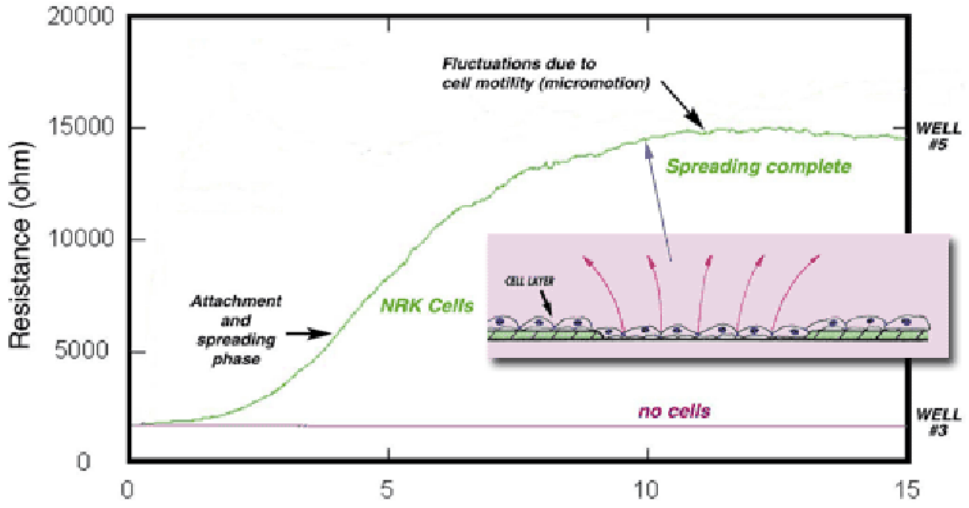


Figure 2.7 Representative Graph generated during ECIS Adapted from (www.biophysics.com)

This technique is a patented technology known as ECIS^{TM}, an acronym for Electric Cell-substrate Impedance Sensing. Cell densities ranging from a heavy confluent layer to very sparse layers can be measured with this approach. The size of the electrodes restricts the maximum number of anchored cells that can be observed from 100 to 1000 cells (dependent upon the type of electrode array used in the instrument), but even a single isolated cell results in impedance changes that can be monitored.

2.13.1 Impedance Measurement

ECIS measurements are made using an AC signal, where the current oscillates in a sinusoidal manner from as low as a few hundred to nearly 100,000 Hz in different measurements. Using AC instead of DC signals to monitor the cells upon electrodes has two important consequences. First, an AC source is used such that the electrolytes in the culture media are not deposited upon the electrode causing the properties of the electrodes to change or polarize. The other important advantage conferred by using an AC signal is described below.

Up until now, reference has only been made to impedance, the AC equivalent to resistance, and this is often the only parameter one may wish to follow to study many aspects of cell behaviour. If one were to apply the AC current (I) to the electrode system and measure the resulting voltage (V) across the electrodes, the impedance (Z) is simply given by the AC equivalent of Ohm's law:

$$Z = V / I$$

The ECIS Zθ instrument, however, is capable of monitoring both the voltage and the phase of the voltage relative to the current. Combining these parameters, the impedance can be broken down into two parts -- one due to pure resistance and the other to the reactance of the system. The reactive part (X_c) in this case is due to the capacitance (C) associated with the metal surfaces in the tissue culture medium (the electrolyte).

We have elected to represent the signal received from the ECIS electrodes as a simple resistor and capacitor in series.

For this simple RC circuit, the impedances of each of the circuit elements are given by:

R=V(in phase)/I

Xc=V(out of phase)/I

and the total impedance is given by,

$Z = (R^2 + X_c^2)\ 0.5$

Xc (the capacitive reactance in ohms) depends upon the AC frequency (f) is given by:

$Xc = 1/(2\Pi\ f\ C)$

Since we know the frequency, we can obtain the capacitance (C) from this term.

With this information, it is possible to state more regarding the cells than simply the time changes in impedance. We, of course, can now report changes over time in the pure resistive (R) as well as the capacitive portions (C) of the impedance, and these are very useful. We shall also see that these data can be further refined using a model that gives back information on the barrier function of cell layers, the spacing beneath the cell and its substratum, and the capacitance of the cell's plasma membranes.

2.13.2 AC Phase measurements

The ECIS Zθ applies an approximately constant current through the ECIS electrodes of about 1 microampere or less. This current result in a voltage across the electrodes that varies in a sinusoidal fashion at the same frequency as the applied current. Were this simply a measurement of a pure resistance, the two sine waves- that of the voltage and of the current-would be exactly in phase -- the waveforms would have different values, but would coincide exactly.

Were this a measurement of a pure capacitance, the two sine waves would be said to be 90 degrees (one quarter wavelength) out-of-phase -- the voltage lagging the current. In this case, when the current is at its peak, the voltage is zero, and when the current is at zero, the voltage is at a peak. We refer to the voltage in this case as the out-of-phase voltage.

In the actual ECIS measurement, since we have resistance and capacitance, the voltage and current are somewhere in between these two situations. The ECIS instrumentation in the 1600R model -the model used in this study- measures this phase difference and breaks this voltage down into two pure in and out-of-phase components that together -add up to the actual signal. These are the voltages used in finally calculating the resistance and capacitance of the ECIS electrodes

2.13.3 Time Course Measurements

The impedance can be sampled as often as several times per sec to as slowly as desired. Each impedance reading is plotted as a point, in Ohms or Nano-farads (nF), versus time. The total acquisition time is user controlled and can range from

a fraction of an hour to several days. The following graph [Figure 2.7] is a measurement of cells attaching and spreading in two different wells of an array. Curve no.4 are BCS cells and curve no.5 are NRK (Normal Rat Kidney) cells. The electrode in each well was sampled at a few second intervals for 15 hours.

Following inoculation at time zero, impedance increases as the cells attach to the electrode and begin spreading. The impedance continues to increase until the cells reach confluence at 2 hours for the BCS cells and 10 hours for the NRK cells. The small fluctuations in the curves are due to micro motion from the constant movement of the monolayer of cells on the electrode.

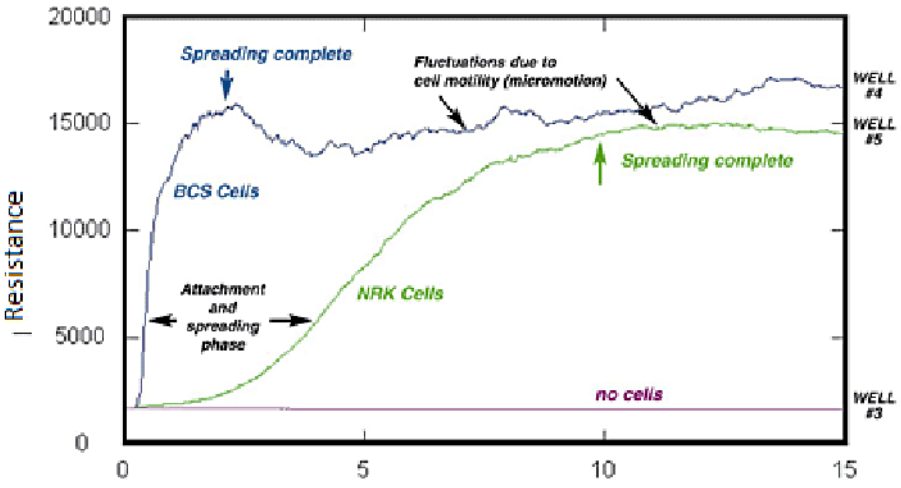


Figure 2.8 Representative Graph generated during ECIS-Time Course Measurements. Adapted from (www.biophysics.com)
The above graph is a measurement of cells attaching and spreading in two different wells of an array. Curve no.4 are BCS cells and curve no.5 are NRK cells. The electrode in each well was sampled at a few second intervals for 15 hours.

2.13.4 Wounding/Electroporation

With the wounding option, an elevated voltage is applied to the electrode for several seconds. The cells on the electrode are killed, presumably from severe electroporation. Closer analysis of the graph reveals that the impedance falls back from its peak value at cells confluence to that of an open electrode because the cells no longer offer a resistance to the current flow.

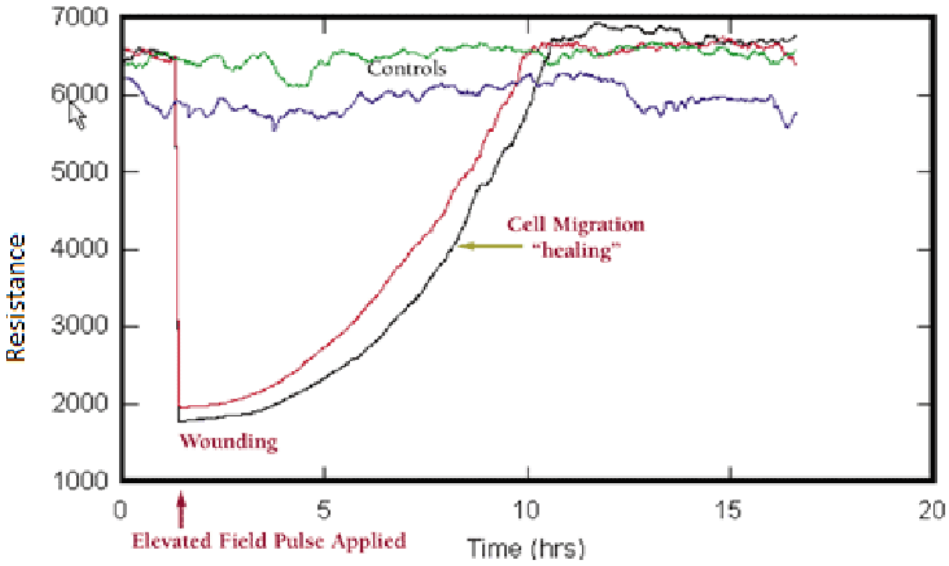


Figure 2. 9 Representative Graph generated during ECIS-Wounding study Adapted from (www.biophysics.com)

The time and intensity of the elevated voltage can be user controlled and reduced so the cells can also be electroporated without cell death.

2.14 Statistical Analysis

Statistical analysis was performed by MINITAB version 13.32 (Minitab Inc. State College, PA, USA) using a two-sample student t-test and the non-parametric Mann-Whitney confidence interval and test, where appropriate. Statistical analysis was carried out using Mann-Whitney U test and the Kruskal-Wallis test for tissue samples and the Pearson Correlation between related molecules.

Chapter 3

Expression of NWASP [Neuronal Wiskott Aldrich Syndrome Protein] Gene Products in Human Breast Cancer

3.1. Introduction

Wiskott-Aldrich Syndrome [WAS] is an X-linked recessive condition with immunodeficiency as the underlying problem. Currently, it is characterised by the triad of recurrent bacterial infections of the sinuses and lungs, eczema that appears atopic in nature, and a bleeding tendency due to thrombocytopenia and platelet dysfunction.

The exact function of NWASP is not fully elucidated, but it seems to function as a bridge between signalling and movement of the actin filaments in the cytoskeleton. It has been recognized that the Wiskott-Aldrich protein provides a link between CDC42, a member of the RHO family of GTPases and the actin cytoskeleton (Symons et al., 1996). The WAS protein interacting with GTP-dependent CDC42 was detected in cell lysates in transient transfections, and with purified recombinant proteins (Kolluri et al., 1996), indicating that the WAS protein could function as a signal transduction adaptor downstream of CDC42, and that cells with 5 domains involved in signaling, cell cytoskeletal abnormalities may result from a defect in CDC42 signaling.

NWASP has been found to be a key regulator of actin polymerization in hematopoietic motility/migration, in immune synapse formation and in facilitating the nuclear translocation of nuclear factor kappaB (Ochs and Thrasher, 2006, Welch and Mullins, 2002). Mutations of NWASP are located throughout the gene and either inhibit or dysregulate normal NWASP function. Classic NWAS occurs when NWASP is absent, X-linked thrombocytopenia is present when mutated NWASP is expressed, and X-linked neutropenia occurs when mis-sense mutations occur in the CDC42-binding site (Ochs and Thrasher, 2006, Notarangelo et al., 2002).

Miki and co-workers first described a 65 kDa protein from brain that bound to the SH3 domains of Ash/Grb2 (Miki et al., 1996). The sequence was homologous to Wiskott-Aldrich syndrome protein (NWASP) and was designated NWASP [neural-WAS]. Stimulated actin assembly is responsible for membrane ruffling (Zalevsky et al., 2001), a process that actively involves the cytoskeletal associated protein family ERM [ezrin-moesin-radixin] (Bretscher, 1989, Hiscox and Jiang,

1999), and is, therefore, important for changes in cell motility and spread. We have recently reported that two major complexes that are linked to the NWASP family, namely the ERM family and RhoGTPases are aberrantly expressed in human breast cancer (Martin et al., 2003, Jiang et al., 2003).

Presumably, NWASP mutations interfere with the proper signalling and growth of cells of the haematopoietic lineage, resulting in the platelet and immune defects observed clinically, although the exact mechanisms and defective pathways remain largely unknown. The aim of this part of the study was to determine which cells found in breast tissue express NWASP. In order to determine which cell types in breast tissue express these molecules, it was necessary to use other cell lines in addition to breast tissue. Breast tissue contains epithelial cells lining the ducts, stromal cells including fibroblasts, endothelial cells lining the blood and lymphatic vessels, components of blood (red and white blood cells and platelets), and nerve cells. The following representative cell lines were chosen: MDA-MB-231, MRC-5, MCF7 and human umbilical vein endothelial cells (HUVECs). These cells are described below.

MRC-5: These cells are fibroblasts which originated from the normal lung tissue of a 14 week old male foetus. These cells replicate rapidly, making them useful for cell culture.

MCF7: This cell line consists of human breast adenocarcinoma cells that were originally obtained from the pleural tap of a 69 year old female Caucasian. The cells exhibit some of the features of differentiated breast epithelium including oestriol synthesis. They are oestrogen receptor positive.

MDA-MB-231: These are also human breast adenocarcinoma cells extracted from a pleural effusion. They are highly anaplastic cells, which form grade 3 tumours in nude mice. They are oestrogen receptor negative.

HUVECs: These cells, as their name indicates, derive from human umbilical vein taken from a full term baby shortly after delivery. The lumen is emptied of blood. Various enzymes, including trypsin and collagenases, are infused into the vein. These enzymes cause the endothelial cells to become detached from their

underlying basement membrane. The cells are subsequently collected and purified. HUVECs are, therefore, relatively immature cells which express a variety of endothelial markers.

3.2 Materials and Methods

RNA extraction and subsequent synthesis of equal amounts of DNA were performed using RNA extraction (RNAZol) and Avian Reverse Transcription kits which were obtained from Abgene Ltd (Epson, UK). PCR primers were designed using Beacon Designer software (Palo Alto, California, USA) and synthesised by Invitrogen Ltd. (Paisley, UK). Molecular-biology grade agarose and DNA ladder were from Invitrogen. Master mix for routine PCR and quantitative PCR was from Abgene.

Breast Cancer cell lines (MCF-7, MDA-MB-231) were obtained from European Collection of Animals Cultured Cell (EACC, Salisbury, England). Cell lines were routinely maintained in DMEM1 F12 medium (Cambrex bioscience, Verviers Belgium) supplemented with 10% foetal calf serum (PAA Laboratories, Yeovil, UK) and antibiotics (Penicillin with Streptomycin).

3.2.1 Cell culture conditions

Control cells were grown in DMEM F12 medium (supplemented with 10% foetal calf serum and antibiotics). NWASP transfected breast cancer cells ($MDA^{MBNWASP+}$) were grown in medium containing amoxycillin derivative to which $MDA^{MBNWASP+}$ cells carried the resistance marker. Cell cultures were routinely incubated in a humidified incubator maintained at 37°C.

3.2.2 Collection and storage of cells

The cells-153 frozen archival breast samples from 127 patients- were collected between June 1991 and July 1997 with the approval of the local ethical committee (BroTaf Health Authority and South East Wales Local Research Ethics sub-committees). All tissues were randomly numbered, and the details were only made known after all the analyses were completed. Frozen sections were cut from each

specimen and 25-30 adjacent 10μm sections were subsequently used for RNA extraction.

3.2.3 RNA extraction and RT-PCR for NWASP detection in breast cancer

Total cellular RNA was isolated from either cell line monolayers or homogenised breast tissues using RNA-Zol reagent (Abgene, Epsom, UK) according to manufacturer's instructions. cDNA was generated from 1μg of RNA using the AMV-reverse transcription kit (Abgene, Epsom, Surrey, UK). Conventional PCR Primers were designed using Beacon Designer software (Paolo Alto, California, USA) to allow amplification of regions that have no overlap with known genes and span at least one intron. PCR was carried out using the following Primer pairs:

NWASPEXF1 atgagctccgtccagcag (forward primer)
NWASPEXR1 tcagtcttcccactcatcatc (reverse primer)

PCR reactions were performed using a GENE Amp PCR system 9700 (PE Biosystems, Warrington, UK) thermal cycler. Amplification of cDNA was carried out at the following conditions: 94ºC for 15 seconds, 56ºC for 15 seconds, 72ºC for 45 seconds, followed by a final extension phase of 7 minutes at 72ºC for 36 cycles. The PCR products, including a 1-KB ladder were separated on 0.8% agarose gels. PCR products were then visualised using ethidium bromide (10mg/ml) and a UV transilluminator (UVItec, Cambridge, UK). Resulting stained agarose gels were photographed using a Unisave camera (Wolf Laboratories) and subsequent images documented using Adobe Photoshop Elements software.

3.2.4 Real Time Quantitative PCR analysis of NWASP in breast cancer

The quantity of NWASP transcripts were assessed using RT-PCR and RT-QPCR. RT- QPCR was carried out using iCycler IQ^{TM} system (Bio-Rad, Camberley, UK), which incorporated a gradient thermocycler and a 96-channel optical unit. Universal probe system (UniPrimer ^{TM}) was used in this study (Intergen, Oxford, UK). The internal standards used in this study were specific plasmids generated using Pcr2.1-cloning vector (Invitrogen, Paisley, Scotland). Conditions for the

QPCR were as follows: 50 cycles of denaturing at 95ºC for 15seconds; annealing at 54ºC for 20 seconds and extension at 60ºC for 40 seconds.

3.2.5 Immunofluorescent staining of NWASP protein

For immunofluorescence staining, cells were grown in 16- well chamber slides (LAB-TEK) (30,000 cells/well) in the presence or absence of HGF (50 ng/ml) and incubated in a 37ºC 5% CO_2 incubator for a set period of time (0–24 h). After incubation, the culture medium was aspirated, the wells rinsed with balanced salt solution (BSS) buffer and the cells fixed in methanol for 20 min at 20ºC. After fixation, the cells were washed twice using BSS buffer and permeabilised by the addition of 200 µl of 0.1% Triton X-100 (Sigma) detergent in Phosphate buffered solution (PBS) for 5 min at room temperature. Cells were rinsed twice with BSS buffer and 200 µl of blocking buffer (10% horse serum in TBS) was added to each well, and the chamber slide incubated for 40 min at room temperature on a bench rocker. The wells were washed once with wash buffer (3% horse serum in TBS buffer containing 0.1% Tween20) and 100 µl of primary antibodies prepared in wash buffer was added to the appropriate wells. The chamber slide was incubated on the rocker for a further 60 min at room temperature. Wells were washed twice with TBS buffer (with 0.1% Tween20), and cells were incubated in 100 µl of secondary antibodies (TRITC) (diluted in the same manner as the primary antibodies) for 50 min. The chamber slide was wrapped in foil to prevent light reaching the conjugate. Finally, the wells were rinsed twice with wash buffer, once in BSS buffer mounted with FluorSave (Calbiochem-Novabiochem Ltd, Nottingham, UK) reagent and visualised using an Olympus BX51 microscope with a Hamamatsu Orca ER digital camera at 9100 using oil immersion lens.

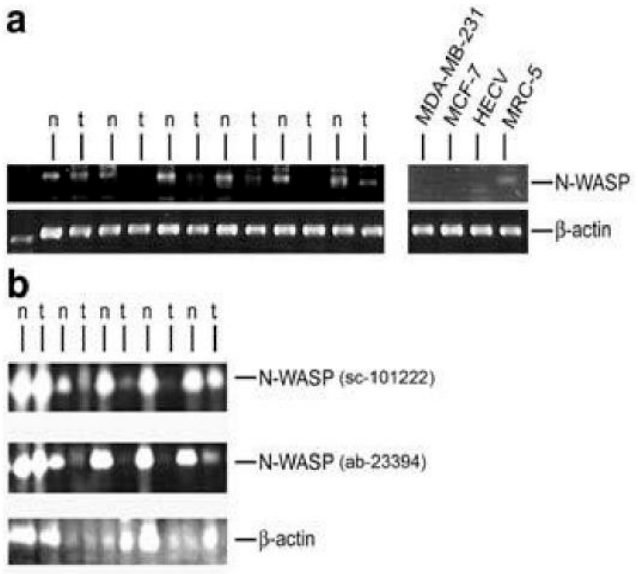
Total tissue or cell lysates were prepared as follows: cell or tissue were lysed in HCMF buffer plus 0.5% SDS, 0.5% Triton X-100, 2 mM CaCl2, 100 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 10 mM sodium orthovanadate for 40 min, sample buffer was added and the protein boiled at 100˚C for 10 min before clarification at 13000g for 10 min. Equal amounts of protein from each sample were added onto an 8% polyacrylamide gel. Following electrophoresis, proteins were blotted onto nitro-cellulose sheets and blocked in

10% horse serum for 60 min before probing with primary antibodies, following with peroxidase-conjugated secondary antibody (1:2,000). Protein bands were visualised with Supersignal West Dura Extended Duration Substrate chemiluminescent system (Perbio Science UK Ltd., Cramlington, UK) and detected using a CCD UVI prochemi system (UVItec Ltd., Cambridge, UK).

