THE UNIVERSITY OF HULL

PROTEOMIC IDENTIFICATION AND VALIDATION OF BIOMARKERS ASSOCIATED WITH RESISTANCE TO RADIOTHERAPY IN BREAST CANCER

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By

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Dedicated to the memory of my late father, who would have been so proud.

Abstract

Background:

Breast cancer is an important health issue. The majority of patients present with early stage cancer and are therefore candidates for breast conserving surgery and radiotherapy. A proportion will suffer from local recurrence, which may be secondary to radiotherapy resistance. Though extensive research has been carried out into molecular markers of resistance, none has been applied to clinical practice, which suggests that the search for such markers is wanting.

Materials and Methods:

The principle of the biomarker discovery pipeline was applied and cancer cell lines were utilised for the first two phases of this project. Protein expression in radiosensitive and radioresistant cell lines was compared using, first antibody microarray technology (AbMA), as a screening tool, and secondly, western blot (WB) technique as a verification tool. The final stage was clinical validation. A clinical series of archival breast cancer tissue was identified; one representing a radiosensitive group, and a second representing a radioresistant group. Immunohistochemistry (IHC) was then employed to compare the differential protein expression between the two.

Results:

The AbMA technology was successfully utilised to yield 63 potential biomarkers of radioresistance. Of these, zyxin, PIASx and DR4 were confirmed using WB. Clinical validation showed no association between zyxin and radioresistance; this protein had been previously suggested to be associated with cellular stress. DR4 has been clinically validated using IHC, and has therefore been identified as a putative biomarker using all three techniques. In addition, the association between radioresistance and the 26S proteasome was clinically validated.

Discussion:

This work supports the role of zyxin as a stress associated protein. The under-expression of DR4, a pro-apoptotic factor, and 26S proteasome, a major effector in the protein proteolysis machinery and cell cycle has been proven. These two proteins present putative markers of radioresistance. The possibility of pre-treatment definition of the expected response to radiation therapy would improve patients' outcome. Radiation can be offered only to those expected to respond to it, while others would be offered other treatment modalities.

Publications

1. A possible role for the 26S proteasome in radiotherapy resistance and loco-regional recurrence following breast conserving therapy for early breast cancer. **D ELFadl**, V Hodgkinson, ED Long, L Scaife, P Drew, M Lind, L Cawkwell. Breast 2011;20(4):334-337.

2. Repeatedly identified differentially expressed proteins (RIDEPs) from antibody microarray proteomic analysis: the importance of cautious data interpretation. V Hodgkinson, **D ELFadl**, P Drew, M Lind, L Cawkwell. J Proteomics 2011;74(5):698-703.

Published abstracts

1. Immunohistochemistry confirms down-regulation of protein markers associated with resistance to radiotherapy. **D ELFadl**, V Hodgkinson, E Long, AW Beavis, PJ Drew, MJ Lind, L Cawkwell. Br J Surg 2011;98(Suppl.2):6-39.
2. Antibody microarray technique identifies protein markers associated with radioresistance in three breast cancer cell lines. **D ELFadl**, L Smith, O Qutob, MB Watson, AW Beavis, V Garimella, PJ Drew, MJ Lind, L Cawkwell. Br J Surg 2009;96(Suppl. 2):1-26.
3. Antibody microarray analysis identifies biomarkers associated with radioresistant breast cancer cell lines. **D ELFadl**, L Smith, O Qutob, MB Watson, AW Beavis, V Garimella, PJ Drew, MJ Lind, L Cawkwell. Cancer Res 2009;69 (2 Suppl):5072.
4. Proteomic identification of putative biomarkers of radiotherapy resistance. L Scaife, VC Hodgkinson, **D ELFadl**, S Mehmood, IA Hunter, GP Liney, AW Beavis, PJ Drew, MJ Lind and L Cawkwell. Radiotherapy and Oncology 103 (suppl 1): S216-S217 (2012).