3.3 Results

3.3.1 Expression of NWASP in normal breast and breast cancer tissues.

We screened two breast cancer lines (MDA-MB 231, MCF 7) and two other cell lines (MRC 5 and HUVECs) to assess the expression of NWASP using RT-PCR. The mRNA level of NWASP in these cell lines are shown in Fig. 3.1a and Fig.3.2. From the initial RT-PCR experiments, it became evident that most of the tumour cells exhibited low levels of NWASP transcript. Further confirmation was obtained by using two NWASP antibodies and confirming the lower protein expression in the same tissue samples using Western blotting technique (Fig. 3.1b), β-actin was used as a house keeping control.



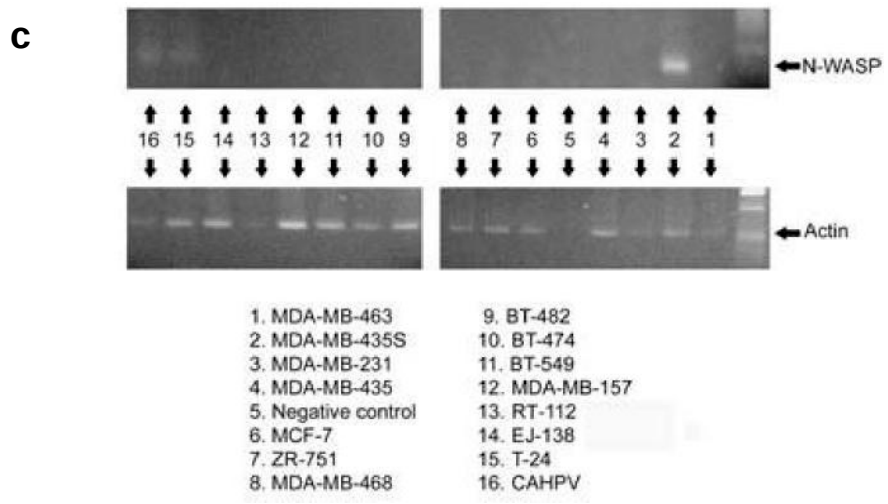


Figure 3. 1 Expression of NWASP in cancer cell lines and in breast tissues.
(a) Tumour tissues showed low levels of NWASP transcript as shown by RT-PCR. (b)This is supported by the reduced protein expression in the same tissue samples using Western blotting (2 NWASP antibodies used for confirmation). β-actin is shown for comparison. The figures (a) and (b) refer to a different set of patients; i.e., they are not matching pairs. (c) The human breast cancer cell lines [3 (MDA-MB-231) and 6 (MCF 7)] included in the screening did not show a signal for NWASP as demonstrated using RT-PCR. Note: the other numbered cells were lines being concomitantly studied in the department and are not part of this research work.

3.3.2 Correlation of NWASP gene transcript with clinical and pathological features in Breast cancer using Q-RTPCR

The levels of NWASP transcript in tumour and normal tissues were compared. Figure 3.2 shows a significant difference in the level of NWASP transcript expressed in normal breast tissue and breast cancer tissue. This difference was statistically significant [$p<0.05$]. In the current study, the Nottingham Prognostic Index (NPI) was used as a prognostic indicator, taking into consideration the size of tumours, number of lymph nodes and tumour grade. Patients were divided into those with good prognosis (NPI-1), with moderate prognosis (NPI-2) and those with poor prognosis (NPI-3). There was a stepwise decrease of the levels of N-NWASP transcript from good to poor prognosis, with the difference between good and poor prognoses being statistically significant (Fig. 3.3). The difference between moderate and good, or moderate and poor did not reach statistical significance ($P<0.05$). The Kruskal Wallis test of the levels of NWASP transcript against the prognostic index did not reveal a significant correlation. This suggests

that a linear correlation between these two parameters does not exist. However, overall, the very low levels indicate poor prognosis. There was an observable difference between node negative and node positive tumours (Fig. 3.3b) but again, statistical significance was not reached.

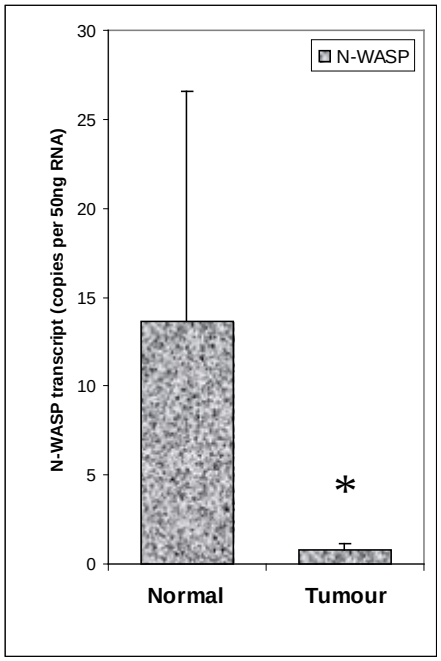


Figure 3.2 NWASP transcript in mammary tissues, number of NWASP transcripts/50 ng RNA. The levels of NWASP transcript were significantly lower in breast cancer tissues compared with normal background mammary tissues * $P < 0.05$ [Mann Whitney U Test].

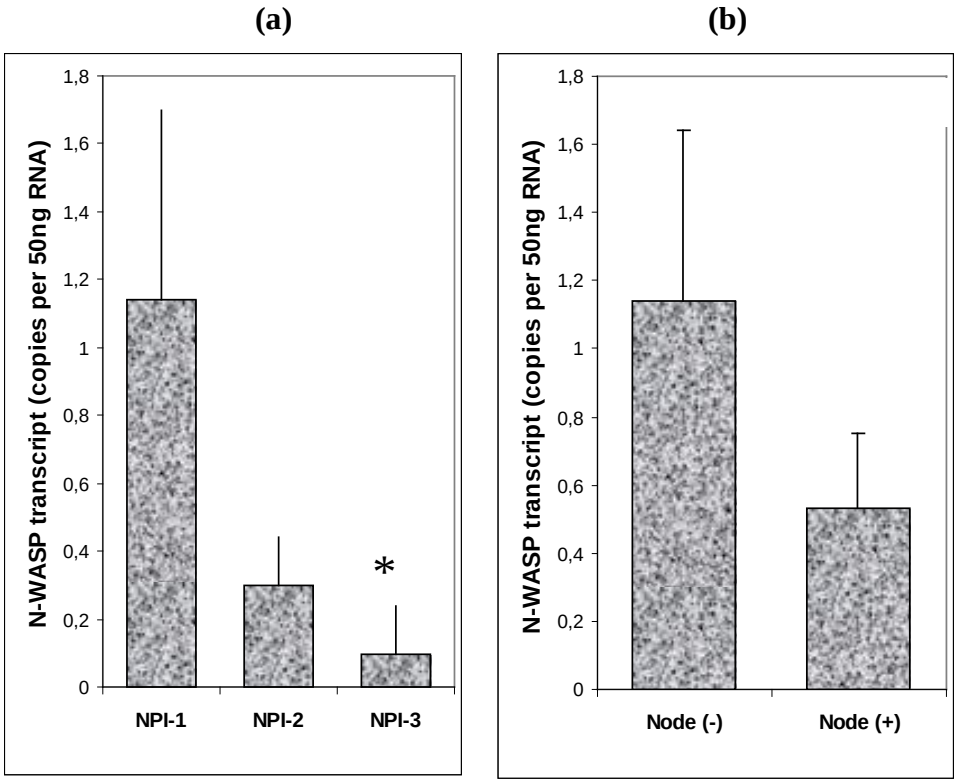


Figure 3.3 NWASP transcript levels were associated with Nottingham Prognostic Index (a) and Nodal involvement (b) NPI3 (NPI > 5.4) tumours had significantly lower levels of NWASP than those of NPI1 status (NPI < 3.4), * p < 0.05.

Table 3.1 Transcript levels of NWASP in human breast cancer tissues; TNM staging, Grade and tumour type

Prognostic Indicator		
TNM staging		
	TNM 1	0.788 ± 0.443
	TNM2	1.116 ± 0.584
	TNM3	0.605 ± 0.406
	TNM4	0.198 ± 0.124
Grade		
	1	0.279 ± 0.2
	2	0.253 ± 0.07
	3	1.45 ± 0.68
Tumour type		
	All	0.83 ± 0.303
	Ductal	0.932 ± 0.38
	Lobular	0.0531 ± 0.04
	Others	1.351 ± 0.813

3.3.3 Levels of NWASP transcript correlated with clinical outcome

The current analysis was based on a patient follow-up period of 72 months. It revealed that patients with metastatic disease and patients who died of breast cancer (excluding those whose died of diseases unrelated to breast cancer) had significantly lower levels of NWASP than those who remained disease free (Fig. 3.4).

Although patients with local recurrences also had lower levels of the transcript, the difference is yet to be statistically significant (P = 0.06). In addition, survival

curves showed that higher levels of NWASP expression were associated with disease- free survival, P = 0.0498 (Fig. 3.5a) but that decreased levels of NWASP expression were associated with poor overall survival in patients with breast cancer (Fig. 3.5b). We did not find a significant correlation between tumour grade, TNM staging and histological type (Table 3.1).

NWASP transcript is one variable in a disease [breast cancer] that has a number of factors which influence survival. Therefore, it is possible that Fig. 3.5 represents patients who died of breast cancer [with metastases as it does not state that they did not have metastases, merely that they died of breast cancer] because of other poor prognostic variables of the disease [not looked at or accounted for in this study]. The small number of patients in each category may have also introduced an artificial bias accounting for the visualised discrepancy in the transcript levels between patients with metastatic disease and those having died from breast cancer.

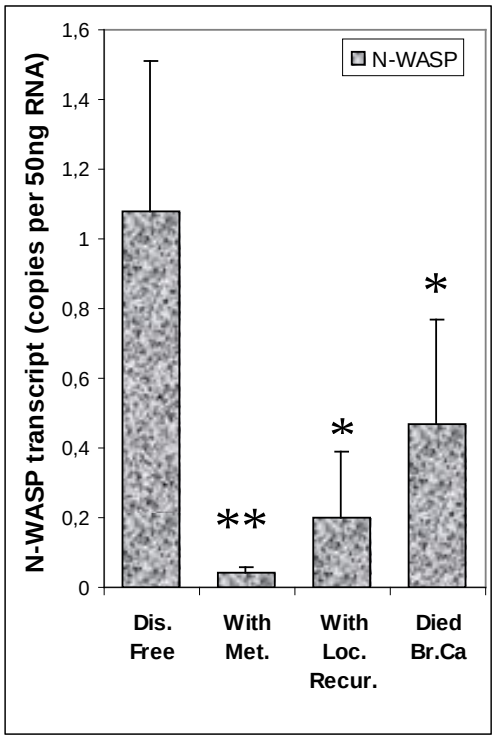


Figure 3.4: Significantly lower levels of NWASP Transcript were seen in those patients with metastatic disease (P<0.01) and in those patients who died of breast cancer (* P<0.05).**

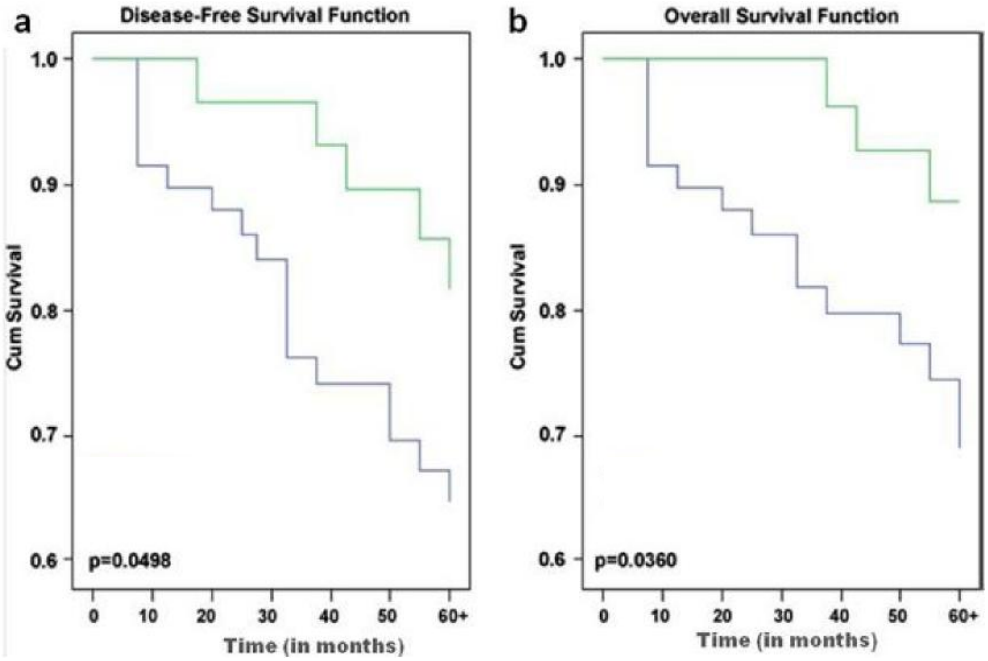


Figure 3.5 NWASP association with survival. Fig 3.5a indicated that NWASP was associated with disease-free survival in patients with breast cancer. Fig 3.5b indicated reduced levels of NWASP were associated with poor overall survival of patients. The Green line represents patients with higher levels of NWASP, and the blue line represents patients with a lower level of NWASP [based on the median level of NWASP in the NPI 2 group (NPI 3.5-5.4)]

Survival was analysed using the Cox-proportion-Hazardous test and showed a 0.0360 probability of low NWASP levels indicating low survivability. The apparent discrepancy of transcript levels between patients with metastatic disease and those having died from breast cancer may be an artificial bias due to the relatively low numbers of these patients in each category.

3.3.4 Immunohistochemical staining of NWASP in normal cells and breast cancer cells

Although the principles underlying Immunohistochemistry [IHC] were well established in the 1930's, the first published report was not until 1942 (Coons, 1971). Subsequent improvements in protein conjugation, tissue fixation, detection labels and microscopy have made IHC an essential tool in research and diagnostic

laboratories. The far reaching applications of IHC now encompass biological research, disease diagnosis and drug development.

We examined the presence of NWASP in normal breast tissue by immunostaining. This was then compared to the staining pattern in breast tumour tissue. The staining of NWASP was most abundant in the normal breast epithelial cells, and moderate in the stromal tissue. By comparison, breast cancer cells had low to moderate NWASP staining.

In Fig. 3.6 we have captured the immunostaining of NWASP in human breast cancer tissue at increasing powers of magnification. The serial images a1-a3 show the background tissue at magnification levels of 40, 100 and 200 respectively. NWASP staining is primarily seen in the cytoplasmic region of the normal epithelial cells, with low degree of nuclear staining. Staining was visible in the cytoplasmic regions of the cancer cells as well, but was much weaker in comparison to normal epithelial cells. Fig. 3.7 panels b1 and b2 further show matched tumour tissue at magnification as above, further showing the immunostaining characteristics of NWASP in human breast cancer cells. Fig. 3.7 panels b3 and b4 further show a second magnified section (X200) of the same tumour tissue. Finally, in Fig. 3.8 panels c1 (X100) and c2 (X100) respectively, the negative and positive controls for NWASP are displayed. There was a reduction in the staining in cancer cells with increased location of the NWASP protein to the cell periphery.

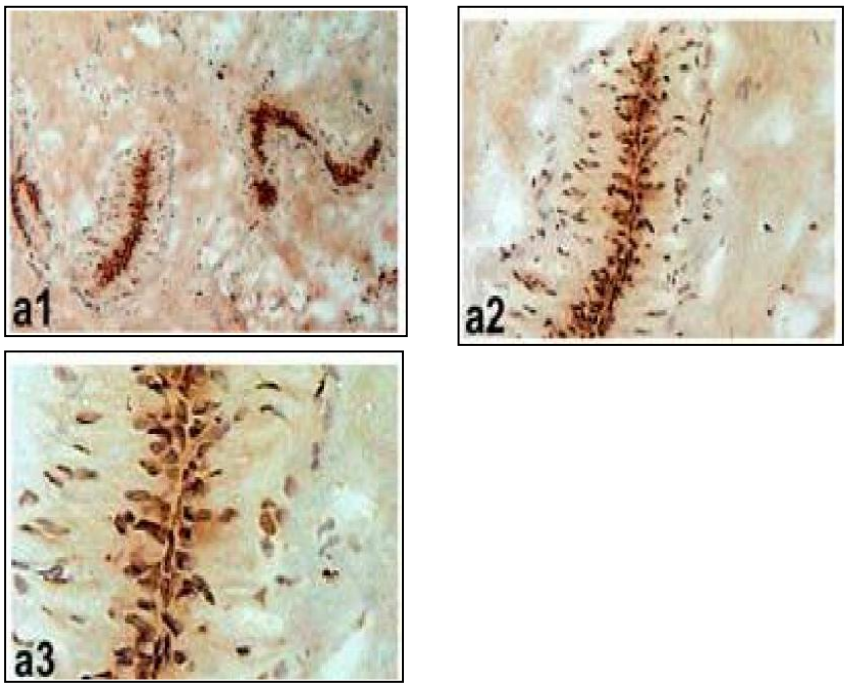


Figure 3. 6 Immunohistochemical staining of NWASP in human breast tissue. Panels a1– a3 show background tissue at increasing magnification (X40, X100 and X200).

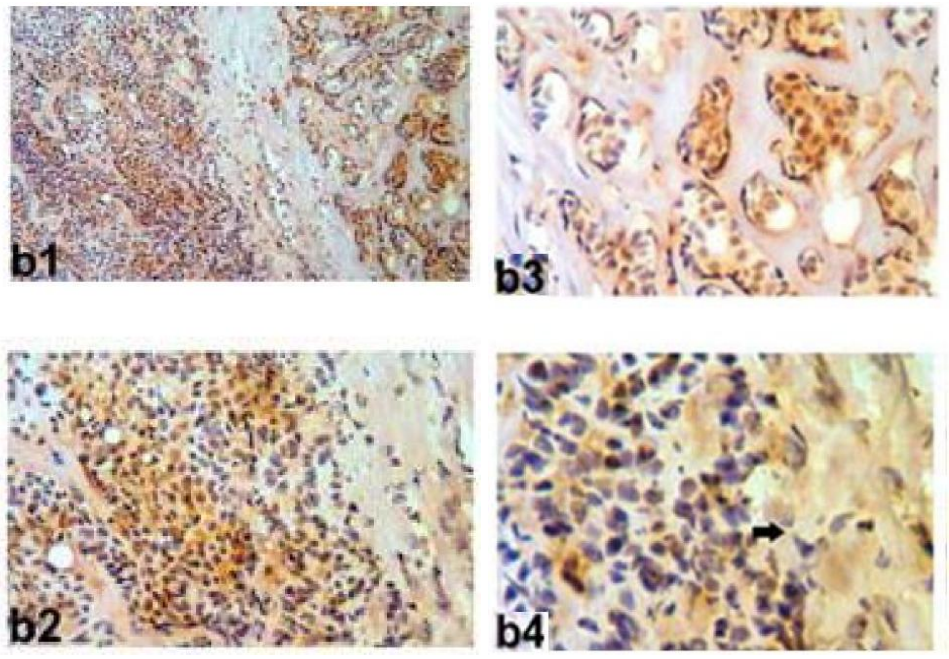


Figure 3.7 Immunohistochemical staining of NWASP in human breast cancer tissue. Panels b1–b2 show matched tumour tissue at the magnification (X40, X100). Panels b3–b4 showing a further magnified section (X200) of the same tumour tissue.

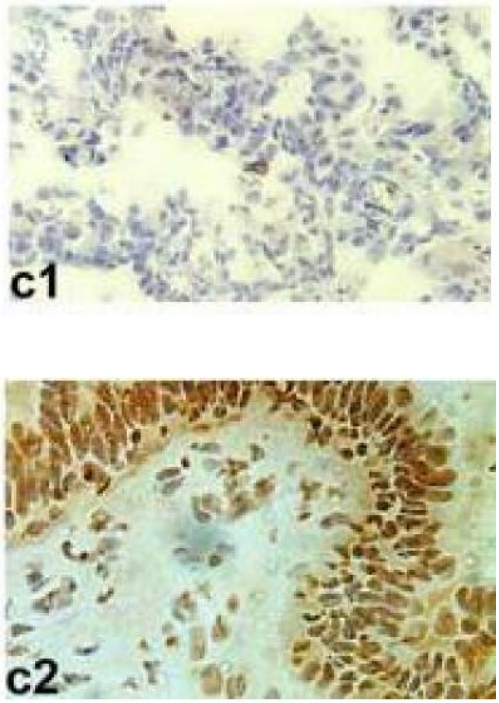


Figure 3. 8 Immunohistochemical staining of NWASP in human breast cancer tissue. Panels c1 (X100) and c2 (X100) show the negative and positive controls for NWASP respectively. There was a reduction in the staining in cancer cells with increased location of the NWASP protein to the cell periphery.

3.4 Discussion

This study has confirmed that NWASP is found in both normal and aberrant [tumour] tissues. The levels of NWASP were lower in tumour tissues. NWASP is primarily a cytoplasmic protein, and the levels of staining were lower in breast tumour tissue as compared to normal epithelial tissue.

We are aware that NWASP is involved in regulation of the actin cytoskeletal remodelling. Cell migration which is essential in any metastatic process is a complex process, one of the key steps of which is the formation of protusive structures-lamellipodia and filopodia. Actin polymerisation is essential in the formation of these structures and is mediated by the Arp 2/3 complex, which in turn is regulated by NWASP. The subcellular relocalisation of NWASP-from nuclear/peri nuclear region to the cytoplasm would serve to promote actin polymerisation in the leading edge of the cells. Upon activation, there is a

reduction in the peri nuclear concentration of NWASP accompanied with its relocation to the leading edge of the lamellipodia (Sukumvanich et al., 2004). Suetsugu and Takenawa demonstrated that unphosphorylated NWASP is located at the nucleus, and this causes suppression of HSP90, HSE and Src family kinase (Suetsugu and Takenawa, 2003). Phosphorylation of NWASP converts it to its active form with subsequent relocation to the cell periphery where CDC42 and Src are activated, thus regulating events involving the actin cytoskeleton i.e. motility, migration. The differential expression of NWASP in human breast cancer could incur aberrant activation in cancer cells and may lead to changes in regulation of migration and hence to metastatic spread. This would require further investigation and would form the basis of a novel research project.

The aberrant expression of the NWASP at mRNA levels in human breast cancer was reported for the first time. Additionally, this possibly has a significant bearing in the clinical outcome of the patients. Comparing the levels of NWASP against the known prognostic factors showed a significantly lowered level of NWASP in patients with either nodal involvement or metastatic disease, both indicators of poor prognosis. The lower levels of NWASP correlated well with a shorter disease free interval, thereby further illustrating the link between NWASP levels and mortality. The findings from the immunohistochemical analysis mirror those of the quantitative transcript analysis. However, owing to a paucity of samples for subset histological analysis, the immunohistochemical analysis was carried out on only a limited number of samples. This precluded any statistical analysis on the Nottingham Prognostic Indicator and Grade subgroups. Although small, the favourable results from the immunohistochemical tests suggest that it would be a worthwhile exercise to conduct a more in-depth analysis on a suitable cohort of breast tumour tissues in the future.

Chapter 4

Cloned NWASP tumour Suppressor gene reduces the invasive and migratory capacity of human breast cancer cells

4.1 Introduction

One of the key features of malignant cells is their ability to locally invade surrounding tissues and also to metastasise to distant sites. Different tumours show a propensity to metastasise to different sites. For example, breast tumours to bone, liver, and lung and colonic cancers to liver and lungs. Lung cancer cells have a very high rate of metastasis with cells invading virtually every organ in the body with a predilection to brain, bone, bone marrow and liver. Key events in the complex process of metastasis include cell transformation and proliferation, basement membrane degradation at primary site with associated vascular transport through capillary or lymphatic vessels, adherence or attachment of the tumour cells to endothelial or sub-endothelial structures at the secondary site, and the subsequent growth of the secondary tumour mass.

In order to metastasise to various organs, tumour cells need to acquire a number of highly differentiated characteristics that define the metastatic phenotype. The metastasising cells show functions that are similar to those seen in the normal processes of embryonic development, tissue remodelling, mammary gland involution or trophoblast implantation. The main distinction between the normal processes and those in the cancer pathway is dysregulation. This can be attributed to high mutation rates and genetic instability. The net result of a high mutation rate is the development of clonal populations with metastatic capabilities. It is further observed that the neoplastic element must expand considerably prior to invasion or metastasis occurring.

Numerous studies have compared the similarities between metastasis and the responses of inflammatory cells, an essential component of both processes being the initial recruitment and subsequent migration of cells through basement membranes and connective tissues. In inflammation, the trigger for migration has been identified as signalling initiated by bacterial or complement-mediated signalling. The responsible factors and triggers are less well defined in metastasis.

4.1.1 Models of invasion and Motility

Essentially, the models are classified into *in vitro* and *in vivo*. Major emphases on cancer invasion motility have focused on *in vivo* models, in which tumour cells are injected intravenously or subcutaneously into mice, and the number of metastases determined. However, this process is relatively expensive, and involves sacrifice of the subject in order to determine results. Therefore, more emphasis is now being placed on the *in vitro* studies. The *in vitro* models used in this study were the following four assays:

1. Matrigel Invasion Assay
2. Cell Growth assay
3. Cytodex 2-bead Motility Assay
4. Cell Matrix Attachment Assay

The full details regarding the various assays are dealt within the relevant sections in Chapter 2. They are briefly touched upon in the following section. Their main advantage is the cost effectiveness and relatively low level of expertise required to perform the assays. Their main disadvantage relates to the lack of reproducibility, the human error and the laborious nature of the experiments. These disadvantages are minimised by using the Electrode Cell-substrate Impedance Sensor (ECIS) - a bio-morphological Assay method which is described in the next chapter.

4.1.2 DNA cloning

DNA cloning produces many identical copies (amplification) of a foreign DNA fragment that may be of particular interest. Ideally, it would be possible to isolate a single DNA fragment and purify it prior to cloning. Although this is feasible in a small number of cases, more commonly, in order to clone a particular nucleotide sequence (DNA fragment), the total cellular DNA is first cleaved with a specific restriction enzyme, resulting in hundreds of thousands of fragments. Each of the resulting DNA fragments is joined to a DNA vector (carrier) molecule to form a hybrid molecule. Following this, each hybrid recombinant DNA molecule conveys its inserted DNA fragment into a single host cell, e.g. a bacterium, where it is replicated (amplified). Subsequent multiplication of the

host cell leads to the formation of a clone in which every bacterium carries copies of the same inserted DNA fragment. This technique is, therefore, referred to as “cloning” (Champe et al., 2005). The cloned DNA can eventually be released from its carrying vector by cleavage using the appropriate restriction endonuclease, followed by its isolation.

4.1.3 Vectors

A vector is a molecule of DNA to which the fragment of foreign DNA of interest to be cloned is joined. Vectors in common use are:

1. Plasmids
2. Bacterial viruses
3. Animal viruses

A vector must have certain essential properties. These are listed below:

1. It must be capable of autonomous replication within a host cell.
2. It must carry at least one gene that confers the ability to select for the vector, such as an antibiotic resistance gene.
3. It must contain at least one specific nucleotide sequence recognised by a restriction endonuclease.

Prokaryotic organisms contain single, large, circular chromosomes which behave as a plasmid would. Additionally, most species of bacteria contain small, circular extra-chromosomal DNA molecules called plasmids. Plasmids may carry genes that confer upon the host bacterium properties such as antibiotic resistance.

We isolated full length human NWASP DNA fragment and then cloned it using pCR3-GFT-NT expression vector using the chemically competent *Escherichia coli* bacteria. The cloned NWASP gene was then transfected into highly malignant MDA-MB-231 breast cancer cells and grown in G418 selection medium. These cells were then used for function studies (invasion and migration capacity) with wild type and GFP MDA-MB-231 breast cancer cells as controls. Following this, the above cells were subjected to immunocytochemical and immunofluoroscopic analysis as detailed previously.

4.2 Materials and Methods

Four assays were used to determine what, if any, effect over-expression of N-NWASP would have on the human breast cancer cell line MDA-MB-231. HGF at 25 ng/ml was used as a stimulator of motility and invasion. There were no obvious differences in cell morphology between the wild type and transformed cells.

4.2.1 Invasion Study

Invasiveness of MDA-MB-231 breast cancer cell line was assessed using the following in vitro assay. Transwell chambers equipped with 6.5 mm diameter polycarbonate filter (pore size 8 μm) (Becton Dickinson Labware, Oxford, UK) were pre-coated with 50 mg/membrane of solubilised basement membrane in the form of Matrigel (Collaborative Research Products, Bedford, MA). After membrane re-hydration, 15,000 cells were aliquoted into each insert with/without HGF/SF (25 ng/ml). After 96 hours, co-culture non-invasive cells were removed with cotton swabs. Invaded cells on the underside of the insert were fixed and stained with crystal violet, followed by microscopic counting (20 fields/insert).

4.2.2 Cell Motility Study

A cell motility assay was carried out. Briefly, cells were pre-coated onto cytodex-2 carrier beads (Sigma-Aldrich, Poole, UK) for 2 hours in complete medium. After the medium was aspirated, and the cells washed (X2 in complete medium), they were aliquoted into wells of a 96-well plate in triplicate (300 μl/well). HGF (25 ng/ml) was added, and the cells incubated over-night. The beads were washed off in medium, and the cells that had migrated onto the floor of the well fixed (4% formaldehyde) and stained with crystal violet. The cells were counted microscopically (940).

4.2.3 Cell Adhesion Assay

The cell-matrix attachment assay was carried out as previously reported. Briefly, Matrigel (10 μg/well) was added to 96-well plates, which were incubated for 24 hours to allow binding of matrix protein to the surface of the well. The plates were then washed and blocked with 5% Bovine serum albumin (BSA). Cells were

added at 10^4/well for 30 min, followed by aspiration and washing. The number of attached cells was determined by direct counting under microscope (7 counts per experimental setting).

4.2.4 Cell growth Assay

The principle behind *in vitro* cell growth assay is based on the ability of a mitochondrial de-hydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT [MTT stock solution 5mg/ml, 40µl/well, thus equaling 200µg MTT/well] to form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals, which are solubilised. The number of surviving cells is directly proportional to the level of the formazan product created. The colour can then be quantified using a simple colorimetric assay.

The cells were then seeded onto a 96 well plate (Nunc, Denmark) at a density of 7,000/well and incubated at 37ºC for 72 hours. MTT was added in solution to the cells (200 µg/well) and incubated for 4 hours at 37ºC. The cells were then lysed with Triton (10%) and the intensity of the colour released was determined by a plate reader (Titertek Multiskan, Eflab, Finland). The number of cells was shown as absorbance units.

4.2.5 *In vivo* growth assay analyzing NWASP gene transformed breast cancer cells

The *in vivo* tumour progression model was adapted from similar previously described protocols. Briefly 2 x 10^6 cells in 100 µl were mixed in a 0.5 mg/ml Matrigel suspension and subcutaneously injected into the left and right flanks of 4–6 weeks old athymic nude mice (CD-1; Charles River Laboratories, Kent, England, UK) and allowed to grow. The mice were maintained in filter top units and were weighed on a weekly basis. The tumour size was measured weekly using Vernier calipers under sterile conditions. Humane end points were:

(a) mice which suffered 25% weight loss

(b) development of tumours exceeding 1 cm^3 (subject to schedule 1 method according to the United Kingdom Home Office and the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines).

At the conclusion of the experiment, the animals were weighed, humanely killed under schedule 1 and the tumours were removed and stored. Tumour volume was determined using the following formula; tumour volume $mm^3 = 0.523 \times width^2 \times length$.

4.3 Results

4.3.1 Over expression of NWASP in breast cancer tissues

The human breast cancer cell line MDA-MB-231 was chosen to study the forced expression of NWASP as these cells did not show a signal for this molecule. Initial detection of the NWASP protein was performed using RT-PCR, and confirmation was obtained using western blotting (Fig.4.1a) and immunofluorescence (Fig. 4.1b).

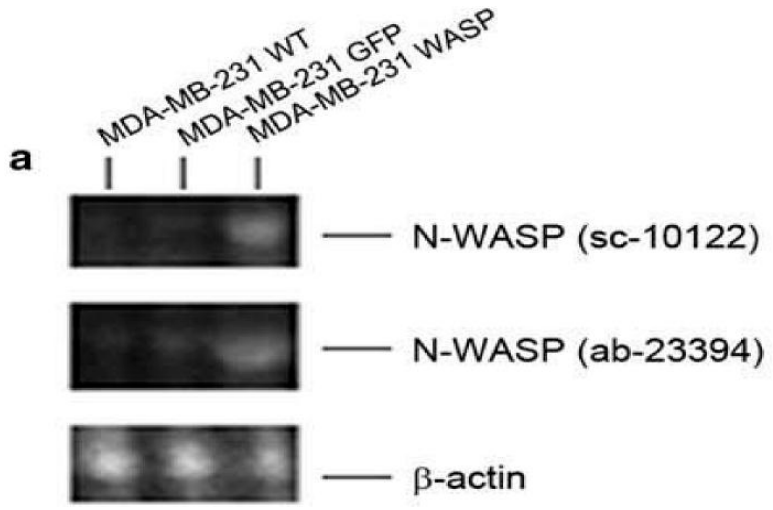
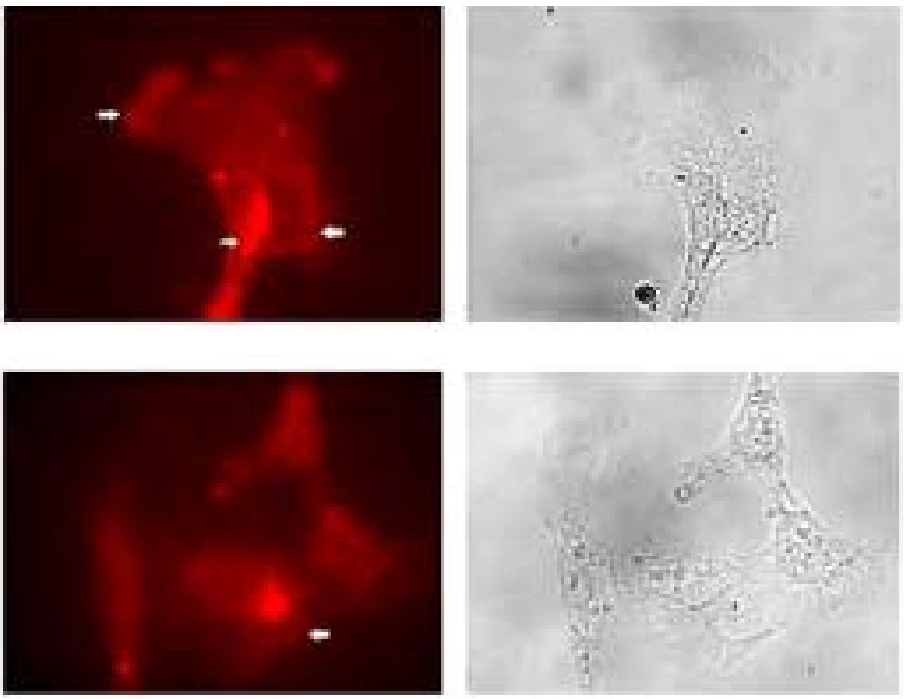
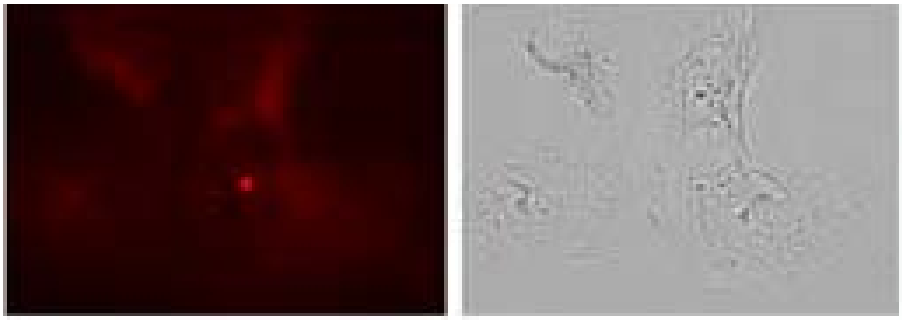


Fig. 4.1a: Over-expression of NWASP in human breast cancer, western blotting. NWASP was successfully over-expressed in MDA-MB-231 cells (MDA-MB-231 NWASP). The successful expression of NWASP protein in transfected MDA-MB-231 cells (MDS-MB-231^{NWASP}) is shown by western blotting. β-actin was used as the loading control.

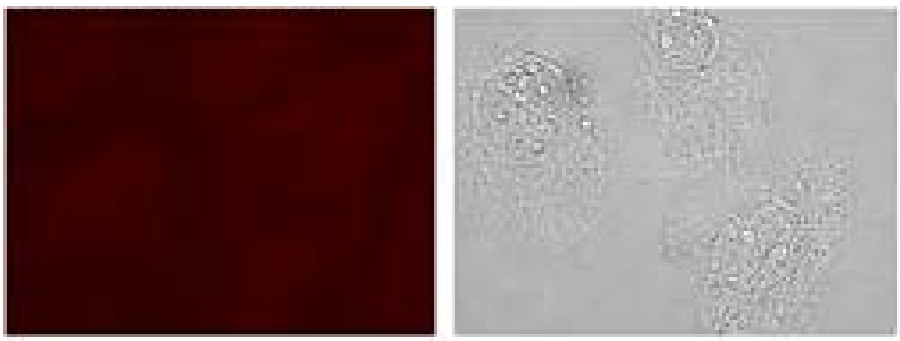
MDA-MB-231 WASP



MDA-MB-231 GFP

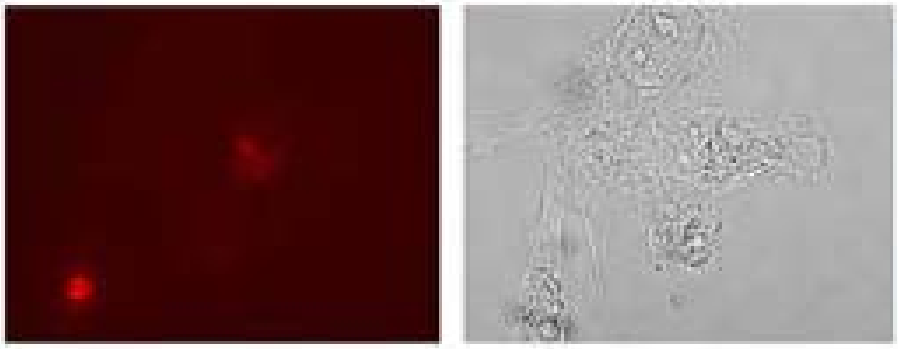


MDA-MB-231 WT

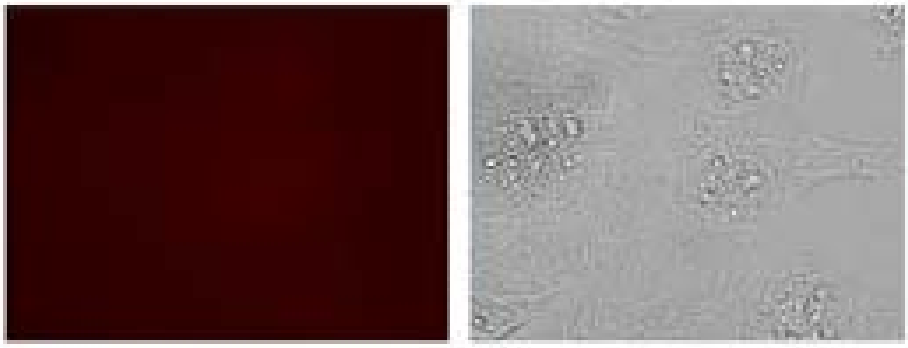


No primary control

MDA-MB-231 WASP



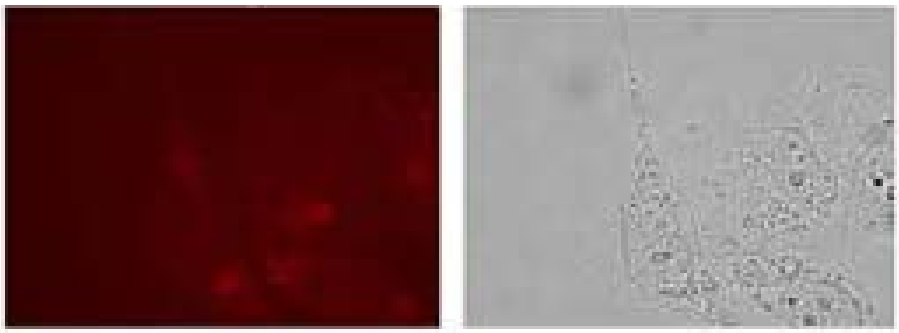
MDA-MB-231 GFP



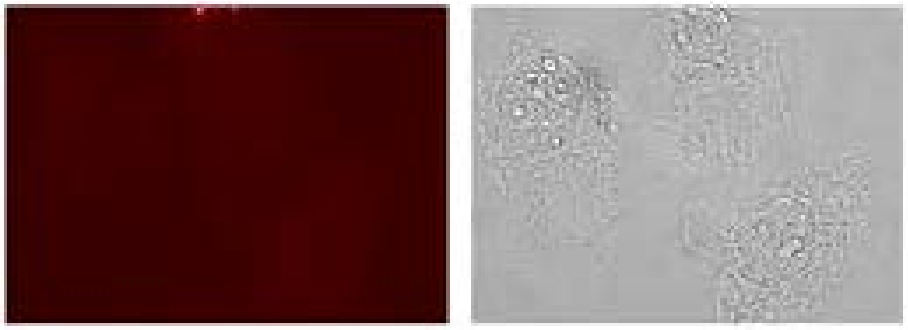
MDA-MB-231 WT

No secondary control

MDA-MB-231 WASP



MDA-MB-231 GFP



MDA-MB-231 WT

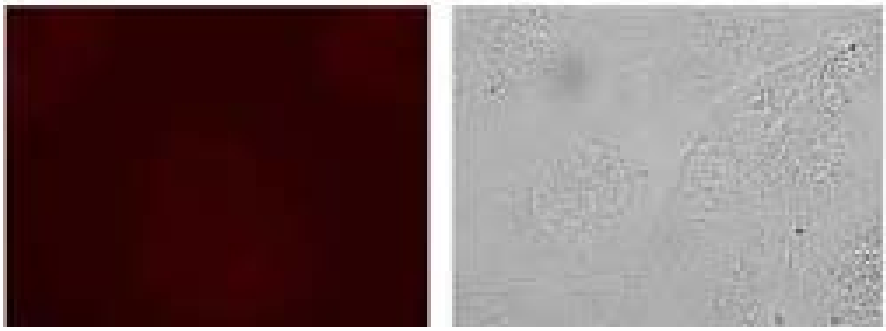


Fig. 4.1b: Over-expression of NWASP in human breast cancer, fluorescence microscopy. NWASP was successfully over-expressed in MDA-MB-231 cells (MDA-MB-231^{NWASP+}). The successful expression of NWASP in transfected cells (MDS-MB-231^{NWASP+}) is shown in the fluorescent microscopy images (X100). The figure clearly shows the expression of the protein at both a sub cellular and peripheral location within MDA-MB-231^{NWASP+}cells. The absence of NWASP in both GFP control plasmid cells (MDA-MB-231^{GFP}) and MDA-MB-231^{WT} cells is also demonstrated. Bright field images are shown to indicate the presence of cells (X100).