Presentations

Oral:

1. Immunohistochemistry confirms down-regulation of protein markers associated with resistance to radiotherapy. SARS annual meeting, Dublin, January 2011.
2. Antibody microarray technique identifies protein markers associated with radioresistance in three breast cancer cell lines. SARS annual meeting. Bristol, UK, January 2009.

Poster:

3. Proteomic identification of putative biomarkers of radiotherapy resistance. European Society for radiotherapy and oncology conference. Barcelona, Spain, May 2012.
4. DR4, identified by antibody microarray analysis, may be a novel biomarker of radioresistance. European Molecular Biology Organization conference. Dublin, Ireland, June 2011.
5. Antibody Microarray identification of putative biomarkers associated with radioresistance in head and neck cancer. European Molecular Biology Organization conference. Dublin, Ireland, June 2011.
6. Protein biomarkers in radioresistant cancer cells identified using antibody microarrays. HUPO 8th Annual World Congress (Human Proteome Organization), Toronto, Canada, September 2009.
7. Antibody microarray technique identifies protein markers associated with radioresistance in breast and oral cancer cell lines. The Milan 11th Breast Cancer Conference. Milan, Italy, June 2009.
8. Antibody microarray analysis identifies biomarkers associated with radioresistant breast cancer cell lines. San Antonio Breast Cancer Symposium. San Antonio, Texas, December 2008.

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List of abbreviations

AbMA	Antibody Microarray
ATM	Ataxia Telengectasia Mutated
ATR	ATM and Rad-3 related
AJCC	American Joint Committee on Cancer
ANC	Axillary Node Clearance
BASO	British Association of Surgical Oncology
BCS	Breast Conserving Surgery
BCT	Breast Conserving Therapy
BSA	Bovine Serum Albumin
CA IX	Carbonic-anhydrase IX
Cdk	Cyclin Dependent Kinase
Chk1	Check Point Protein 1
Chk2	Check Point Protein 2
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CT	Computed Tomography
DAB3	3`-diaminobenzidine tetrahydrochloride
DCIS	Ductal Carcinoma in situ
DDR	DNA Damage Response
DFS	Disease Free Survival
DR4	Death Receptor 4
DR5	Death Receptor 5
EBCTCG	Early Breast Cancer Trialists Collaborative Group
EGFR	Epidermal Growth Factor Receptor
EIC	Extensive Intraductal Component
ER	Oestrogen Receptor

FFPE	Formalin Fixed Paraffin Embedded
GFI-1	Growth Factor Independent 1
HER-2	Human epidermal growth factor receptor-2
HIF-1	Hypoxia Inducible factor-1
HR	Homologous Repair
HRP	Horse Radish Peroxidase
IDC	Invasive ductal carcinoma
IGF1R	Insulin-like Growth Factor Receptor
IHC	Immunohistochemistry
IORT	Intra-Operative RadioTherapy
LABC	locally Advanced Breast Cancer
LCIS	Lobular carcinoma in situ
LOH	Loss of Heterozygosity
LR	Local Recurrence
LRR	Locoregional Recurrence
LVI	LymphoVascular Invasion
MAPK	Mitogen Activated Protein Kinase
MDT	Multi-Disciplinary Team
MRN complex	Meiotic recombination 11, Rad50, NBS1
NAC	NeoAdjuvant Chemotherapy
NHEJ	Non- Homologous End Joining
NHSBSP	National Health Service breast screening programme
NICE	National Institute for Clinical Excellence
NLS	Nuclear Localization Signal
NSABP	National Surgical Adjuvant Breast and Bowel Project
OS	Overall Survival
PABI	Partial Accelerated Breast Irradiation
PG	Prostaglandins
PI3K	Phosphotidylinositol 3-kinase
PIAS	Protein Inhibitor of Activated STAT
PKC	Protein kinase-C
PLC-γ	Phospholipase C
PN	Parent radiosensitive sublines
PR	Progesterone Receptor
PTV	Planning Target Volume
RC-DC	Reducing agent Compatible – Detergent Compatible
RIDEP	Repeatedly Identified Differentially Expressed Proteins
RPA	Replication Protein A
RR	Radioresistance
RT	Radiotherapy
SCC	Squamous Cell Carcinoma
SLNB	Sentinel Lymph Node Biopsy
Spred	Sprouty Related EVH1 Domain
STAT	Signal Transducer and Activator of Transcription
SUMO	Small Ubiquitin-related MOdifier
TBS	Tris Buffered Saline
TGF	Tumour Growth Factor

TNFα	Tumour Necrosis Factor α
TRAIL	Tumour-necrosis-factor Related Apoptosis Inducing Ligand
UBAE1	Ubiquitin Activating Enzyme 1
UICC	International Union against Cancer
UPP	Ubiquitin-Proteasome Pathway
VEGF	Vascular Endothelial Growth Factor
WB	Western blot
WBRT	Whole Breast Radiotherapy
WLE	Wide Local Excision

CHAPTER I

Introduction to Breast Cancer

1.1. Epidemiology of breast cancer

Breast cancer is the most common cancer in women. More than 48,000 people were diagnosed in 2008 in the UK, less than 1% of them were men (Cancer Research UK 2012). More than 80% of cases occur in women aged 50 years or more. The reported incidence has increased by more than 50% in the last twenty years, possibly in part following the introduction of the National Health Service breast screening programme (NHSBSP), (Figure 1.1). It is difficult to explain this increase in aetiological terms, as it is likely to be influenced by more efficient diagnosis and record keeping of cancer registries. However, the upward trend continues with a further increase of 6% reported in the last 10 years. Breast cancer remains the second most common cause of female cancer deaths after lung cancer – 11, 650 women died from it in 2009, 10% of them being younger than 50 years (Cancer Research UK 2012)

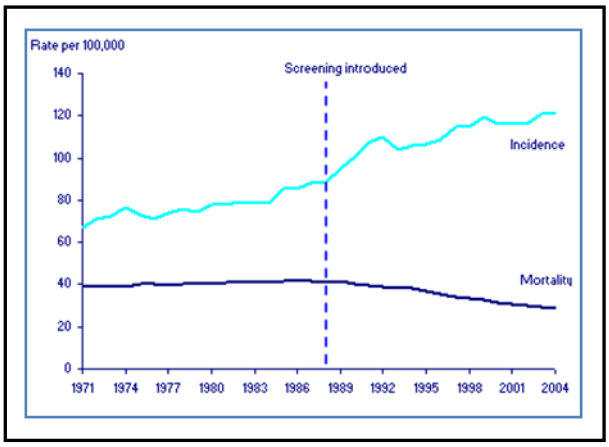

Figure 1.1 Incidence and mortality of breast cancer in the UK, and the effect of introducing the NHSBSP. In the 25 years following the introduction of NHSBSP, there was a steady decline in breast cancer mortality in spite of a documented increase in disease incidence. From: Cancer Research UK.

Earlier diagnosis and advancement in therapy have resulted in incremental improvements in survival in the last 20 years. This is a continuous process, with recent figures of 3 year survival showing an upward trend in the period 2004-2006 when compared with the period 1996-2000 (Rachet et al. 2009). The NHS cancer plan was published by the Department of Health in 2000, and implementation started after 2001. It has recommended different strategies to improve cancer care and outcomes in England and Wales, and could explain the superior survival figures reported above. The data for this study was obtained in 2008, and therefore the second comparative period (2 years) was shorter than the first period (4 years).