4.3.2 *In vitro* studies of MDA-MB-231^{NWASP+} cells

Subsequent assay on the stable MDA-MB-231^{NWASP+} cells was carried out to determine the effects of NWASP on this invasive human breast cancer cell line. Changes in invasiveness were assessed, with MDA-MB-231^{NWASP+} cells showing significantly reduced invasion compared to the wild type cells (MDA-MB-231^{WT}) and GFP-plasmid control cells (MDA-MB-231^{GFP}) (Fig. 4.2) ($P<0.0001$).

This invasiveness remained reduced even in the presence of HGF (25 ng/ml), which actively induces invasion in this cell type ($P<0.0001$). Motility of the cells was also significantly reduced ($P<0.001$) compared to the wild type and plasmid control cells (Fig. 4.3), which is consistent with the role of NWASP as a regulator of the actin cytoskeleton, and again remained reduced in the presence of HGF. The adhesion of these cells was increased (MDA-MB-231^{NWASP+} 10.4: 3.8 versus MDA-MB-231^{WT} 7.6: 2.2; $P = 0.05$), as shown in Fig. 4.4. This would support the argument that the cells that express NWASP show an increased adhesiveness, and therefore, a decreased potential for invasiveness or metastasis.

The effect of HGF on the temporal and spatial distribution of NWASP was determined by immunofluorescence over 90 min (Fig. 4.5). It appears that the nuclear and cytoplasmic expression of NWASP was modified upon treatment with HGF by 30 min incubation, with the redistribution of NWASP towards the cell periphery. This was clearly evident by 60 min incubation, with NWASP clearly located at the ruffling, leading edge of the cell. By 90 min incubation, the effect was beginning to abate. There was no loss of expression of NWASP, merely a redistribution of this protein.

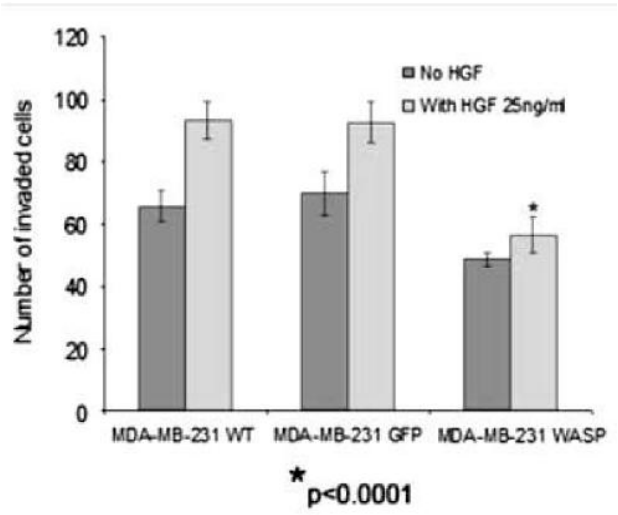


Figure 4.2 Forced expression of NWASP resulted in cells with significantly lower levels of invasiveness (P<0.0001), in response to HGF (25 ng/ml)

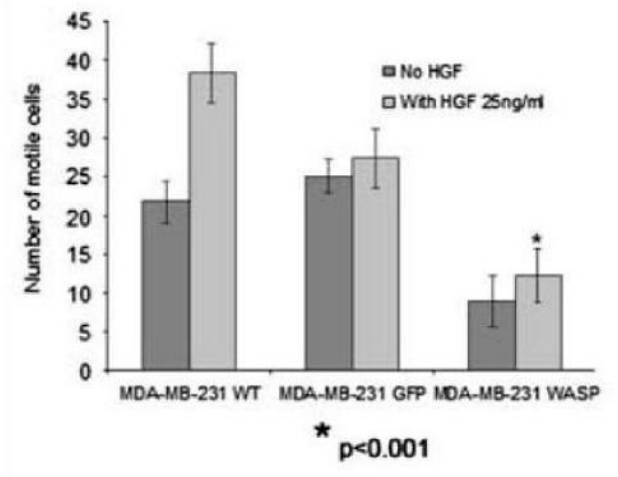


Figure 4.3 Forced expression of NWASP resulted in cells with significantly lower levels of motility (P<0.001), in response to HGF (25 ng/ml)

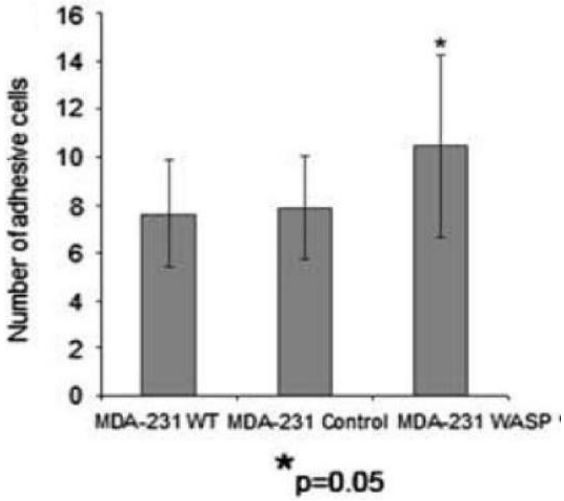


Figure 4.4 Forced expression of NWASP resulted in cells with increased adhesiveness to basement membrane (P = 0.05) in response to HGF (25 ng/ml)

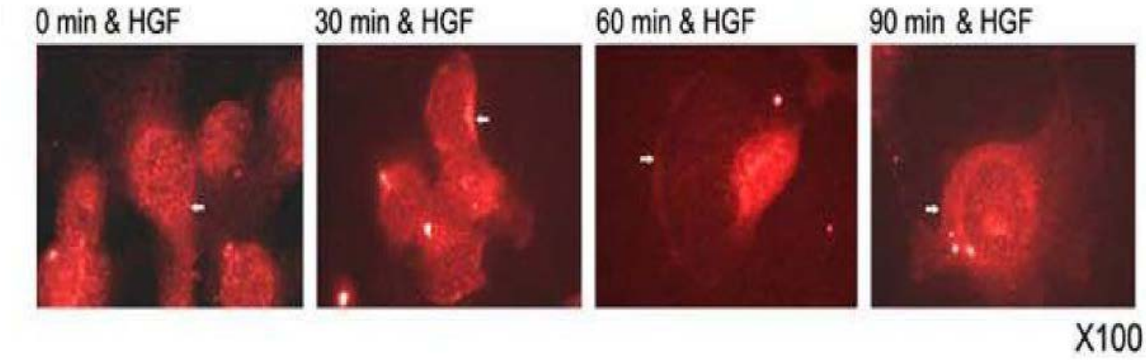


Figure 4.5 HGF (50 ng/ml) effected a temporal and spatial re-location of NWASP in the transfected cells, as indicated by arrows. A nuclear/peri nuclear expression gave way to increased cytoplasmic and cell peripheral location by 60 min incubation with HGF.

4.3.3 *In vivo* tumour growth of MDA-MB-231^{NWASP+} cells

The tumours induced in the athymic nu/nu female mice were measured over 28 days. During the growth period and at the experimental end point, the MDA- MB-231^{NWASP+} tumours were consistently slower growing compared to the MDA- MB-231^{WT} and MDA- MB-231^{GFP} control cells (Fig. 4.6). After 28 days, the tumour volume of the MDA MB-231^{NWASP+} cells was significantly smaller than both control cell groups, indicative of an inhibitory effect of NWASP in these human breast cancer cells (Final tumour volume mm^3: MDA-MB-231^{NWASP+} 132.57 ±

48.69 versus MDA-MB-231^{WT} 390.72 ± 125.66 and MDA- MB-231^{GFP} 431.84 ± 162.79; P = 0.002).

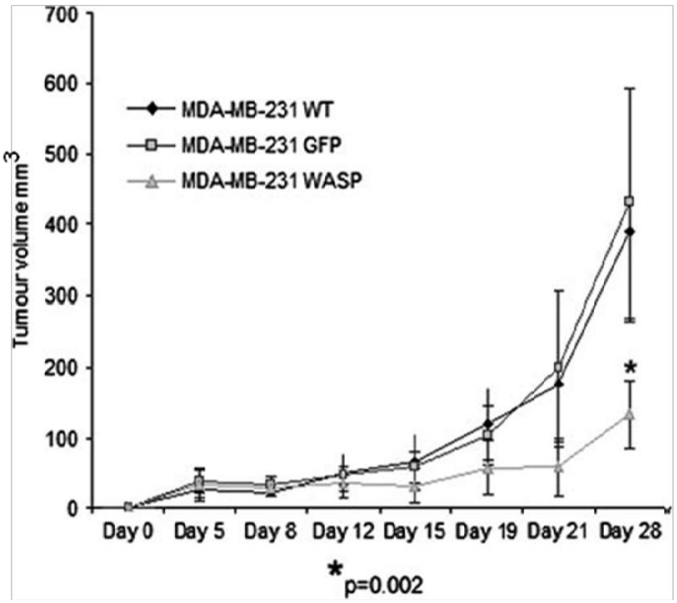


Figure 4.6 The over expression of NWASP in human breast cancer cells and *in vivo* tumour growth. The growth of tumours was significantly reduced in MDA-MB-231^{NWASP} cells compared to the wild type (MDA-MB-231^{WT}) and plasmid control cells (MDA-MB-231^{GFP}) (p=0.002).

4.4 Discussion

An extensive literature review provided an extreme paucity of quality data as regards NWASP and its role in breast cancer. This stimulated interest and was pronounced in paving the way for this project.

In the previous chapter, we had shown that low levels of expression of NWASP were associated with a poor prognosis in Breast cancer, both in terms of disease free and overall survival. In this chapter, the aim was to study the effect of NWASP in relation to the invasive and migratory capacity of breast cancer cells. The hypothesis being that NWASP expression should correlate with reduced migration and invasive capacity, and thereby lower levels of NWASP expression correlated with poorer outcomes in individuals with breast cancer.

Towards this end, the invasive and migratory capacity of breast cancer cells MDA-MB-231 were compared both prior to forced expression of NWASP [MDA-MB-231^{WT} (Fero et al., 1998), and post expression of NWASP [MDA-MB-231^{NWASP+}].

As shown in the results section, changes in invasiveness were observed with MDA-MB-231^{NWASP+} cells showing significantly reduced invasion compared to the wild type cells (MDA-MB-231^{WT}) and GFP-plasmid control cells (MDA-MB-231^{GFP}) ($P<0.0001$). This invasiveness remained reduced even in the presence of Human Growth Factor, which, under normal circumstances, actively induces invasion in this cell type ($P<0.0001$). Additionally, the motility of the cells was also significantly reduced ($P<0.001$) compared to the wild type and plasmid control cells, and motility remained reduced in the presence of HGF ($P<0.001$). These findings would be consistent with the perceived role of NWASP as a regulator of the actin cytoskeleton. As a corollary of the study, it was observed that the adhesion of these cells was expectedly increased.

An additional finding was the redistribution of the NWASP over time, within the cells, when treated with HGF. The expression which was initially found to be nuclear and peri nuclear, was found to be within the cytoplasm and the periphery of the cells. The exact significance of this is unclear, and perhaps further studies will elucidate the same. Finally, the *in vivo* studies in the athymic mice showed the growth of tumours to be significantly reduced in the MDA-231 NWASP cells as compared to the MDA-231 WT and plasmid control cells.

Collectively, these findings suggest that the NWASP, and therefore, by inference, the gene that codes for NWASP could have a role as tumour suppressor gene in human breast cancer. This study serves to ignite further research into the beneficial role that NWASP and its encoding gene could potentially play in therapy of breast cancer in the future. This is particularly relevant in times such as these where the entire human genome has been deconstructed, and commercially viable Gene Expression Profiling Kits such as MammaPrint (Agendia Inc., Irvine, California) and Oncotype DX (Genomic Health, Redwood, California) are readily available.

Chapter 5

Electric Cell Substrate Impedance Imaging Assays

5.1 Introduction

5.1.1 The ECIS™ Model

Application of a mathematical model of the impedance changes due to the presence of a cell layer, where the impedance data can be used to calculate cell morphological parameters including the barrier function of the cell layer, the spacing between the ventral side of the cell and the substratum, and the cell membrane capacitance. In the seminal paper by Giaver and Keese, the validity of the model was confirmed (Giaever and Keese, 1991). To employ the model to refine ECIS data, the resistance and capacitance of the cell free electrode is measured at several different AC frequencies. In the model, cells are represented as disk shaped objects having insulating membrane surfaces and filled with conducting electrolyte.

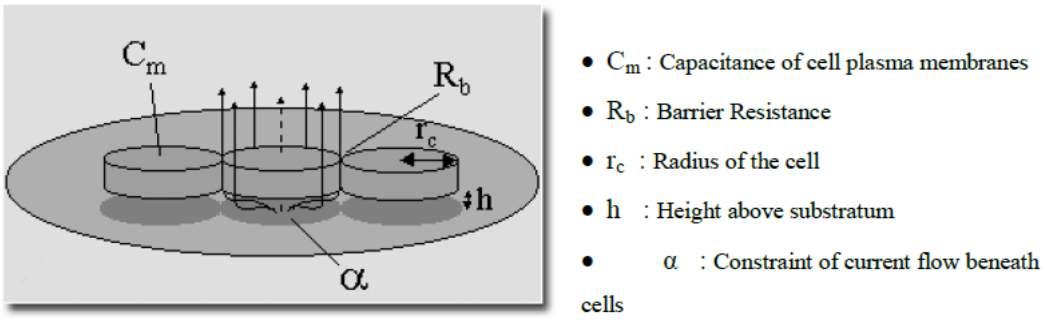


Figure 5. 1 Cells modelled as disks. Courtesy (www.biophysics.com)

Since the focal adhesion plaques of cells represent a very small fraction of the total ventral surface of the spread cell, we treat the cells as hovering a small distance above the electrode which serves as their substratum. In calculating how the impedance changes due to this cell coverage, we assume that the resistance and capacitance of the gold surface itself does not change but that the measured capacitance and resistance change because the cells alter the path of the current flow. The main source of the impedance change can be attributed to the fact that some current must flow through the narrow spaces between the ventral surface of the cells and the electrode, and that current flows out through the narrow spaces

between the cells (barrier resistance). AC current can pass directly through the cells since their insulating membranes serve as capacitors themselves.

ECIS enables us to perform a fully automated wound healing assay that generates quantitative data in real time without the need for reopening the incubator door once the experiment has been set up. The advantages of this are self-explanatory. Additionally, ECIS measurements are label free and, as explained earlier, they are highly reproducible.

5.1.2 ECIS Wounding Assay

The ECIS wound healing assay replaces the traditional “scratch” or “scrape” assay. Traditionally, the cell layer was disrupted mechanically using a toothpick, pipette or a needle. A microscope followed the subsequent migration of cells to “heal” the wound. ECIS employs electric signals to both wound and monitor the healing process.

ECIS electrical wounding is highly targeted - it only affects a small population of cells in contact with the active 250µm diameter ECIS electrode, producing a well-defined 250µm wound. This can be verified both by ECIS staining and by ECIS measurement. The benefits of this method are the reproducibility and the lack of observer bias and variability. Additionally, the protein coating of the cells is unaffected by the current, and it remains fully intact.

5.1.3 ECIS cell migration measurements

Upon electrically wounding the cells, ECIS returns to its normal mode to immediately follow the neighbouring healthy cells as they migrate inward to replace the killed cells. Traditionally, as described in earlier chapters, these measurements are carried out with extensive labour involving microscopy and researcher quantification. The ECIS wound healing/cell migration assay is a completely automated assay requiring a minimum amount of labour. Computer control enables both cell wounding and measurements of the subsequent healing process.

ECIS has now been modified (Patent pending) such that automated assays of this sort can be performed. In normal ECIS measurements, a current of less than a microampere is normally used. This is undetected by the cells, and, in its measuring mode, ECIS essentially eavesdrops on cell behaviour electrically. When the current is boosted 1000 fold to a milliampere, the resulting voltages across the cell membranes result in electroporation. If this is applied for only a few milliseconds, the cells recover, and it is possible to insert impermeable molecules including DNA constructs into the cytoplasm. When the high current is applied for several seconds, cell death ensues due to severe electroporation and possible local heating effects.

The ECIS wound is very well defined, as it includes only those cells on the 250 µm diameter electrode. Death can be verified both with the ECIS measurement and vital staining. Typical ECIS data involving this assay is shown in the figure 5.2. Here cells were first grown as complete monolayers and the impedance traces from confluent wells can be seen on the graph. At the red dot, an elevated field was applied to two of the wells, wounding the cells on the small electrode and causing the impedance to drop to that of an open electrode. Over time, these two traces return to control values, as the healthy cells outside of the small electrode migrate inward to repopulate the wounded area and replace their dead cohorts (healing). These types of data are highly reproducible and respond to culture conditions. This is evident in the graph showing the similar pattern following the second wounding.

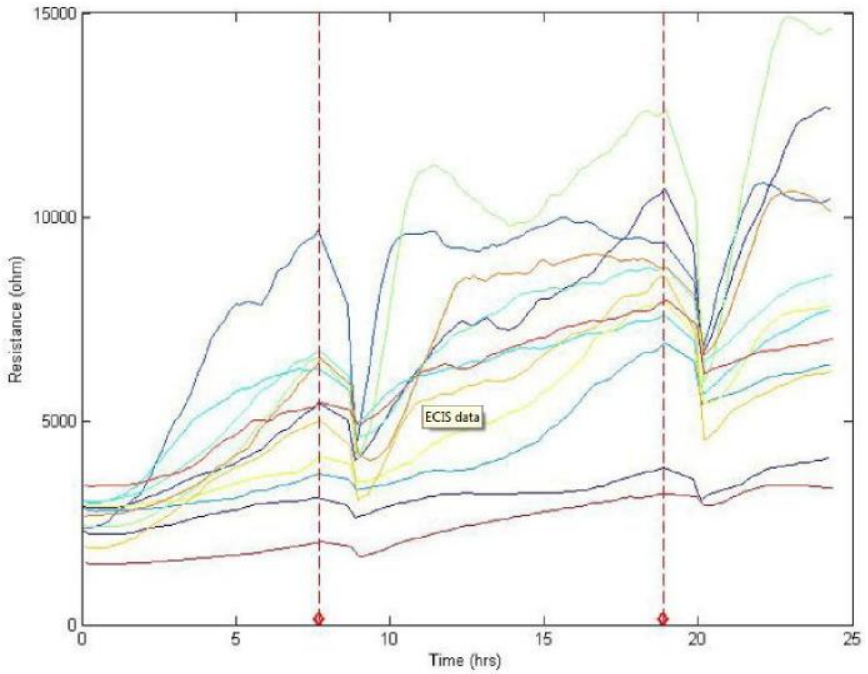


Figure 5.2 Typical ECIS data generated during a wounding assay.

The term *impedance* was coined by Oliver Heaviside in July 1886 (Heaviside, 1971). It is the measure of the opposition that a circuit offers to a current when a voltage is applied. In other words, it is the complex ratio of the voltage to the current in an alternating circuit. Impedance extends the concept of resistance to Alternating Current circuits. Resistance only has magnitude, and hence differs from Impedance which has both magnitude and phase. The symbol for impedance is usually *Z*. In general, impedance will be a complex number, with the same units as resistance, for which the Standardised International unit is the Ohm (Ω)

Table 5. 1 Comparison of Traditional "Scratch" and newer ECIS methods

Scratch Method	**ECIS Wound Healing Assay**
The scratch assay is traditionally carried out with simple tools such as needles, rakes, pipette tips or even toothpicks.	The ECIS Wound is carried out by the ECIS Software without the need for technician manipulation.
The scratch itself often varies and is not highly reproducible.	The ECIS Elevated Field Module produces a 250 um wound every time.
The scratch method requires hands-on measurements.	ECIS measurements are automated, quantifying data in real-time.
The traditional scratch method almost always "scraped" off the cell's protein coat.	With the ECIS Wound the cell's protein coating is unaffected by the current, it is not "scraped" off.

5.2 Materials and Methods

In our department, we have available the ECIS 9600 and ECIS 1600R purchased from Applied Bio-physics. In the current study, the ECIS 1600R was used for the experiments.

The electronics are consolidated in a single case that sits beside a tissue culture incubator. Two leads connect the electronics to an electrode array holder within the incubator space – this, in turn, makes contact with up to two consumable arrays. A USB cable provides communication to a PC that controls all data acquisition, storage and analysis. In addition to the ECIS electronics and software, the system is supplied with a state-of-the-art PC/monitor, colour inkjet printer and an initial supply of electrode arrays. An optional compact CO2 tissue culture is also available. The ECIS software runs on a Window's TM platform with user-friendly software for all operations.