1.2 Aetiology of breast cancer

Breast cancer has a multi-factorial aetiology; hormonal, environmental and genetic components being contributory to its development. Female sex and increasing age are the most well documented aetiological factors. Prolonged exposure to oestrogen is a known risk factor; early menarche, late menopause, nulliparity, and older age at first pregnancy (MacMahon 2006). A survey of more than 6000 women, half of which had breast cancer reported that age at menarche of ≤ 11 years and age at first pregnancy of ≥35 years were significantly associated with breast cancer development (Magnusson et al. 1999). In addition, prolonged use of oestrogen containing contraceptive pills has been associated with post-menopausal breast cancer (Magnusson et al. 1999). However, a recent meta-analysis including 13 prospective studies that compared users of the pill with non users has concluded that the risk of developing breast cancer was not significant (Zhu et al. 2012). Women affected by breast cancer at an earlier than average age (<35), are more likely to have genetic mutations, namely BRCA1 and BRCA2 (Krainer et al. 1997). Though the exact frequency in the general population is

not known, BRCA1 has been reported to contribute to around 50% of all familial breast cancers (Krainer et al. 1997). BRCA1 mutation is associated with ovarian cancer, while BRCA2 mutation is associated with male breast cancer. Mutations in p53 (discussed in section 3.3.2.4) are also known to be associated with an increased risk of developing breast cancer. Ionizing radiation has being reported to increase the risk of breast cancer; a linear dose - effect relationship has been reported. Chest radiotherapy for the treatment of Hodgkin's lymphoma is associated with a higher risk of developing breast cancer. Furthermore, mantle field irradiation (mediastinal, axillary and cervical) is associated with an even higher risk than mediastinal radiotherapy alone (De Bruin et al. 2009). It is difficult to accurately estimate the risk secondary to low dose exposures, such as encountered in daily life, and most estimates are extrapolations from reported risks following high dose exposures (Brenner et al. 2003). RT for the treatment of breast cancer has been associated with a 1.8 relative risk of contralateral breast cancer (Clarke et al. 2005a).

1.3 Anatomy of the Breast

The adult female breast is a subcutaneous organ of the anterior thoracic wall. It extends from the sternal edge to just anterior to the mid axillary line, and from the second to the sixth ribs. An axillary tail is a subcutaneous extension of breast tissue towards the axilla.

The breast consists of breast parenchyma (the duct lobular system) and supportive tissue (adipose tissue and connective tissue stroma). Each breast contains 15-20 lobes, each of them drained by a lactiferous duct which dilates distally into a lactiferous sinus before terminating at the nipple (Figure 1.2). Each lobe consists of a group of lobules,

containing terminal ductules and acini. Lactiferous ducts are lined with simple columnar or cuboidal epithelium and an outer layer of myoepithelial cells.

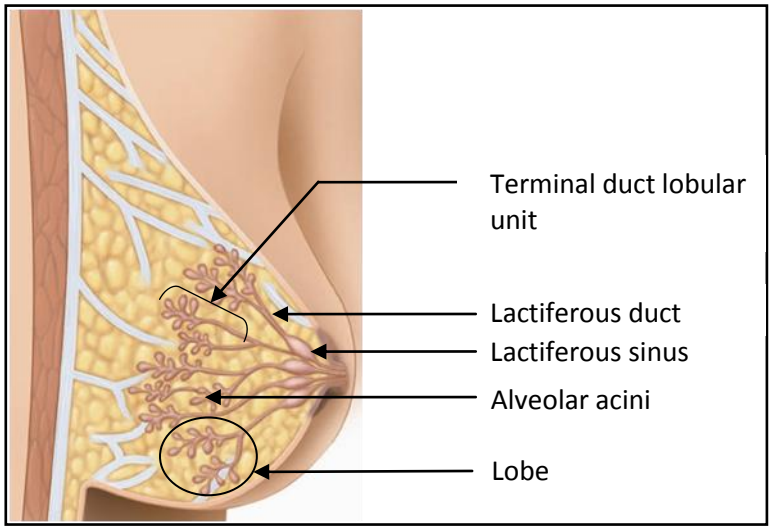

Figure 1.2 Anatomy of the breast. The major structural units of the breast are highlighted including the terminal duct lobular structures, and the distal duct systems at the nipple.