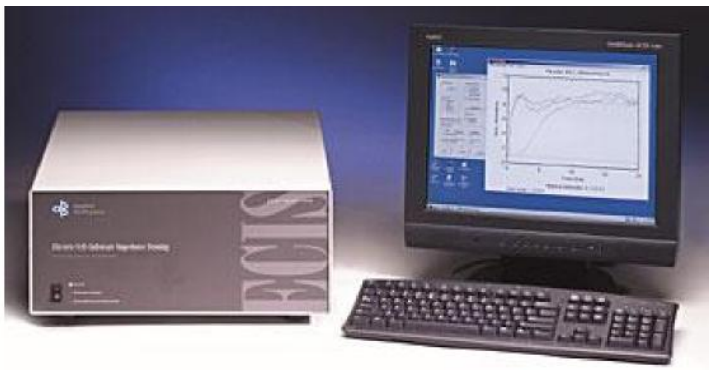


Figure 5.3 ECIS Working Assembly

Specifications

Maximum number of wells: 16

Frequency range: continuous from 100Hz to 100kHz

Operating System: Windows XP

System Controller: Desktop or laptop PC

Front Panel Controls: Power on/of

Front Panel Indicators: Power, Measurement in Progress,

Wounding/Electroporation

Mode Activated (optional)

PC to 1600R communications: USB

Power: 120-240 VAC 50/60Hz

Dimensions: 17.25 x 8.5 x 20 inches (WxHxD)

Weight: 40 lbs

System includes: dual slot array holder, colour matrix printer, laptop or desktop PC

Array Holder dimensions: 5.5x 1.5x 6.0 inches (WxHxD)

Array Holder material: Lexan ® polycarbonate

Array holder to instrument cable length: 4.5 ft (a port in the incubator is desirable, 5/8 inch diameter minimum).

As mentioned in detail in chapter 2, the core working element is the 8 chamber well with the gold electrodes. In order to ensure that no deposits accrue on the electrodes, these need to be cleaned. Deposits would cause added impedance, and hence erroneous readings. Cleaning was achieved using 200 μl of 10 mM L-cysteine into each of the 8 wells and allowing the same to stand for 40 minutes. Care must be taken to avoid contact with the electrodes as this may damage them.

This was followed by washing the wells with 300 μl of DMEM F12 (Dulbecco's Modified Eagles Medium). Three rinses ensured an adequately cleaned well. The wells were now ready for use for the experiment. The 8 wells were allocated the various constituents for the attachment, wounding, and migration studies as is described in the following section. The 8 chamber well was now placed in the ECIS chamber and subjected to the studies as detailed in the following pages.

For the attachment study the order of the wells are as follows:

Attach 1:
3=empty plasmid
4=$NWASP^+$
5=empty plasmid + HGF at 25 ng/ml
6=$NWASP^+$ + HGF at 25 ng/ml

Attach 2:
2=empty plasmid
3=$NWASP^+$

6=Empty plasmid + Wiskostatin at 50 ng/ml
7=$NWASP^+$ + Wiskostatin at 50 ng/ml

Attach 3:
2=empty plasmid+ HGF at 25 ng/ml
3=$NWASP^+$ + HGF at 25 ng/ml
6=Empty plasmid + Wiskostatin at 50 ng/ml + HGF at 25 ng/ml
7= $NWASP^+$ + Wiskostatin at 50 ng/ml+ HGF at 25 ng/ml

For the Wounding study, the order of the wells is as follows:

Wounding 1:
3=empty plasmid
4= $NWASP^+$
5=empty plasmid + HGF at 25 ng/ml
6= $NWASP^+$ + HGF at 25 ng/ml

Wounding 2:
2=empty plasmid
3= $NWASP^+$
6=Empty plasmid + Wiskostatin at 50 ng/ml
7= $NWASP^+$ + Wiskostatin at 50 ng/ml

Wounding 3:
2=empty plasmid+ HGF at 25 ng/ml
3= $NWASP^+$ + HGF at 25 ng/ml
6=Empty plasmid + Wiskostatin at 50 ng/ml + HGF at 25 ng/ml
7= $NWASP^+$ + Wiskostatin at 50 ng/ml+ HGF at 25 ng/ml

5.3 Results

ECIS-based assays have been established to measure the cell morphologic, adhesive, and invasive properties in a real-time, automated, and quantitative fashion. Monolayer disruption via the retraction of endothelial cell or loosening of tight cell junctions induced by tumour cells may be the major or at least part of the causes for the observed electrical resistance changes. The ability of tumour cells to induce these changes reflects their invasive potential. It is important to note that to date, there is no consensus of opinion for universally analysing ECIS data. Typically, data is presented using images derived from ECIS software

It has been shown that when endothelial cell layers were challenged with highly metastatic cell lines, binding occurred to the endothelial cell layer, followed by the retraction of the endothelial cell junctions, and finally the penetration of the cells through the endothelial monolayer. This sequence of events has been documented using scanning and transmission electron microscopy as well as phase-contrast time-lapse microscopy. It has been postulated that this *in vitro* sequence of events mirrors similar invasive activities that occur during the metastatic process in vivo. These activities have also been monitored using the ECIS system. As the metastatic cells invade the endothelial layer, they break down its barrier function, resulting in large drops in impedance. These impedance changes are automatically followed over time, and used to quantify the *in vitro* invasive activities if the cells.

The ECIS 1600R performs real time data measurement and subsequent analysis.

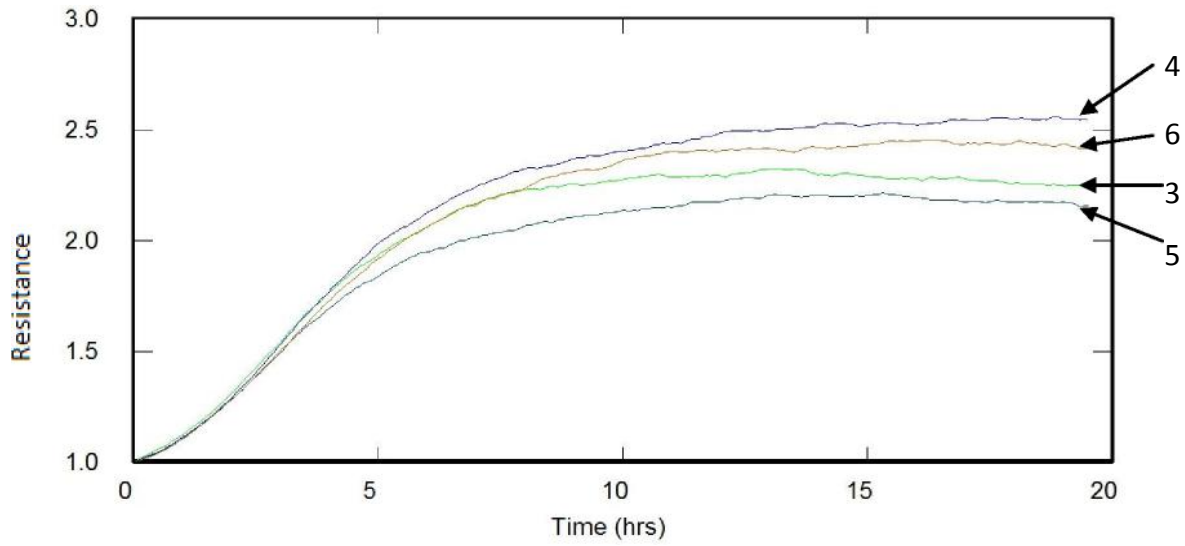


Figure 5. 4 Attachment study 1

Attach 1:
3=empty plasmid
4= $NWASP^{+}$
5=empty plasmid + HGF at 25 ng/ml
6= $NWASP^{+}$ + HGF at 25 ng/ml

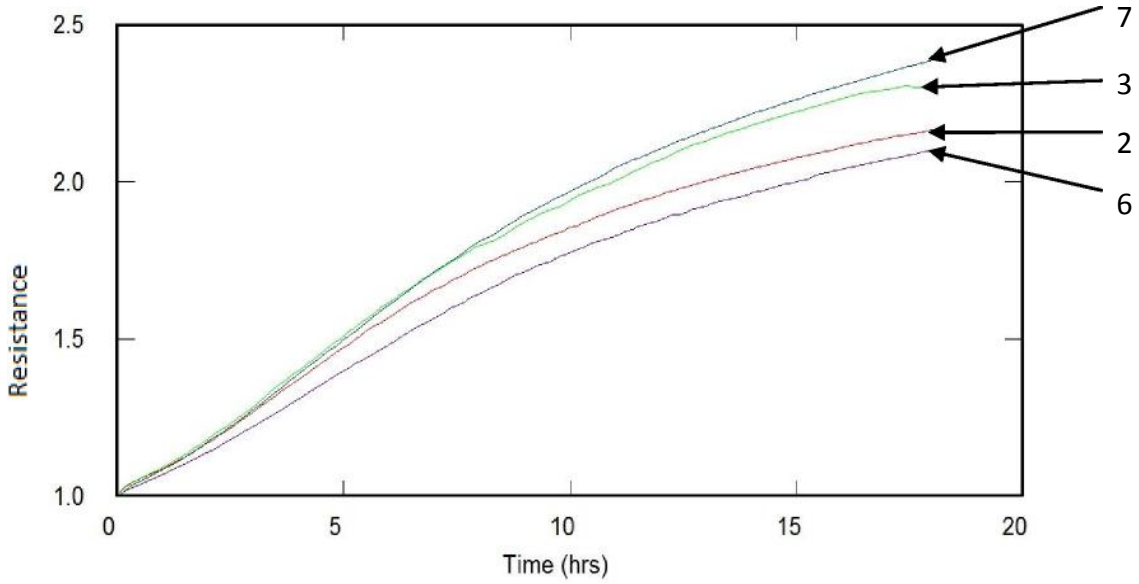


Figure 5. 5 Attachment study 2

Attach 2:

2=empty plasmid

3= NWASP^{+}

6=Empty plasmid + Wiskostatin at 50 ng/ml

7= NWASP^{+} + Wiskostatin at 50 ng/ml

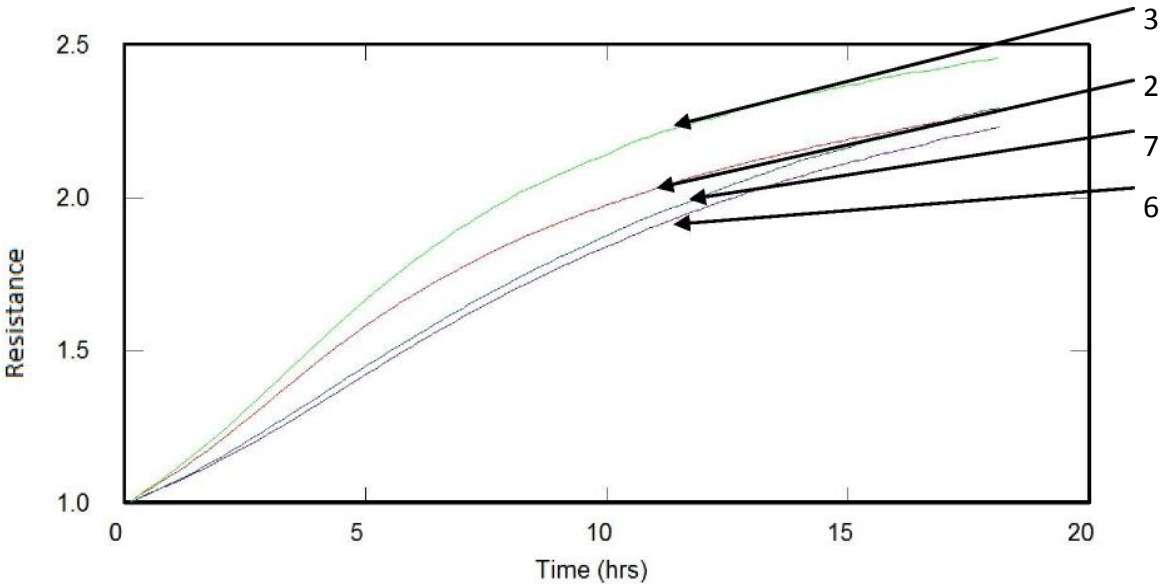


Figure 5. 6 Attachment study 3

Attach 3:

2=empty plasmid+ HGF at 25 ng/ml

3= NWASP^{+} + HGF at 25ng/ml

6=Empty plasmid + Wiskostatin at 50 ng/ml + HGF at 25 ng/ml

7= NWASP^{+} + Wiskostatin at 50ng/ml+ HGF at 25 ng/ml

The resistance change of the electrode−electrolyte interface increased linearly with the number of cells attached on the detecting electrode. The slope of the linear relationship appeared to depend on the type of coating protein. As the surface area occupied by the cells was also proportional to the cell number, the resistance change was in turn proportional to the area covered by the cells.

Wiskostatin- a cell-permeable N-alkylated carbazole derivative that selectively blocks actin filament assembly-was used. It acts as a selective, reversible inhibitor of NWASP (neural Wiskott Aldrich syndrome protein), a signal integrating protein. Appears to bind to NWASP, stabilize the auto inhibited conformation and prevent the activation of Arp2/3 (actin-related protein 2/3) complex.

Human tumours express large amounts of growth factors and their receptors. A tumour will not grow beyond the size of a pinhead without new blood vessels to supply oxygen and nutrients. Growth factors are significant because they can induce angiogenesis, the formation of blood vessels around a tumour. These growth factors also encourage cell proliferation, differentiation, and migration on the surfaces of the endothelial cells.

From the first attachment study (Figure 5.4), it is evident that the $NWASP^+$ cells attach even faster when treated with Human Growth Factor [HGF]. The attachment study 2 (Figure 5.5) demonstrates that even with the Wiskostatin inhibition, the $NWASP^+$ cells have better attachment. In the graph generated in the attachment study 3 [Figure 5.6], $NWASP^+$ cells have an increased attachment even when treated with HGF and Wiskostatin.

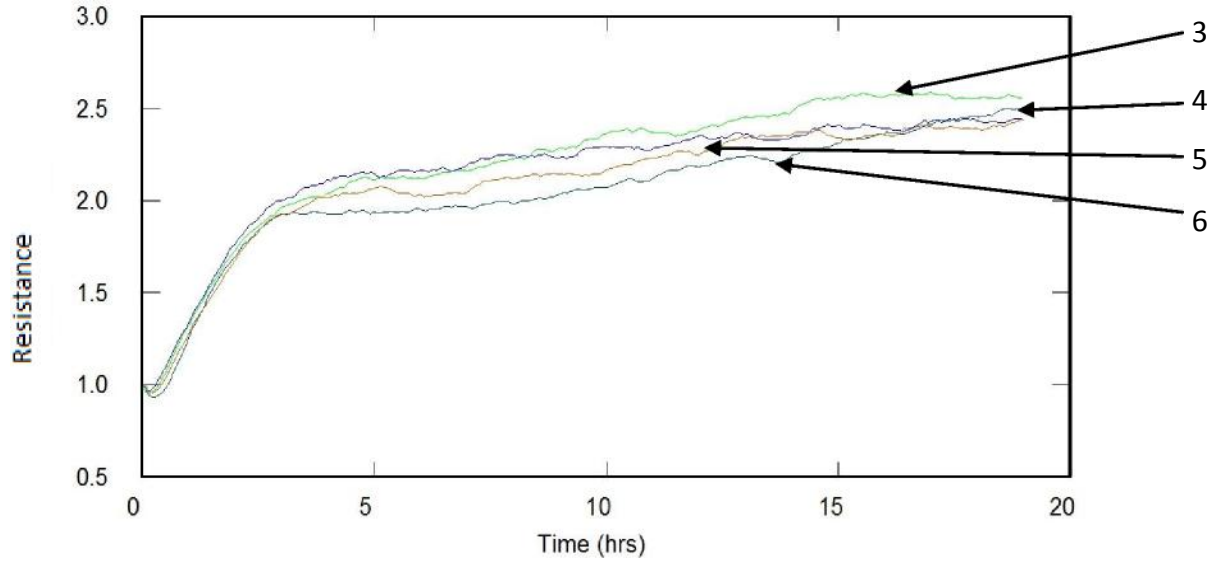


Figure 5.7 Wounding 1

Wounding 1:
3=empty plasmid
4= NWASP^{+}
5=empty plasmid + HGF at 25 ng/ml
6= NWASP^{+} + HGF at 25 ng/ml

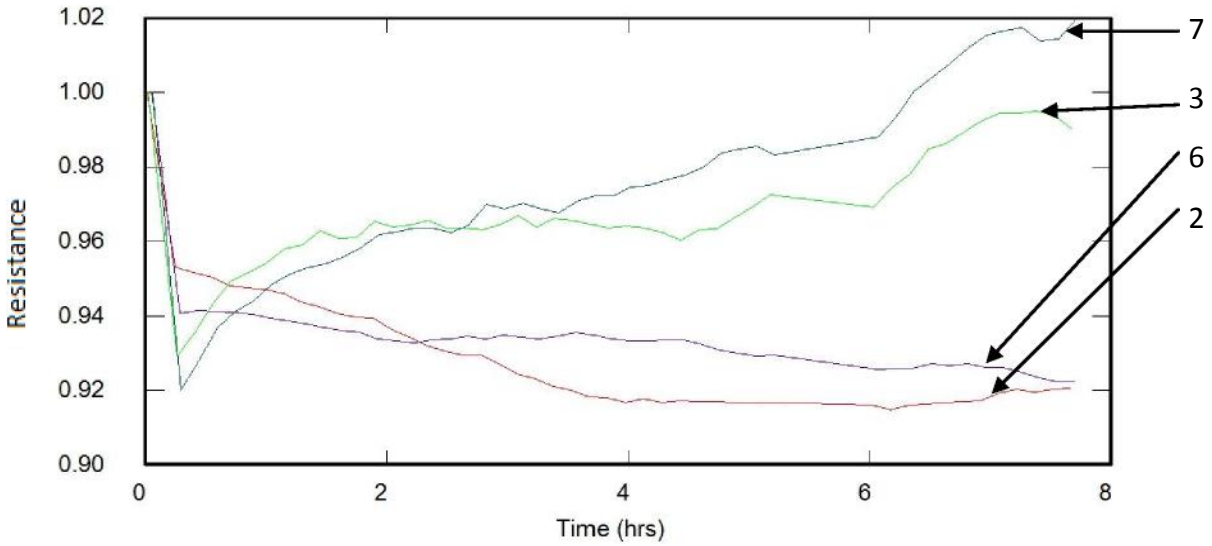


Figure 5.8 Wounding 2

Wounding 2:
2=empty plasmid
3= NWASP^{+}
6=Empty plasmid + Wiskostatin at 50 ng/ml
7= NWASP^{+} + Wiskostatin at 50 ng/ml

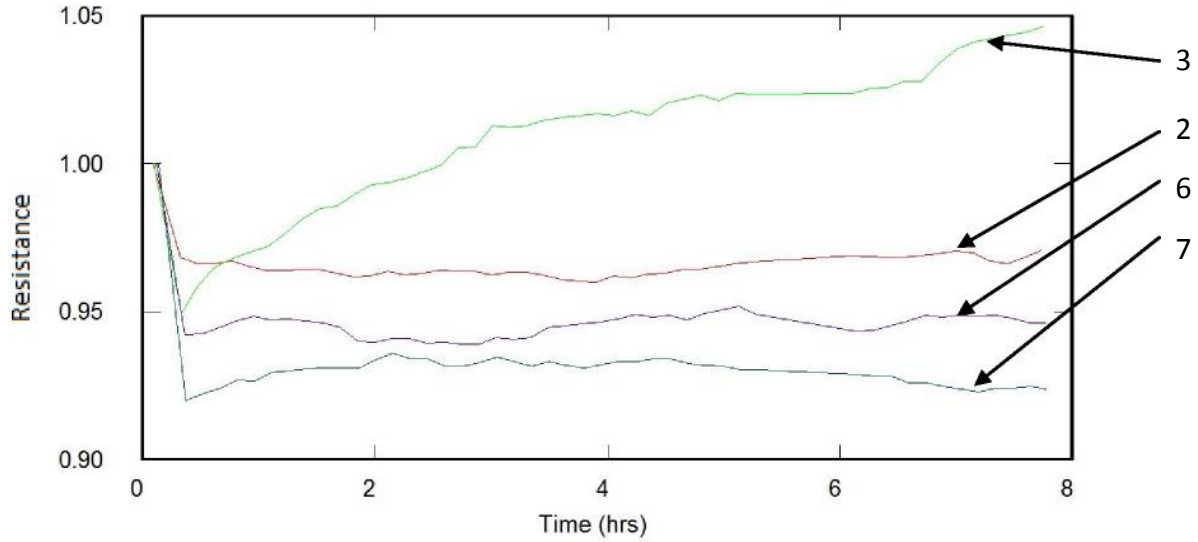


Figure 5.9 Wounding 3

Wounding 3:
2=empty plasmid+ HGF at 25ng/ml
3= NWASP^{+} + HGF at 25ng/ml
6=Empty plasmid + Wiskostatin at 50ng/ml + HGF at 25 ng/ml
7= NWASP^{+} + Wiskostatin at 50ng/ml+ HGF at 25 ng/ml

Post wounding, with the attachment and spreading of cells on the gold electrode, the impedance increased because the cells acted as insulating particles to restrict the current flow. After the cells were fully spread, the measured impedance continued to fluctuate to reflect the constant motion and metabolic activity of the cells.

In the wounding experiment (Figure 5.7), it is apparent that there is not much difference in migration speeds of the various cells. The graph for the wounding experiment 2 (Figure 5.8) shows that Wiskostatin [inhibitor] depressed the migration of the empty plasmid, but not the NWASP^{+} cells. The graph for the wounding experiment 3 [Figure 5.9] is more difficult to interpret as it increased the migration of the NWASP^{+} cells, but in combination with Wiskostatin, decreased it.

Essentially, the ECIS experiments and subsequent data generated with the self-generated attachment measurements and wounding measurements [generated in

real time] lend further support to the previously performed biological assays described in the earlier chapters.

5.4 Discussion

In the experiments conducted, we have used Wiskostatin as the inhibitor and human growth factor [HGF] as a stimulator to act as additional adjuncts to further study attachment and migration [wounding].

Wiskott-Aldrich syndrome protein (NWASP) stimulates actin-related protein (Arp) 2/3-mediated actin polymerization. This leads to diverse downstream effects, including the formation and remodelling of cell surface protrusions, modulation of cell migration, and intra-cytoplasmic propulsion of organelles and pathogens. Selective inhibitors of individual Arp2/3 activators would enable more exact dissection of NWASP dependent cellular pathways. Wiskostatin is a recently described chemical inhibitor that selectively inhibits NWASP-mediated actin polymerization *in vitro*. A growing number of recent studies have utilized this drug *in vivo* to uncover novel cellular functions for NWASP. However, the selectivity of Wiskostatin in intact cells has not been carefully explored. Studies with this drug have shown rapid and dose-dependent inhibition of NWASP-dependent membrane trafficking steps. It has also been seen that the addition of Wiskostatin inhibited numerous other cellular functions that are not believed to be NWASP dependent. Further studies have revealed that Wiskostatin treatment causes a rapid, profound, and irreversible decrease in cellular ATP levels, consistent with its global effects on cell function.

There exists significant experimental data that suggests that hypophyseal hormones influence certain phases of cancer induction and growth (Ball, 1932). Human growth hormone has clearly been implicated as one of the factors that favour the chemical induction of cancer, and increasing the rate of appearance and growth of a variety of cancers (Moon et al., 1950). For these reasons, Wiskostatin and Human growth factor were selected to aid this part of the experiments.

Referring to the results section, the first attachment study shows that the $NWASP^{+}$ cells attach even faster when treated with Human Growth Factor [HGF]. The attachment study 2 demonstrates that even with the Wiskostatin inhibition, the

$NWASP^+$ cells have better attachment. In the graph generated in the attachment study 3, $NWASP^+$ cells have an increased attachment even when treated with HGF and Wiskostatin. In order for cells to leave the primary tumour mass and disseminate, there must be a reduction in the normal adhesion to other cells as well as changes in their adhesion to the surrounding extracellular matrix. The migration of cells through the matrix and across the vessel walls allows such dissemination. This migration is dependent upon stable adherence and traction produced by adhesion molecules and their ligands. This would support the hypothesis that NWASP has a role to play in decreasing the metastatic potential of cells by enhancing attachment capability.

In the wounding experiment, a very minor difference in migration speeds of the various cells was observed. The graph for the wounding experiment 2 shows that Wiskostatin [inhibitor] depressed the migration of the empty plasmid, but not the $NWASP^+$ cells. This was contrary to the expected finding that the NWASP cells – should they be inhibited by Wiskostatin- should also show a decreased migratory capacity. The graph for the wounding experiment 3 poses even more difficulty in interpretation, as it increased the migration of the $NWASP^+$ cells, but in combination with Wiskostatin, decreased it. These results suggest that there are more than one and possibly a myriad of complex interactions that will govern the net effect of external forces on behaviour. Further studies would be needed to elucidate the same.

In summary, the results support the idea that the measured resistance changes reflect the ability of cells to adhere, and also to migrate. This, in turn, is a marker of the metastatic potential of the cells. Attachment and Wounding assays conducted using the ECIS system represented several advantages and/or unique features when compared with other invasion measurement methods used. First, attachment and migration of breast cancer cells – which are important to assess metastatic potential- is measured, which better mimics in vivo events compared with Matrigel-coated chamber assays. Second, the ECIS system has high sensitivity. Finally, ECIS follows real-time changes during cell invasion, illustrating the kinetics of this process, whereas the Matrigel method only measures one end-point result at a time.

Chapter 6

GENERAL DISCUSSION

6.1 General Discussion

This is the first study to show aberrant expression of NWASP transcript in human breast cancer. The results demonstrate that NWASP is significantly down-regulated in human breast cancer, being reduced in node positive tumours and having a negative correlation with the NPI status. Moreover, reduced expression is associated with poor outcome for patients, i.e. metastatic disease, local recurrence and death from breast cancer. These data are supported by the reduced expression at the protein level of NWASP, particularly in invasive tumours as deduced from immunohistochemistry. *In vitro* studies determined that over-expression of NWASP led to human breast cancer cells having reduced motility and invasion but with increased adhesion. The over-expression of the NWASP protein was spatially modulated by HGF over time (90 min incubation) from a nuclear/peri nuclear location to the periphery of the cell-particularly at the leading edge. *In vivo* studies revealed that these altered breast cancer cells had significantly reduced tumour growth. These data collectively indicate that NWASP is a potential tumour suppressor in breast cancer.

Increased motile behaviour in cancer cells is a prerequisite for the successful metastasis. Therefore, proteins involved in the regulation of cancer cell motility could be the key in the understanding of how this process occurs, might be prevented and could then be used as potential prognostic indicators. NWASP family proteins, including WASP, NWASP and Scar/WAVE, are potent activators of the Arp2/3 complex (Kowalski et al., 2005) and together they are important nucleators of new actin filaments in response to signals causing cell shape and motility changes (Parsons et al., 2005). GTP-loaded CDC42 and phosphatidylinositol-4, 5-bisphosphate [PtdIns (4, 5) P2] synergistically enhance NWASP-induced actin nucleation by the Arp2/3 complex (Higgs and Pollard, 2000). Although identified arising from the Wiskott-Aldrich Syndrome, which gives rise to a number of malignancies (Perry et al., 1980, ten Bensel et al., 1966, Sullivan et al., 1994), NWASP has not been greatly investigated in clinical samples of other tumour types with most of the work being concentrated on the signalling/control of actin reorganisation at the molecular level (Jones et al., 2002). Previously in breast cancer cells, phosphoinositide 3-kinase (PI3K), small

Rho GTPases, such as CDC42 and Rac1, and NWASP have been demonstrated as vital effectors in the regulation of dynamic changes to the actin cytoskeleton and during cell migration. Divergent signalling occurs downstream of PI3K in MDA-MB-231 cells after uPA- and EGF-stimulated chemotaxis, cytoskeletal rearrangements and activation of CDC42, Rac1 and NWASP. The activity of PI3K was found to be unnecessary for these uPA-induced chemotactic responses. However, EGF induced changes were entirely dependent upon PI3K. The PI3K-independent chemotactic signalling by uPA involved the disruption of interactions between β1-integrins and NWASP and translocation of NWASP to the actin cytoskeleton (Linder et al., 1999).

The data presented here indicates that the loss of NWASP in human breast cancer samples may indicate a dysregulation in the regulation of changes in the actin cytoskeleton and hence result in increased migratory potential of the tumour cells. As an initial event in the metastatic cascade, it is not surprising that the loss of NWASP is, therefore, associated with patients that have metastatic disease, local recurrences and eventual death from the disease.

NWASP is involved mostly in the formation of podosomes (cell-substrate adhesion sites) (Yamazaki et al., 2005), or invadopodia (in oncogenic cells) which are consisted of filamentous actin-rich cores and surrounding ring structures containing adhesive proteins such as vinculin and talin. These are also involved in the degradation of the ECM (Yamaguchi et al., 2006) and it has been shown that EGF receptor signalling regulates invadopodium formation through the NWASP-Arp2/3 pathway, with cofilin being necessary for the stabilization and maturation of invadopodia in invasive carcinoma cells (Sarrio et al., 2006).

Interestingly, given that ezrin, one of the ERM protein family (ezrin, merlin, radixin, moesin), is associated with malignant progression and metastasis in human neoplasias (Martin et al., 2003, Manchanda et al., 2005); it has been discovered that merlin and the ERMs can interact with and regulate NWASP (Sukumvanich et al., 2004). Merlin and moesin were found to inhibit NWASP mediated actin assembly *in vitro*, a function that appears independent of their ability to bind actin. In addition, exogenous expression of constitutively active ERM inhibits NWASP dependent Shigella tail formation, suggesting that ERMs may function as inhibitors

of NWASP function *in vivo*. This novel function of merlin and the ERMs illustrates a mechanism by which these proteins directly exert their effects on actin reorganization. This also provides new insight into the regulation of NWASP and provides evidence to support the potential importance of changes in NWASP in human breast cancer and how they may relate to cancer progression and metastatic disease. We have recently reported that the ERM family and Rho GTPase, are also aberrant in the same cohort of patients (Jiang et al., 2003, Martin et al., 2003), providing additional evidence that NWASP and its regulatory proteins are amongst the molecular complexes that are frequently dysregulated in breast cancer and contribute to disease progression in this malignancy. It was interesting to note that IHC revealed that, in tumour sections, NWASP showed localisation at the cell periphery, rather than the nuclear/peri nuclear location observed in the background tissue sections. This may have a bearing on the activation or conformation of the NWASP protein as NWASP has been shown to re-locate to the leading edge of extending lamellipodia after activation with accompanying reduced peri nuclear concentration (Sukumvanich et al., 2004). Moreover, it has been demonstrated that unphosphorylated NWASP is located at the nucleus which causes suppression of HSP90, HSE and Src family kinases (Suetsugu and Takenawa, 2003). The active, phosphorylated form of NWASP is then re-located to the cell periphery where CDC42 and Src are activated, thus regulating events involving the actin cytoskeleton i.e. motility, migration. The differential expression of NWASP in human breast cancer could incur aberrant activation in cancer cells and may lead to changes in regulation of migration and hence to metastatic spread.

In recent years, much work has centered on the various proteins in the cytoskeleton and the complex interactions that govern the pivotal role that these play in the two way communication between a cell and its extracellular matrix or other cells. Integrins are transmembrane receptors that mediate attachment of cells to its surroundings cells or the extracellular matrix. They play important roles in cell adhesion, migration, proliferation and survival. Integrins work alongside other proteins such as cadherins, selectins and syndecans to mediate cell–cell and cell–matrix interaction and communication. Integrins bind cell surface and ECM components such as fibronectin, vitronectin, laminin, and collagen. Talin and

Vinculin are cytoskeletal proteins that form part of a macromolecular complex on the cytoplasmic face of integrin mediated cellular junctions with the extracellular matrix. The enormous data – genetic, biochemical and structural- generated by recent studies have shown that talin is essential for the assembly of such junctions, whereas vinculin appears to be important in regulating adhesion dynamics and cell migration.

Among the cytoskeletal proteins that interact directly with the beta-chain cytoplasmic domain, talin has emerged as playing a critical role in integrin activation and linkage to the actin cytoskeleton. Talin is an elongated flexible antiparallel dimer, with a small globular head connected to an extended rod. The talin head contains a FERM (4.1/ezrin/radixin/moesin) domain with binding sites for several beta integrin cytodomains and the talin rod contains a second lower-affinity integrin-binding site, a highly conserved C-terminal actin-binding site and also several binding sites for vinculin (Ziegler et al., 2008). Vinculin can interact with F-actin both in recruitment of actin filaments to the growing focal adhesions and also in capping of actin filaments to regulate actin dynamics (Golji and Mofrad, 2013).

In conclusion, NWASP is significantly down-regulated in human breast cancer, particularly in aggressive and node positive tumours and in patients with poor prognosis. Over expression leads to altered phenotype of human breast cancer cells, from an invasive type to that of a significantly less motile and invasive but more adhesive phenotype. Moreover, these cells produced tumours of significantly reduced growth and volume *in vivo*. These data suggest that the loss of expression of NWASP in breast cancer could be an important step in the progression of this disease and as such should warrant further investigation as a tumour suppressor in human breast cancer partly by inhibition of motility and invasion.

6.2 Limitations of the current study

The current study, although comprehensive in itself as regards studying of clinical samples and genetic based analysis, merely forms a preliminary phase of a possible major investigation into the possibility of NWASP being a putative

tumour suppressor gene in relation to breast cancer. While the study has thrown up exciting leads, a number of insufficiencies arise, and these could certainly be addressed in future studies. The main limitation of this study was the number of breast cancer specimens available for study. In order for the data and results to be more robust, a larger cohort of samples would be needed. It is realized that the "normal breast tissue" samples used in the study, by virtue of being obtained from the ipsilateral breast as the cancer, is not the ideal specimen. It lends itself open to criticism due to the possibility of field change that may occur in a breast that has a cancer within it. The period of follow up is limited to 7 years. It would be interesting and more scientifically valid to have a longer period of follow up. This would indicate if trends in the initial period of follow up remained or results changed dramatically with time. A clearer link needs to be elucidated between mRNA and protein levels. This is because this study has measured mRNA expression, which although strongly correlated with protein synthesis, the association is not 100%. Moreover, the conclusions of the current study were largely drawn from the analysis of the gene transcripts, and additionally from limited information that came from protein analysis, i.e., immunohistochemistry (IHC).

The IHC study was limited here in the following two aspects:

1. Some samples in the cohort were small tumours, and, therefore, it was not possible to obtain additional sections for all the samples. This means that, in comparison to the gene transcript data, the IHC data is less complete. Also, there were rigid time constraints on the research period. A future study encompassing larger samples with emphasis on both types of analysis would be more desirable.
2. The type of the IHC analysis and lack of quantitative data from the IHC studies.

At the time of IHC staining, the staining was not prepared in a way for quantitative analysis during which the sections were already stained with Haematoxylin. This presented difficulties during image analysis of the staining. The number of available sections at the time of the study did not permit the

making of additional runs. A semi-quantitative score for assessment of cellular staining by ICC and IHC techniques was, therefore, not carried out, although a subjective description of the slides was narrated. There is scope to redress this in future studies.

The establishment of cell lines has been indispensable for biological research for several decades. However, recognized disadvantages of cell lines are that they are usually derived from tumors and have adapted to growth in culture. Cell culture media -by adding appropriate amounts of salt, glucose, amino acids, vitamins, and serum-try to mimic the natural physiology. However, the lack of a heterogeneous population of cell types and tissue architecture often abolishes cell-cell interaction and other functions based on tissue context. Cells in culture are prone to genotypic and phenotypic drifting. As a consequence, cell lines can lose tissue-specific functions and acquire a molecular phenotype quite different from cells *in vivo*. Acceptance of cell lines as model for biological function varies between fields. Cell biological studies on basic mechanisms, such as the cell cycle are routinely and overwhelmingly carried out in long-established cell lines. In contrast, there is substantial controversy of how well cell lines preserve aspects of the disease and whether or not they should be used in cancer drug development. Thus, animal experiments are often preferred despite their added complexity. Accurate molecular phenotypes to determine whether the function to be investigated is preserved in cell lines would enable a rational choice of the most appropriate experimental system.

The main focus from the outset was to focus on NWASP in breast cancer, and the functional studies of breast cancer cells transfected with cloned normal NWASP gene (MDA-231^{NWASP+}). Nevertheless, the IHC and ICC studies have generated a host of high quality photographs which in themselves are of significant value.

6.3 Future work

In order to design and deliver targeted and specific therapeutic interventions, elucidation of the cellular and molecular mechanisms of breast cancer and its spread is of paramount importance. In this light, NWASP and its potential as a tumour suppressor may have implications as a potential therapeutic intervention. The ongoing studies in molecular biology continue to shed light on the

mechanisms underlying carcinogenesis. Much work has centered on the exciting possibilities opened up due to newer agents and the promises afforded by Gene Therapy. A detailed discussion of this is beyond the scope of this work, but a mention of them must be made in light of the subject matter.

Gene therapy-the use of DNA as a pharmaceutical agent to treat disease- was first advocated with caution as early as 1972 (Friedmann and Roblin, 1972). This has now mushroomed with over 1700 clinical trials being conducted using a number of techniques for gene therapy. In somatic gene therapy, the therapeutic genes are transferred into the somatic cells. In germline gene therapy, the desirable functional genes are introduced into the germ cells and are subsequently integrated into their genomes. The delivery of DNA into the cells is accomplished using either recombinant viruses (viral vectors) or using naked DNA or DNA complexes (non-viral methods). The most common form of gene therapy involves inserting a desired gene at a location within the host genome. The use of engineered nucleases-such as the zinc finger nuclease-knocks out specific genes. This technique is currently being employed in the knockout of human CCR5 gene in T cells to control Human Immunodeficiency Virus infection (Perez et al., 2008). Of particular reference to this Thesis is the recent report coming out of Italy that children with Wiskott Aldrich Syndrome had been treated with a partially deactivated lentivirus to replace a faulty gene and that the results at 7-32 months were promising (Aiuti et al., 2013) .

It is hoped that future research on NWASP and its coding gene will make it an integral part of chemo-immunotherapeutic intervention for breast cancer, and indeed for other hematological malignancies which individuals who suffer from this condition are susceptible to. These appear far reaching and long term goals, but the potential that they afford serves as more than a catalyst to drive further research.

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APPENDIX

Appendix 1 Poster presentation

27th Annual San Antonio Breast Cancer Symposium, December 8 - 11, 2004; San Antonio, Texas

Filename: 550284

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Title: WASP is a putative tumour suppressor in human breast cancer cells and is associated with clinical outcome

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Introduction: WASP, the Wiskott-Aldrich syndrome protein (WASP) is a protein whose abnormality contributes to WAS condition. The WASP which belongs to a large protein family, is key regulators to the actin cytoskeleton

structure and is central to cell membrane ruffling, adhesion and migration. However, the role of WASP in cancer, particularly in breast cancer is not clear. The aim of the current study was to examine the impact of WASP on breast cancer cells and in clinical breast cancer.

Methods: Full length human WASP cDNA was isolated, cloned and used to transfect breast cancer cells. The invasiveness and migration of cancer cells were investigated using a Matrigel invasion and a cyto-carrier motility assay. The distribution of WASP in human mammary tissues was studied using immunohistochemical analysis and expression of WASP transcript quantitative reverse transcription PCR.

Results: MDA MB 231 cells stably transfected with WASP (MDA-231^{WASP+}) exhibited a significantly reduced in vitro invasiveness compared with control and wild type cells ($p=0.011$). MDA-231^{WASP+} cells migrated at a slower rate compared with control cells, and most notably lost its response to the migration inducer, hepatocyte growth factor/scatter factor (HGF/SF) (13.3±1.7 for MDA-$_{231}{}^{WASP+}$ and 23.2±2.3 in control cells, following stimulation with HGF/SF), suggesting that over-expression of WASP in breast cancer cells confer cells to low invasive and low migration phenotype. In human mammary tissues, epithelial cells stained strongly positive for WASP, whereas stromal cells and endothelial cells had little staining. In contrast, cancer cell in breast tumour tissues stained very weakly compared with normal epithelial cells. WASP transcripts were regularly detected in normal mammary tissues, but were frequently lost in tumour tissues, as revealed by RT-PCR. This was reflected in the quantitative analysis which showed a significantly lower levels of WASP in tumour tissues compared with normal background mammary tissues (0.83±0.3 vs 13.6 ±13, p-0.04/Although no significantly correlation between levels of the transcript and tumour grade, and TNM staging was seen, lower levels of WASP transcript were, however, correlated with the clinical outcome following a six year follow up. Patients who developed metastatic disease and who died of breast cancer had significantly lower levels of WASP transcript compared with those who remained disease free (0.04±0.02 and 0.47±0.3, vs 0.79±0.44, $p=0.01$ and $p<0.05$ respectively). Patients with local recurrence had lower levels of the transcript, however the difference was not significant ($p>0.05$, vs disease free).

Conclusion: It is concluded that WASP, a member of the WASP family acts as tumour progression suppressor in human breast cancer, partly by inhibition of migration and invasion of cancer cells.

Signature of presenting author

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Gordon A Pereira

Appendix 2: Published paper from research

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RESEARCH PAPER

N-WASP is a putative tumour suppressor in breast cancer cells, in vitro and in vivo, and is associated with clinical outcome in patients with breast cancer

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Abstract N-WASP is a key regulator of cell migration and actin polymerisation. We examined the correlation of N-WASP, with human breast cancer, in vitro, in vivo and in clinical breast cancer tissue. Immunohistochemical study of frozen sectioned human breast mammary tissues ($n = 124$) revealed that mammary epithelial cells stained positively for N-WASP and that cancer cells in tumour tissues stained very weakly. Quantitative RT-PCR revealed that breast cancer tissues had significantly lower levels of N-WASP compared with normal background mammary tissues (0.83 ± 0.3 vs 13.6 ± 13, $P = 0.03$). Although no significantly correlation was found with tumour grade and TNM staging, lower levels of transcript were seen to correlate with clinical outcome following a ten year follow up. Thus tumours from patients with predicted poor prognosis had significantly lower levels than from those with good prognosis (0.098 ± 0.14 vs 1.14 ± 0.56, $P = 0.05$). Patients with metastatic disease/died of breast cancer had significantly lower levels of N-WASP compared to those remaining disease free (0.04 ± 0.02 and 0.47 ± 0.3, vs 0.79 ± 0.44, $P = 0.01$ and $P < 0.05$ respectively). During in vitro experiments, MDA-MB-231 cells stably transfected with N-WASP (MDA-MB-231^{WASP+}) exhibited a significantly reduced in vitro invasiveness and motility compared with control and wild type cells ($P < 0.0001$), had increased adhesiveness ($P = 0.05$) and moreover MDA-MB-231^{WASP+} exhibited reduced in vivo growth ($P = 0.002$). The motogen HGF (50 ng/ml) caused a relocation of N-WASP to the cell periphery in a temporal and spatial response. It is concluded that N-WASP, a member of the N-WASP family may act as a tumour progression suppressor in human breast cancer and may thereforee have significant clinical value in this condition.