1.4 Histological types of breast cancer

Breast cancer can arise from any of the parenchymatous components of breast tissue. The terminal duct-lobular unit (Figure 1.2) is the site of most breast cancers. Invasive ductal carcinoma (IDC) and invasive lobular carcinoma are the two most common types, and together they can account for up to 90% of all breast cancers (Li et al. 2005;van de Vijver 1999). The histologic types and the frequency with which they occur in breast cancer patients are shown in Table 1.1.

Table 1.1 Histological types of breast cancer. Ductal breast carcinoma of no specific type is the most common type, followed by lobular cancer. Tubular, mucinous and medullary breast cancers are much less common. (Abeloff et al. 2004)

Histological type	Frequency (%)
Ductal carcinoma, no specific type	70-75
Lobular carcinoma	10-15
Tubular carcinoma	1-5
Mucinous carcinoma	2-5
Medullary carcinoma	1-3
Others	3-5

The majority of breast cancers are infiltrating ductal cancers of no specific type. These arise from the epithelial lining of the duct system, form tubules or nests, breach the basement membrane and infiltrate the surrounding tissue (Figure 1.3 A). They typically induce a strong stromal reaction. The resulting dense collagen deposition causes the familiar hard consistency of the cancer and the white macroscopic morphology. More than 50% of IDC are associated with ductal carcinoma in situ (DCIS) (Wong et al. 2010); the presence of epithelial malignant cells which are confined within the basement membrane. DCIS constitutes up to 25% of all diagnosed breast cancers (Virnig et al. 2010). Most cases of DCIS are clinically occult and are radiologically diagnosed during screening.

Lobular cancers arise from the epithelial cells of the lobular acini, and account for about 10% of breast cancers (Arpino et al. 2004). The epithelial cells are small and extend by forming single, linear files of cells (Figure 1.3 C). If the basement membrane remains intact, lobular carcinoma in situ (LCIS) is said to be present. The overall prognosis of early stage lobular cancers remains similar to that of IDC (Peiro et al. 2000), and the recommended treatment is the same; breast conserving surgery and adjuvant radiotherapy. There is a higher incidence of multifocality (multiple foci within

the same breast quadrant), multicentricity (multiple foci within different breast quadrants), and preoperative inaccuracy in assessing the size of the index lesion (Biglia et al. 2007;Hussien et al. 2003). The National Institute for Clinical Excellence (NICE) has therefore recommended the use of preoperative MRI in diagnosed lobular cancers, to identify occult tumours (NICE 2012).

Mucinous, tubular and papillary cancers are less common types of infiltrating breast carcinoma. They are known to exhibit less aggressive phenotypes than IDC (Li et al. 2005).

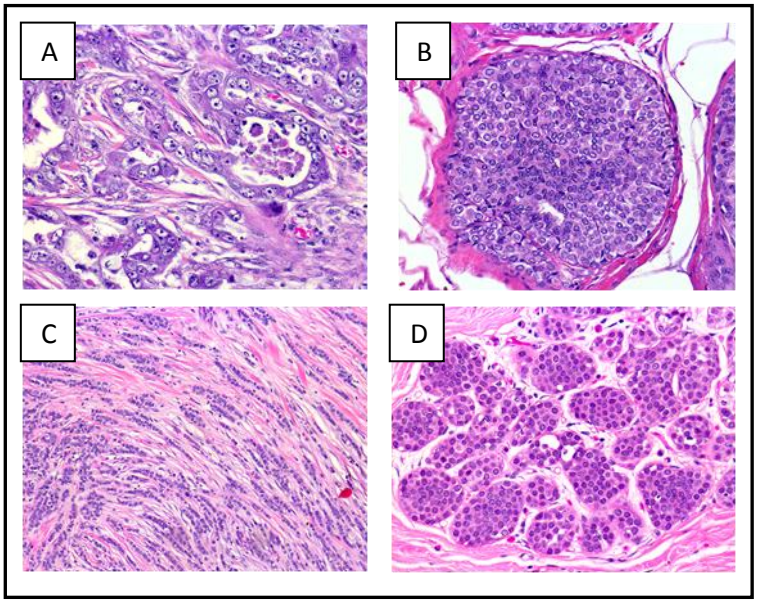

Figure 1.3 Common histologic types of breast cancer. Haematoxylin and eosin staining of histologic sections; A: IDC. B: DCIS, showing an intact basement membrane and cancer cells being contained within the duct lumen. C: Infiltrating lobular carcinoma, showing the characteristic linear sheets of cells. D: LCIS. From John Hopkins Medicine; Malignant tumours - an Atlas of Pathology. http://www.hopkinsbreastcenter.org/pathology/malignant/.

Mucinous carcinoma displays extracellular mucin deposition around tumour cells (Figure 1.4). Tubular cancers are usually well differentiated and characterized by the appearance of small glands that resemble ducts.

1.5 Histological grading

Breast cancers are graded into low, intermediate and high grades (corresponding to grade I, II and III). This is based on the Nottingham modification of the Scarff-Bloom-Richardson system (Mallon et al. 2000). This method has been proven to be reproducible, and to provide prognostic information thus guiding treatment choice (Contesso et al. 1987;Dalton et al. 1994). Three morphological features are scored; the percentage of tubular formation (reflecting glandular differentiation), the degree of nuclear pleomorphism and the mitotic count per ten high power fields. The scores are then added (Figure 1.5), and the collective final score used to indicate the grade. High grade tumours show poor tubule differentiation, high nuclear pleomorphism and a high mitotic index. High grade cancers are associated with more aggressive breast cancers, and poor prognosis.

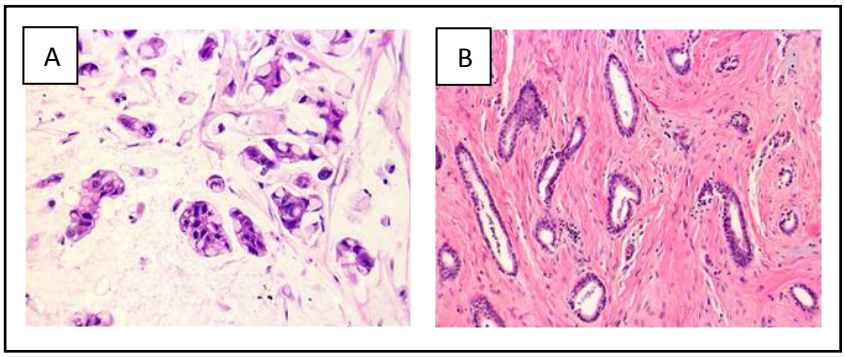

Figure 1.4 Less common histologic types of breast cancer; haematoxylin and eosin staining of histologic sections; A: Mucinous carcinoma, with abundant mucin deposition. B: Tubular carcinoma, showing well differentiated duct-like structures. From John Hopkins Medicine; Malignant tumours – an Atlas of Pathology.
http://www.hopkinsbreastcenter.org/pathology/malignant/

1.6 Staging of breast cancer

Staging systems are used as means to categorize patients and guide management decisions. The most commonly used system is TNM staging system. The TNM system is devised by the American Joint Committee on Cancer (AJCC) and the International Union against Cancer (UICC) (Singletary and Connolly 2006). It describes the tumour size in centimetres (T), lymph node involvement (N) and the presence or absence of distant metastasis (M).