Keywords Breast cancer · Metastasis · Nodal spread · N-WASP · Prognosis

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Introduction

The ability of tumour cells to spread from primary and metastatic tumours is the major cause of death in patients with cancer. Such spread of tumour cells relies on cell motility, resulting in invasion, intravasation and extravasation of tumour cells to secondary foci—the metastatic cascade. The motility of cancer cells is thereforee an important problem in elucidating factors that may be responsible for metastatic spread. The microenvironment of the tumour cell is a major factor in the outcome of tumour cell motility during metastasis [1]. Thus, when developing the strategies that can prevent cancer spread, or determining potential prognostic indicators for the patient understanding the subtle changes resulting in increased motility are key.

Wiskott-Aldrich syndrome (WAS), also described as Werlhof's disease by Van den Bosch and Drukker [2], was originally described in American kindred where it was manifesting as eczema, thrombocytopenia, proneness to infection, and bloody diarrhoea [3], with death usually occuring before 10 years of age. Subsequently, causes of

death were found to be mainly infections or bleeding, but also due to the development of malignancies such as lymphoreticular tumours, leukaemia and reticuloendothelial system malignancies [4–6]. It has been recognised that the Wiskott-Aldrich protein provides a link between CDC42, a member of the RHO family of GTPases and the actin cytoskeleton [7]. Moreover, T lymphocytes of affected males with WAS exhibit a severe disturbance of the actin cytoskeleton, suggesting that the WAS protein may regulate its organization. The WAS protein interacting with GTP-dependent Cdc42 was detected in cell lysates in transient transfections, and with purified recombinant proteins [8], indicating that the WAS protein could function as a signal transduction adaptor downstream of Cdc42, and that cytoskeletal abnormalities may result from a defect in Cdc42 signalling. It has further been demonstrated WAS to be rare X-linked disorder with variable clinical phenotypes that correlates with the type of mutations in the WAS protein (WASP) gene [3, 6, 9].

WASP has been found to be a key regulator of actin polymerization in hematopoietic cells with 5 domains involved in signalling, cell motility/migration, in immune synapse formation and in facilitating the nuclear translocation of nuclear factor kappaB [9–10]. Mutations of WASP are located throughout the gene and either inhibit or dysregulate normal WASP function. Classic WAS occurs when WASP is absent, X-linked thrombocytopenia is present when mutated WASP is expressed, and X-linked neutropenia occurs when missense mutations occur in the Cdc42-binding site [9, 11–12].

Miki et al. [13] first described a 65 kDa protein from brain that bound to the SH3 domains of Ash/Grb2. The sequence was homologous to Wiskott-Aldrich syndrome protein (WASP) and was designated N-WASP (neural-WASP). N-WASP has several functional motifs including a pleckstrin homology (PH) domain and a cofilin-homologous region, through which N-WASP depolymerises actin filaments. N-WASP-stimulated actin assembly is responsible for membrane ruffling [14], a process that actively involves the cytoskeletal associated protein family, ERM (ezrin-moesin-radixin) [15–16] and is thereforee important for changes in cell motility and spread. N-WASP activity is regulated by an intramolecular interaction that is assuaged following concomitant binding of Cdc42-GTP to the Cdc42/Rac interactive binding (CRIB) domain and PtdIns(4,5)P_2 to the polybasic region [17]. We have recently reported that two major complexes that are linked to the WASP family, namely the ERM family and Rho GTPases are aberrantly expressed in human breast cancer [18–19].

This present study sought to determine how N-WASP expression is distributed in human breast cancer and if over-expression of this known regulator of motility might affect the in vitro and in vivo behaviour of invasive human breast cancer cells. To this end, a cohort of human breast tissues (background and tumour) were analysed by IHC and Q-PCR to discover both the distribution and change in expression of N-WASP. N-WASP was found to be down-regulated in human breast cancer, at both mRNA and protein levels. The invasive human breast cancer cell line, MDA-MB-231 was used to over-express N-WASP with over-expression resulting in cells with reduced motility and invasion, increased adhesion to basement membrane and more significantly, reduced tumour growth in vivo. This has important implications in understanding the mechanism whereby cancer cells become more motile and presents an interesting tool in analysing the progression of human breast cancer.

Materials and methods

Tissues and patients

Breast cancer cell line MDA-MB-231 was purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, England). Breast cancer tissues (n = 124) and normal background tissues (n = 32) were randomly collected immediately after surgery and stored in the deep freezer until use. Patients were routinely followed clinically after surgery. The median follow-up period was 72 months. The presence of tumour cells in the collected tissues was verified by examination of frozen sections using H & E staining by a consultant pathologist. The data concerning patient tissues is shown in Table 1.

Tissue processing and extraction of RNA and generation of cDNA

Over 20 frozen sections from the each tissue sample were homogenised in a RNA extraction solution using a hand held homogeniser to extract total RNA. The concentrations of RNA were quantified using a UV spectrophotometer. 1μg RNA was used to generate cDNA using a commercially available RT kit (AbGene Laboratories, Essex, England).

Detection of N-WASP using RT-PCR

Routine RT-PCR was carried out using a PCR master mix that was commercially available (AbGene). Primers were designed using the Beacon Designer software (version 2, California, USA), to amplify regions of human N-WASP that have no significant overlap with other known

Table 1 Clinical information for breast tumour tissues analysed ($n = 124$)

Tissue type		Grade		NPI	
Background	*n*	1	*n*	1	*n*
Tumour	33	2	24	2	68
	124	3	42	3	38
			58	Unknown	16
					2

TNM		Histology		Outcome	
1	*n*	Ductal	*n*	Healthy	*n*
2	70	Lobular	94	Metastasis	85
3	40	Other	14	Local recurrence	7
4	7		16	Mortality	5
Unknown	4			Unknown	15
	3			All poor outcomes	12
					27

NPI Nottingham Prognostic Indicator
TNM Tumour/Nodal Status

sequences and that the amplified products span over at least one intron. The primers used to amplify N-WASP were: WASPF1 5′gagctggatgagaacaacac′3 and WASPZR 5′actga acctgaccgtacaaaagaagtggcaggaagagt′3. Reactions were carried out at the following conditions: 94°C for 5 min, 36 cycles of 94°C for 15 s, 55°C for 25 s and 72°C for 15 s. PCR products were separated on a 2% agarose gel and photographed using a digital camera mounted over a UV transluminator. Samples of tissue from the whole patient cohort were used in this analysis ($n = 124$). β-actin was routinely used for normalization.

Quantitative analysis of N-WASP

The levels of N-WASP transcripts from the above-prepared cDNA was determined using a real-time quantitative PCR, based on the Amplifluor™ technology, modified from previous reported [20]. Briefly, pairs of PCR primers were similarly designed using the Beacon Designer software (version 2, California, USA), but to one of the primer, an additional sequence, known as the Z sequence (5′actgaacct gaccgtaca′3) which is complementary to the universal Z probe (Intergen Inc., England, UK). A Taqman detection kit for β-actin was purchased from Perkin-Elmer. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which has the Z sequence, 10 pmol of FAM-tagged probe (Intergen Inc), and cDNA from approximate 50 ng RNA. The reaction was carried out using IcyclerIQ™ (Bio-Rad) which equipped with an optic unit that allows real time detection of 96 reactions, using the following condition: 94°C for 12 min, 50 cycles of 94°C for 15 s, 55°C for 40 s and 72°C for 20 s. The levels of the transcripts were generated from a standard that was simultaneously amplified with the samples.

Immunohistochemical staining of N-WASP protein

Frozen sections of breast tumour and background tissue were cut at a thickness of 6μm using a cryostat. The sections were mounted on super frost plus microscope slides, air dried and then fixed in a mixture of 50% Acetone and 50% methanol. The sections were then placed in "Optimax" wash buffer for 5–10 min to rehydrate. Sections were incubated for 20 min in a 0.6% BSA blocking solution and probed with two primary antibodies for comparison (internal region epitope: goat anti-human N-WASP (sc-10122), Santa Cruz Biotechnologies Inc., Santa Cruz, California, USA and N-terminal epitope: rabbit anti-human N-WASP (ab-23394), Abcam Plc, Cambridge, UK). Following extensive washings, sections were incubated for 30 min in the secondary biotinylated antibody (Multilink Swine anti- goat/mouse/rabbit immunoglobulin, Dako Inc.). Following washings, Avidin Biotin Complex (Vector Laboratories) was then applied to the sections followed by extensive washings. Diamino benzidine chromogen (Vector Labs) was then added to the sections which were incubated in the dark for 5 min. Sections were then counter stained in Gill's Haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a cover slip and evaluated by 4 independent assessors as to intensity of staining.

Immunofluorescent staining of N-WASP protein

For immunofluorescence staining, cells were grown in 16-well chamber slides (LAB-TEK) (30,000 cells/well) in the presence or absence of HGF (50 ng/ml) and incubated in a 37°C/5% incubator for a set period of time (0–24 h). After incubation, the culture medium was aspirated, the wells rinsed with balanced salt solution (BSS) buffer and the cells fixed in methanol for 20 min at −20°C. After fixation the cells were washed twice using BSS buffer and permeabilised by the addition of 200 μl of 0.1% Triton X-100 (Sigma) detergent in Phosphate buffered solution (PBS) for 5 min at room temperature. Cells were rinsed twice with BSS buffer and 200 μl of blocking buffer (10% horse serum in TBS) was added to each well and the chamber slide incubated for 40 min at room temperature on a bench rocker. The wells were washed once with wash buffer (3% horse serum in TBS buffer containing 0.1% Tween20) and

100 µl of primary antibodies prepared in wash buffer was added to the appropriate wells. The chamber slide was incubated on the rocker for a further 60 min at room temperature. Wells were washed twice with TBS buffer (with 0.1% Tween20) and cells were incubated in 100 µl of secondary antibodies (TRITC) (diluted in the same manner as the primary antibodies) for 50 min. The chamber slide was wrapped in foil to prevent light reaching the conjugate. Finally, the wells were rinsed twice with wash buffer, once in BSS buffer mounted with FluorSave (Calbiochem-Novabiochem Ltd, Nottingham, UK) reagent and visualised using an Olympus BX51 microscope with a Hamamatsu Orca ER digital camera at ×100 using oil immersion lens.

SDS-PAGE and Western blotting for N-WASP protein expression

Total tissue or cell lysates were prepared as follows: cell or tissue were lysed in HCMF buffer plus 0.5% SDS, 0.5% Triton X-100, 2 mM $CaCl_2$, 100 µg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 10 mM sodium orthovanadate for 40 min, sample buffer was added and the protein boiled at 100°C for 10 min before clarification at 13000 ×*g* for 10 min. Equal amounts of protein from each sample were added onto an 8% polyacrylamide gel. Following electrophoresis, proteins were blotted onto nitro-cellulose sheets and blocked in 10% horse serum for 60 min before probing with primary antibodies, following with peroxidase-conjugated secondary antibody (1:2,000). Protein bands were visualised with Supersignal West Dura Extended Duration Substrate chemiluminescent system (Perbio Science UK Ltd., Cramlington, UK) and detected using a CCD UVIprochemi system (UVItec Ltd., Cambridge, UK).

Construction of expression cassette for N-WASP

A total of 10 human breast cancer cell lines were screened for endogenous expression of N-WASP. An invasive cell line, MDA-MB-231 (MDA-MB-231^{WT}) which was N-WASP negative in our hands (reported to have some expression [21]) was chosen for introduction of the N-WASP gene. The gene, after amplification from normal breast tissue cDNA (using primer sets: WASPEXF1 atgagctccgtccagcag and WASPEXR1 tcagtcttcccactcatcatc) was T-A cloned into an NT GFP-TOPO (Invitrogen) plasmid (confirmed by routine DNA sequencing) before electroporation into the breast cancer cells, followed by selection using G418. Expression of the gene was confirmed by RT-PCR, with those cells positive for N-WASP designated MDA-MB-231^{WASP+}.

In vitro assays analyzing N-WASP gene transformed breast cancer cells

Four assays were used to determine what, if any, effect over-expression of N-WASP would have on the human breast cancer cell line MDA-MB-231. HGF at 25 ng/ml was used as a stimulator of motility and invasion. There were no obvious differences in cell morphology between the wild type and transformed cells.

Invasiveness of MDA-MB-231 breast cancer cell line was assessed using the following in vitro assay. Transwell chambers equipped with 6.5 mm diameter polycarbonate filter (pore size 8 µm) (Becton Dickinson Labware, Oxford, UK) were pre-coated with 50 µg/membrane of solubilised basement membrane in the form of Matrigel (Collaborative Research Products, Bedford, MA). After membrane re-hydration, 15,000 cells were aliquoted into each insert with/without HGF/SF (25 ng/ml). After 96 h co-culture non-invasive cells were removed with cotton swabs. Invaded cells on the underside of the insert were fixed and stained with crystal violet, followed by microscopic counting (20 fields/insert).

A cell motility assay was carried out. Briefly, cells were pre-coated onto cytodex-2 carrier beads (Sigma-Aldrich, Poole, UK) for 2 h in complete medium. After the medium was aspirated and the cells washed (X2 in complete medium), they were aliquoted into wells of a 96-well plate in triplicate (300 µl/well). HGF (25 ng/ml) was added and the cells incubated over-night. The beads were washed off in medium, and the cells that had migrated onto the floor of the well fixed (4% formaldehyde) and stained with crystal violet. The cells were counted microscopically (×40).

The cell-matrix attachment assay was carried out as previously reported [16]. Briefly, Matrigel (10 µg/well) was added to a 96-well plates, which were incubated for 24 h to allow binding of matrix protein to the surface of the well. The plates were then washed and blocked with 5% BSA (bovine serum albumin). Cells were added at 10^4/well for 30 min, followed by aspiration and washing. The number of attached cells was determined by direct counting under microscope (7 counts per experimental setting).

In vivo growth assay analyzing N-WASP gene transformed breast cancer cells

The in vivo tumour progression model was adapted from similar previously described protocols [22–23]. Briefly 2×10^6 cells in 100 µl were mixed in a 0.5 mg/ml Matrigel suspension and sub-cutaneously injected into the left and right flanks of 4–6 week old athymic nude mice (CD-1; Charles River Laboratories, Kent, England, UK) and allowed to grow. The mice were maintained in filter top units and

were weighed and the tumour size measured weekly using Vernier callipers under sterile conditions. Human end points were (a) mice which suffered 25% weight loss, (b) development of tumours exceeding 1 cm^3 (subject to schedule 1 method according to the United Kingdom Home Office and the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines). At the conclusion of the experiment, the animals were weighted, humanely killed under schedule 1 and the tumours were removed and stored. Tumour volume was determined using the following formula; tumour volume $mm^3 = 0.523 \times width^2 \times length$.

Statistical analysis

Statistical analysis was performed by MINITAB version 13.32 (Minitab Inc. State College, PA, USA) using a two-sample student t-test and the non-parametric Mann-Whitney confidence interval and test, where appropriate. Statistical analysis was carried out using Mann-Whitney U test and the Kruskal-Wallis test for tissue samples and the Pearson Correlation between related molecules.

Results

Expression profiling of N-WASP in human breast cancer cells and tissues

Initially, we tested a number of paired human breast tissue (tumour and normal background) for expression of N-WASP. Both RT-PCR (Fig. 1a) and western blotting (Fig. 1b) demonstrated a reduction in expression of N-WASP at both mRNA and protein levels, leading to out subsequent full study. Additionally, we also investigated the expression of N-WASP in a limited number of cell lines, human breast cancer cell lines MDA-MB-231 and MCF-7, fibroblasts cell line MRC-5 and vascular endothelial cell line HECV. The transcript was detected only in MRC-5 cells (Fig. 1a), possibly due to their embryonic origin. Moreover, later tests revealed that the breast cancer cell line available to us did not show positive signals for N-WASP (Fig. 1c).

N-WASP in human mammary tissues

From immunohistochemistry, N-WASP was seen primarily in mammary epithelial cells (Fig. 2a using goat anti-human N-WASP, sc-10122), with little or no staining in stromal cells and endothelial cells. The protein was located in an obvious subcellular position, in and around the nuclear area, with some cytoplasmic staining. In contrast, cancer sections showed that expression of N-WASP was drastically reduced (Fig. 2b and c). In cancer cells there was only moderate staining of N-WASP. It is interesting to note that in these cancer cells, N-WASP was observable at the cell periphery, perhaps indicated a change in activation/conformation of the protein. These results were supported by staining of the same tissues using an alternative N-WASP antibody (supplementary data).

We next examined the presence of N-WASP mRNA in human breast cancer tissues using quantitative RT-PCR. Matched normal and tumour tissues were tested. The levels of transcript for N-WASP were significantly lower in breast cancer tissues compared with normal tissues (Fig. 3).

Correlation of N-WASP transcripts with prognosis, grade and TNM staging

In the current study, the Nottingham Prognostic Index (NPI) was used as a prognostic indicator, taking into

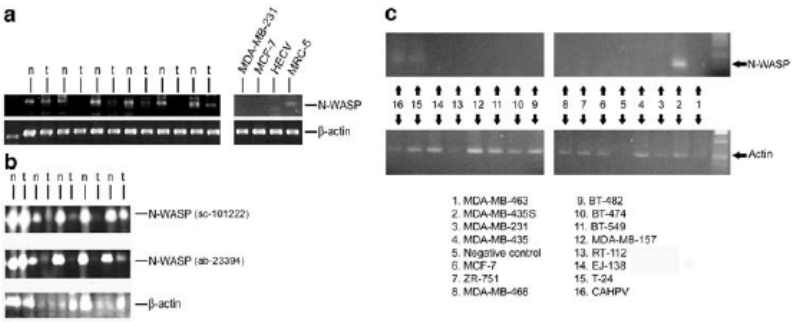

Fig. 1 Expression of N-WASP in cancer cell lines and in breast tissues. Most tumour tissues showed low levels of N-WASP transcript as shown by RT-PCR (**a**). This is supported by the reduced protein expression in the same tissue samples using Western blotting (2 N-WASP antibodies used for confirmation) (**b**). *β*-actin is shown for comparison. The human breast cancer cell lines included in the screening did not show a signal for N-WASP (**a** and **c**) as demonstrated using RT-PCR

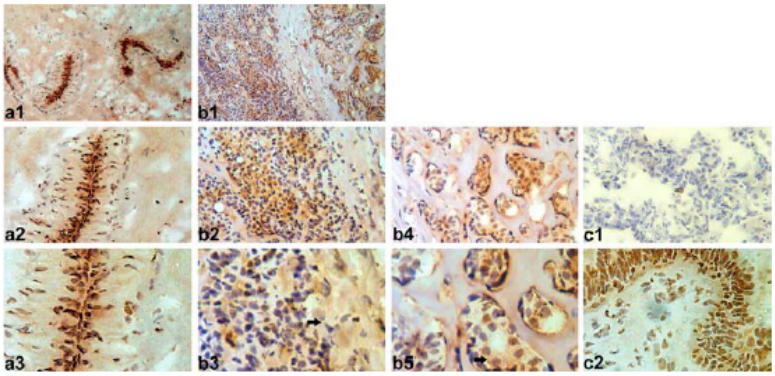

Fig. 2 Immunohistochemical staining of N-WASP in human breast cancer tissue. Panels **a1**–**a3** show background tissue at increasing magnification (×40, ×100 and ×200), panels **b1**–**b3** show matched tumour tissue at the same magnification, with panels **b4**–**b5** showing a second magnified section of the same tumour tissue. Panels **c1** (×100) and **c2** (×100) show the negative and positive controls for N-WASP (sc-10122) respectively. There was a reduction in the staining in cancer cells with increased location of the N-WASP protein to the cell periphery (see arrows). This distribution and change in intensity was confirmed using a second N-WASP antibody (supplementary data)

consideration of the size of tumours, number of lymph nodes and tumour grade. Patients were divided into those with good prognosis (indicated in Fig. 4a as NPI-1), with moderate prognosis (NPI-2) and those with poor prognosis (NPI-3). There was a stepwise decrease of the levels of N-WASP transcript from good to poor prognosis, with the difference between good and poor prognoses being statistically significant (Fig. 4). The difference between moderate and good, or moderate and poor did not reach significance ($P > 0.05$). The Kruskal Wallis test of the levels of N-WASP transcript against the prognostic index did not reveal a significant correlation. This suggests that a linear correlation between these two parameters does not exist, however, overall, the very low levels indicate poor prognosis. There was an observable different between node negative and node positive tumours (Fig. 4b) but again, significance was not reached.

We did not find a significant correlation between tumour grade, TNM staging and histology type (Table 2).

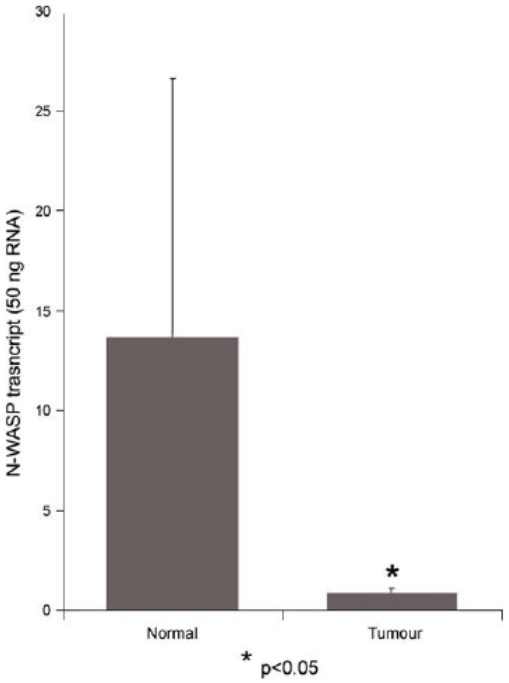

Fig. 3 N-WASP transcript in mammary tissues, number of N-WASP transcripts/50 ng RNA. The levels of N-WASP transcript were significantly lower in breast cancer tissues compared with normal background mammary tissues * $P < 0.05$

Levels of N-WASP transcript correlated with clinical outcome

The current analysis was based on a patient followup period of 72 months. It revealed that patients with metastatic disease and patients who died of breast cancer (excluding those whose died of diseases unrelated to breast cancer), had significantly lower levels of N-WASP than those who remained disease free (Fig. 5a). Although patients with local recurrences also had lower levels of the transcript, the difference is yet to be statistically significant ($P = 0.06$). In addition, survival curves showed that higher levels of N-WASP expression were associated with disease-free survival, $P = 0.0498$ (Fig. 5b) but that decreased

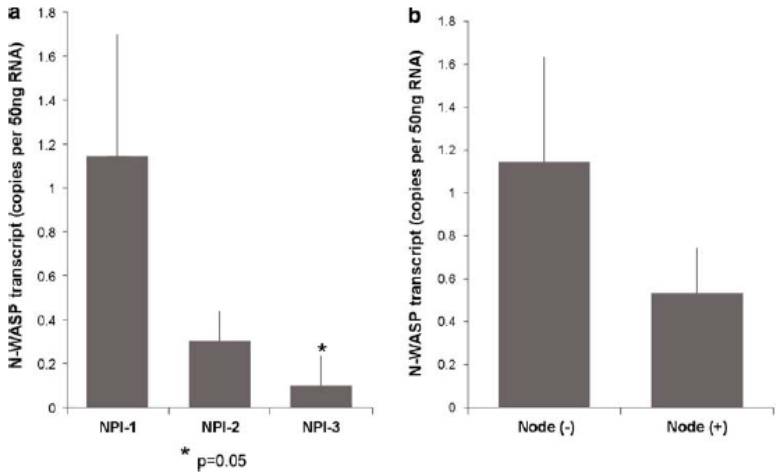

Fig. 4 N-WASP transcript levels were associated with nodal status (**a**) and predicted clinical outcome (**b**). NPI3 (NPI > 5.4) tumours had significantly lower levels of N-WASP than those of NPI1 status (NPI < 3.4), * $P = 0.05$

Table 2 Transcript levels of N-WASP in human breast cancer tissues; TNM staging, Grade and tumour-type

Prognostic Indicator		
TNM staging		
	TNM 1	0.788 ± 0.443
	TNM 2	1.116 ± 0.584
	TNM 3	0.605 ± 0.406
	TNM 4	0.198 ± 0.124
Grade		
	1	0.279 ± 0.2
	2	0.253 ± 0.07
	3	1.45 ± 0.68
Tumour type		
	All	0.83 ± 0.303
	Ductal	0.932 ± 0.38
	Lobular	0.0531 ± 0.04
	Others	1.351 ± 0.813

No significant difference was reached

levels of N-WASP expression were associated with poor overall survival in patients with breast cancer (Fig. 5c). Survival was analysed using the Cox-proportion-Hazardous test and showed a 0.0360 probability of low N-WASP levels indicating low survivability. The apparent discrepancy of transcript levels between patients with metastatic disease and those having died from breast cancer may be an artificial bias due to the relatively low numbers of these patients in each category.

Interestingly, there was no correlation between levels of WASP and other related or associated proteins after Pearson Correlation tests (<0.195) for WISP 1–3, WAVE 1–3 and CCN2-3.

Forced expression of N-WASP in the human breast cancer cell line MDA-MB-231

The human breast cancer cell line MDA-MB-231 was chosen to study the forced expression of N-WASP as these cells did not show a signal for this molecule (Fig. 1a ,c). RT-PCR was used to detect the presence of the N-WASP protein in the transfected cells, which was confirmed using western blotting (Fig. 6a) and immunofluorescence (Fig. 6b). The stably transfected cells were then assayed to determine what the effect of expression of N-WASP was on this invasive human breast cancer cell line. Changes in invasiveness were assessed, with MDA-MB-231^{WASP+} cells showing significantly reduced invasion compared to the wild type cells (MDA-MB-231^{WT}) and GFP-plasmid control cells (MDA-MB-231^{GFP}), Fig. 6c ($P < 0.0001$). This invasiveness remained reduced even in the presence of HGF (25 ng/ml), which actively induces invasion in this cell type ($P < 0.0001$). Motility of the cells was also significantly reduced ($P < 0.0001$) compared to the wild type and plasmid control cells (Fig. 6d), which is consistent with the role of N-WASP as a regulator of the actin cytoskeleton and again remained reduced in the presence of HGF ($P < 0.0001$). The adhesion of these cells was unexpectedly increased (MDA-MB-231^{WASP+} 10.4 ± 3.8 versus MDA-MB-231^{WT} 7.6 ± 2.2; $P = 0.05$), as shown in Fig. 6e.

The effect of HGF on the temporal and spatial distribution of N-WASP was determined by immunofluorescence over 90 min, Fig. 6f. It appears that the nuclear and cytoplasmic expression of N-WASP was modified upon treatment with HGF by 30 min incubation, with the redistribution of N-WASP towards the cell periphery. This was clearly evident by 60 min incubation, with N-WASP

clearly located at the ruffling, leading edge of the cell. By 90 min incubation, the effect was beginning to abate. There was no loss of expression of N-WASP, merely a redistribution of this protein.

In vivo tumour growth of MDA-MB-231^{WASP+} cells

The tumours induced in the athymic nu/nu female mice were measured over 28 days. During the growth period and at the experimental end point, the MDA-MB-231^{WASP+} tumours were consistently slower growing compared to the MDA-MB-231^{WT} and MDA-MB-231^{GFP} control cells (Fig. 7). After 28 days, the tumour volume of the MDA-

Fig. 6 Over-expression of N-WASP in human breast cancer. N-WASP was successfully over-expressed in MDA-MB-231 cells (MDA-MB-231 WASP). The successful expression of N-WASP in transfected MDA-MB-231 cells (MDS-MB-231 WASP) is shown after western blotting (**a**). The fluorescent microscopy (**b**) clearly shows the expression of the protein at both a subcellular and peripheral location within MDA-MB-231 WASP cells. The absence of N-WASP in both GFP control plasmid cells (MDA-MB-231 GFP) and MDA-MB-231WT cells is also demonstrated. Bright field images are shown to indicate the presence of cells. Forced expression of N-WASP resulted in cells with significantly lower levels of invasiveness (**c**) and motility (**d**) ($P < 0.0001$), in response to HGF (25 ng/ml) and increased adhesiveness to basement membrane (**e**) ($P = 0.05$). HGF (50 ng/ml) effected a temporal and spatial re-location of N-WASP in the transfected cells, as indicated by arrows (**f**). A nuclear/peri-nuclear expression gave way to increased cytoplasmic and cell peripheral location by 60 min incubation with HGF

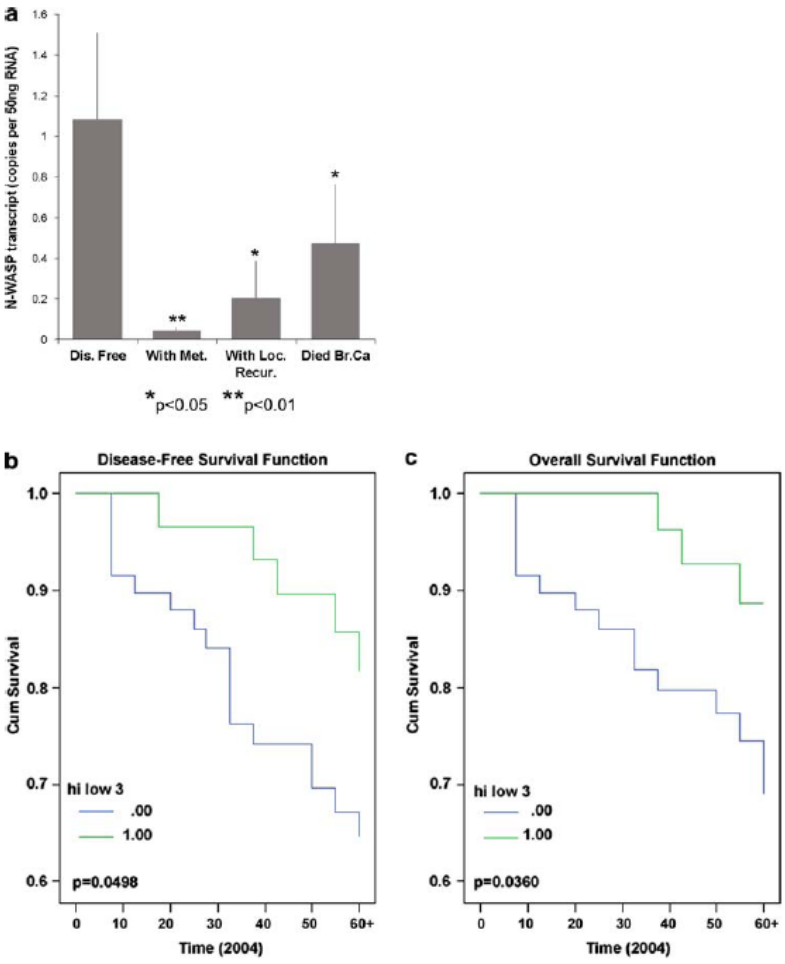

Fig. 5 N-WASP transcript levels were associated with clinical outcome. Significantly lower levels of the N-WASP transcript were seen in those patients with metastatic disease ($P < 0.01$) and in those patients who died of breast cancer ($P = 0.05$) (**a**). Moreover, survival curves indicated that N-WASP was associated with disease-free survival in patients with breast cancer (**b**). Reduced levels of N-WASP were associated with poor overall survival of patients (**c**)

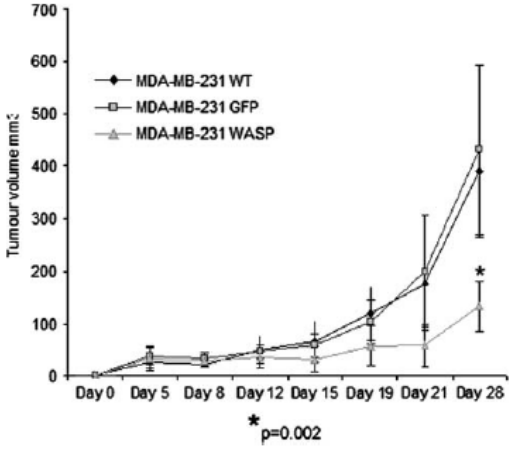

Fig. 7 The over expression of N-WASP in human breast cancer cells and in vivo tumour growth. The growth of tumours was significantly reduced in MDA-MB-231 WASP cells compared to the wild type (MDA-MB-231 WT) and plasmid control cells (MDA-MB-231 GFP) ($P = 0.002$)

MB-231^{WASP+} cells was significantly smaller than both control cell groups, indicative of an inhibitory effect of N-WASP in these human breast cancer cells (Final tumour volume mm^3: MDA-MB-231^{WASP+} 132.57 ± 48.69 versus MDA-MB-231^{WT} 390.72 ± 125.66 and MDA-MB-231^{GFP} 431.84 ± 162.79; $P = 0.002$).

Discussion

This is the first study to show aberrant expression of N-WASP transcript in human breast cancer. The results demonstrate that N-WASP is significantly down-regulated in human breast cancer, being reduced in node positive tumours and having a negative correlation with NPI status. Moreover, reduced expression is associated with poor outcome for patients, i.e. metastatic disease, local recurrence and death from breast cancer. These data are supported by the reduced expression at the protein level, of N-WASP, particularly in invasive tumours as deduced from immunohistochemistry. In vitro studies determined that over-expression of N-WASP led to human breast cancer cells having reduced motility and invasion but with increased adhesion. The over-expression of the N-WASP protein was spatially modulated by HGF over time (90 min incubation) from a nuclear/per-nuclear location to the periphery of the cell-particularly at the leading edge. In vivo studies revealed that these altered breast cancer cells had significantly reduced tumour growth. These data collectively indicate that N-WASP is a potential tumour suppressor in breast cancer.

Increased motile behaviour in cancer cells is a prerequisite for the successful metastasis. Thereforee, proteins involved in the regulation of cancer cell motility could be key in the understanding of how this process occurs, might be prevented and could then be used as potential prognostic indicators. WASP family proteins, including WASP, N-WASP and Scar/WAVE are potent activators of the Arp2/3 complex [24] and together they are important nucleators of new actin filaments in response to signals causing cell shape and motility changes [25]. GTP-loaded Cdc42 and phosphatidylinositol-4,5-bisphosphate (PtdIns (4,5)P2) synergistically enhance N-WASP-induced actin nucleation by the Arp2/3 complex [26].

Although identified arising from the Wiskott-Aldrich Syndrome, which gives rise to a number of malignancies [4–6], N-WASP has not been greatly investigated in clinical samples of other tumour types with most of the work being concentrated on the signalling/control of actin reorganisation at a molecular level [27]. Previously in breast cancer cells, phosphoinositide 3-kinase (PI3K), small Rho GTPases, such as Cdc42 and Rac1, and N-WASP have been demonstrated as vital effectors in the regulation of dynamic changes to the actin cytoskeleton and during cell migration. Divergent signalling occurs downstream of PI3K in MDA-MB-231 cells after uPA- and EGF-stimulated chemotaxis, cytoskeletal rearrangements and activation of Cdc42, Rac1 and N-WASP. The activity of PI3K was found to be unnecessary for these uPA-induced chemotactic responses. However, EGF induced changes were entirely dependent upon PI3K. The PI3K-independent chemotactic signalling by uPA involved the disruption of interactions between $\beta(1)$-integrins and N-WASP and translocation of N-WASP to the actin cytoskeleton [28].

The data presented here indicates that the loss of N-WASP in human breast cancer samples may indicate a dysregulation in the regulation of changes in the actin cytoskeleton and hence result in increased migratory potential of the tumour cells. As an initial event in the metastatic cascade, it is not surprising that loss of N-WASP is thereforee associated with patients that have metastatic disease, local recurrences and eventual death from the disease.

WASP is involved mostly in the formation of podosomes (cell-substrate adhesion sites) [29], or invadopodia (in oncogenic cells) which are consisted of filamentous actin-rich cores and surrounding ring structures containing adhesive proteins such as vinculin and talin. These are also involved in the degradation of the ECM [30] and it has been shown that EGF receptor signalling regulates invadopodium formation through the N-WASP-Arp2/3 pathway, with cofilin being necessary for the stabilization and maturation of invadopodia in invasive carcinoma cells [31].

Interestingly, given that ezrin, one of the ERM protein family (ezrin, radixin, moesin), is associated with malignant progression and metastasis in human neoplasias [32–

tumour suppressor in human breast cancer partly by inhibition of motility and invasion.

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Appendix 3: Control and Vector used in study

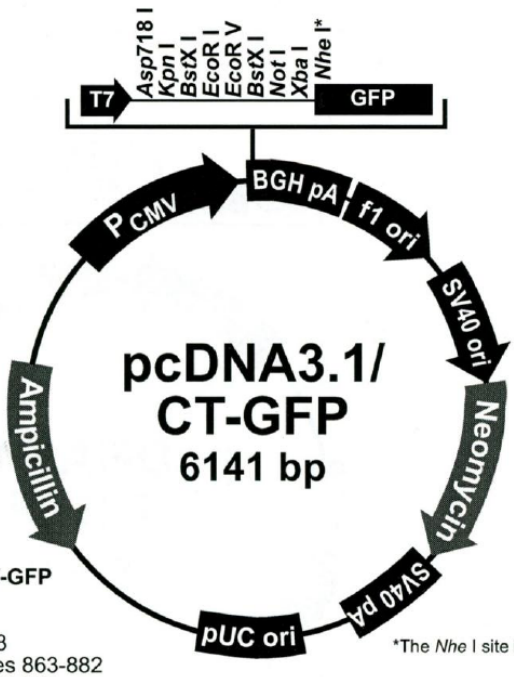
pcDNA3.1/CT-GFP Map

Description

pcDNA3.1/CT-GFP is a 6141 bp control vector containing the gene for Cycle 3 GFP. The vector was constructed by cloning an *Xba* I-*Eco*R I/Klenow fragment containing Cycle 3 GFP (with initiation codon) into pcDNA3.1/V5-His B digested with *Xba* I and *Pme* I. Expression and fluorescence of Cycle 3 GFP has been confirmed.

Map of Control Vector

The figure below summarizes the features of the pcDNA3.1/CT-GFP vector. **The complete nucleotide sequence for pcDNA3.1/CT-GFP is available for downloading from our World Wide Web site (www.invitrogen.com)** or by contacting Technical Service (page 32).



*The *Nhe* I site is at the N-terminus of GFP

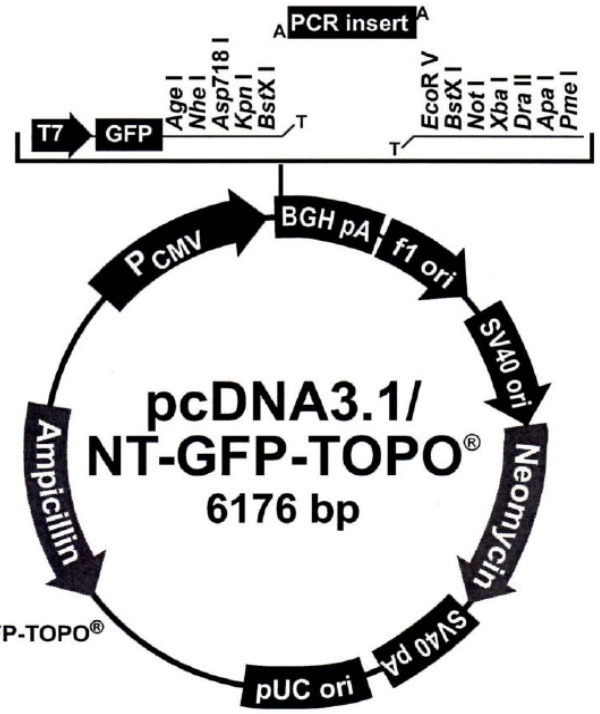
Comments for pcDNA3.1/CT-GFP 6141 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 908-996
GFP ORF: bases 988-1707
BGH reverse priming site: bases 1729-1746
BGH polyadenylation signal: bases 1732-1959
f1 origin of replication: bases 2005-2433
SV40 promoter and origin: bases 2461-2769
Neomycin resistance gene: bases 2844-3638
SV40 polyadenylation signal: bases 3814-3944
pUC origin: bases 4327-5000
Ampicillin resistance gene: bases 5145-6005

pcDNA3.1/NT-GFP-TOPO® Map

Map

The figure below summarizes the features of the pcDNA3.1/NT-GFP-TOPO® vector. The vector is supplied linearized between base pairs 1678 and 1679. This is the TOPO® Cloning site. **The complete nucleotide sequence is available for downloading from our World Wide Web site (www.invitrogen.com)** or from Technical Service (page 32).



Comments for pcDNA3.1/NT-GFP-TOPO®
6176 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
GFP ORF: bases 905-1621
GFP Forward priming site: bases 1512-1529
Multiple cloning site: bases 1645-1754
TOPO® Cloning site: 1690-1691
BGH reverse priming site: bases 1766-1783
BGH polyadenylation sequence: bases 1769-1996
f1 origin of replication: bases 2042-2470
SV40 promoter and origin: bases 2498-2806
Neomycin resistance gene: bases 2881-3675
SV40 polyadenylation sequence: bases 3849-3979
pUC origin: bases 4362-5035 (opposite strand)
Ampicillin resistance gene: bases 5180-6040 (opposite strand)

Appendix 4

Ethical approval used for collection of tissue samples

AWDURDOD IECHYD
BRO TAF
HEALTH AUTHORITY

19 December 2001 AD/JS/JJL

Mr M A Al-Rawi,
Department of Surgery,
University of Wales College of Medicine,
Heath Park,
Cardiff.

Dear Mr Al-Rawi,

01/4303 - The effects of lymphangiogenesis in breast cancer metastasis

The Bro Taf Local Research Ethics Committee (Panel B) reviewed the above application for ethical approval at its meeting on the 19th December 2001. I am pleased to be able to inform you that full ethical approval was granted.

I enclose for your information a copy of the Bro Taf Membership list on which the Members of Panel B, who were present at the meeting on the 19th December 2001, are indicated. I confirm that the Bro Taf Local Research Ethics Committee complies with the ICH Guidelines for Good Clinical Practice as they relate to an Independent Ethics Committee. A copy of the Committee's Constitution and Terms of Reference is available on request.

You will no doubt realise that whilst the Local Research Ethics Committee has given approval for your project on ethical grounds, it is still necessary for you to obtain approval, if you have not already done so, from the relevant Clinical Director and/or Chief Executives of Trusts (or U.W.C.M.) in which the work will be carried out.

The committee attach certain standard conditions to all ethical approval. These are that if staff conducting research should change, any new staff should read the research programme submitted to the committee for ethical approval and this letter (and any subsequent letter I may write concerning this application for ethical approval); that if the procedures used in the research programme should change or the programme itself should be changed you should consider whether it is necessary to submit a further application for any modified or additional procedures to be approved and if the employment or departmental affiliation of the staff should change you should notify me of that fact. Any material changes to the structure or operation of the trial (including the recruitment of subjects) must be submitted to, and approved by, the Committee before being adopted.

☑ HEADQUARTERS:
Churchill House

☐ Temple of Peace and Health
Cathays Park, Cardiff, CF10 3NW

The Committee also ask that if any serious adverse events occur or if you should encounter any unexpected ethical issues, you will inform them of what these are. Full ethical approval needs to be resought if any study does not begin within two years of the date of this letter.

Yours sincerely,

A. Dowden.

Mrs A Dowden,
Chairman, Panel B,
Bro Taf Local Research Ethics Committee