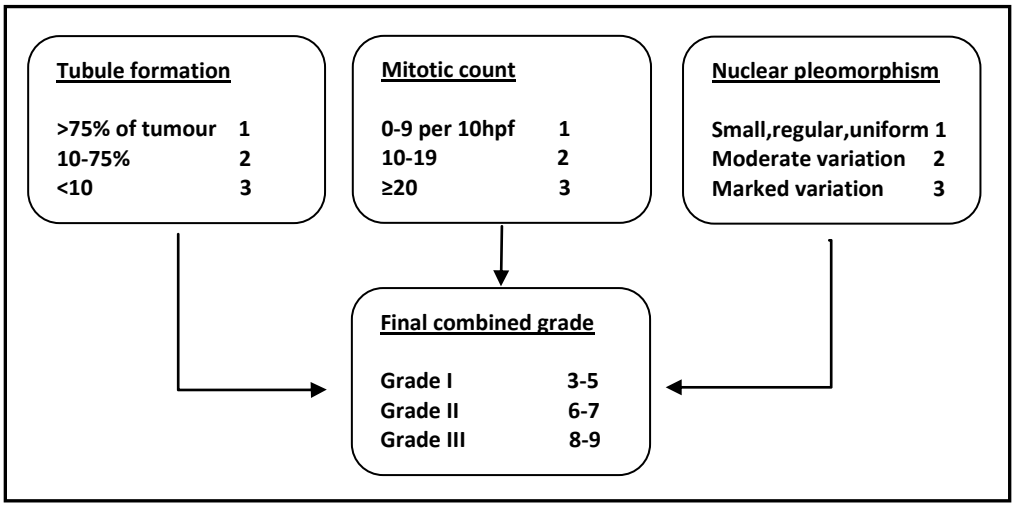

Figure 1.5 Nottingham modification of the Scarff-Bloom-Richardson grading system for breast cancer. Three histological grades exist, I – III, and are based on three microscopic characteristics: tubule formation, mitotic count and nuclear pleomorphism. Scores of 1 -3 are awarded to each histological characteristic, and the final combined score will determine the histological grade of the tumour. Hpf= high power field.

The 7th edition has been published in 2010. Changes in the 7th edition relate to pathological nodal staging and the presence of micro-metastasis, bone marrow micro-metastasis, circulating cancer cells, and neoadjuvant chemotherapy. A shortened version of the AJCC TNM staging is depicted in Table 1.2 (American joint committee on cancer 2010). TNM information can also be used to categorize patients into stages 0 to IV (Table 1.3).

Table 1.2 The AJCC TNM breast cancer staging system. The 7th edition of the AJCC cancer staging manual is the most recent updated edition, and a shortened version is shown. (American joint committee on cancer 2010).

The AJCC TNM staging of breast cancer	
Primary tumour **(T)**	
Tx	Tumour cannot be assessed
T0	No primary tumour
Tis	Insitu carcinoma
T1	Tumour ≤2 cm
T2	Tumour 2.1 – 5 cm
T3	Tumour >5 cm
T4	Tumour of any size with involvement of chest wall, involvement of skin and/or inflammatory carcinoma
Lymph node status **(N)**	
Nx	Regional nodes cannot be assessed
N0	Regional nodes not involved
N1	Involved but mobile ipsilateral axillary nodes
N2	Involved fixed ipsilateral axillary nodes, or involved ipsilateral Internal mammary nodes in absence of axillary involvement
N3	Involved ipsilateral infra-clavicular nodes, or involved ipsilateral supraclavicular nodes, or involved ipsilateral internal mammary and axillary nodes.
Distant metastasis **(M)**	
Mx	Metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 1.3 The four stages of breast cancer based on the AJCC TNM criteria. Tumour size, lymph node status and distant metastasis are the three criteria used to classify patients into four main stages; I-IV. These four stages are indicative of prognosis and act as a guide to management recommendation. (American joint committee on cancer 2010).

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
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
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

1.7 Management of breast cancer

The management of breast cancer depends on the extent of the disease. Nowadays, management is decided by the multi-disciplinary team (MDT) and is based on triple assessment and individual patients' characteristics. The concept of triple assessment is that each patient, whether symptomatic or screen detected, should receive clinical, radiological and histo-pathological diagnosis of the breast lesion in question. Recommendations for the MDT and guidelines about surgical and adjuvant management were first proposed by BASO (British Association of Surgical Oncology) in 1992, and subsequently regular updates have been issued; the latest published in 2009 (BASO 2009). Breast cancer staging follows updated guidelines from the AJCC (Singletary & Connolly 2006). Complete pathological staging for breast cancer is available following the primary surgical treatment, and is important in the consideration of adjuvant treatment. Treatment for breast cancer can be discussed as follows:

1.7.1 Treatment of early stage breast cancer.

The term early breast cancer usually indicates stages I and II invasive cancers. In some institutions, stage IIIA may also be included. The majority of patients will present with early disease, and can be offered breast conserving therapy (BCT). This constitutes breast conserving surgery (BCS) – otherwise known as wide local excision (WLE), followed by adjuvant radiotherapy (RT). BCT is further discussed in section 1.8. RT in the treatment of breast cancer in discussed in section 1.8.2 and chapter II.

1.7.1.1 Surgery to the axilla

Surgery to the axilla is an integral part of the treatment. Clinically negative axillae were traditionally managed with axillary clearance. Nowadays, the concept is that all patients should have axillary staging, and if positive should receive axillary treatment. Sentinel lymph node biopsy (SLNB) is the recommended practice in the UK (NICE 2012). The sentinel node is defined as the first node affected by cancer cells metastasizing to the regional lymphatic drainage territory. The biopsy is obtained using the dual detection method (using both a radioactive isotope tracer and a blue dye). A pathologically positive SLNB will therefore indicate possible involvement of the remaining regional nodes. Patients with negative SLNB will receive no further axillary intervention. Pathologically positive SLNB status will indicate the need for treatment of the axilla; axillary dissection or axillary RT. Most breast units offer axillary clearance. Recently published results from the NSABP trial B-32 (National Surgical Adjuvant Breast and Bowel Project) confirmed that management with SLNB is equivalent to primary axillary node clearance (ANC) in terms of disease free survival (DFS) and overall survival (OS) (Krag et al. 2010). Women with breast cancer were allocated to receive SLNB + ANC (group 1), or SLNB with ANC only if SLNB positive (group 2). The 8 year OS was 91.8% in group 1, and 90.3% in group 2, while the DFS was 82.4% and 81.5% respectively. Most breast units will carry out ANC following the detection of a positive SLN. There is, however, lack of evidence - based recommendations. The results of EORTC-AMAROS (After Mapping of the Axilla: Radiotherapy Or Surgery?) trial are currently awaited. This is a randomised, multicentre, phase III clinical trial comparing axillary RT with ANC in patients with positive SLNB. Trial end-points include loco-regional control, and patient morbidity. Offering no axillary treatment has also been

proposed as a viable option; trial ACOSOG Z0011 randomised 893 patients with T1-T2 clinically node negative cancers to either SLNB alone or SLNB and axillary dissection (Giuliano et al 2011). After a median follow up of six years, there was no significant difference in terms of breast recurrence, regional recurrence or OS. IBCSG 23-01 trial has randomised a similar group of patients but who only had evidence of micrometastasis in SLNB; (≤ 2 mm), to axillary dissection or no further treatment. Interim results after a median follow up of just over 4 years showed no difference between the two groups in terms of OS or DFS (Galimberti et al 2011). The management of lymph node micrometastasis is however still controversial with some studies suggesting that 5 year DFS rates were lower in patients with micrometastasis compared to those with node negative disease (Andersson et al. 2010), and in patients with micrometastasis who received no adjuvant treatment compared to those who did (de Boer et al. 2009).

1.7.2 Treatment of locally advanced breast cancer (LABC)

The exact definition of LABC varies between different institutions; however it usually denotes non-metastatic cancers of advanced local stage. The term includes tumours >50mm (T3), skin or chest wall involvement (T4), fixed ipsilateral axillary lymph nodes (N2), or ipsilateral internal mammary nodes (N3). The term also includes inflammatory breast cancers (T4). Management of these patients will require MDT input, and is likely to include surgical management in the form of mastectomy in addition to radiation therapy (chest wall & supra-clavicular fossa) and chemotherapy, with or without hormone therapy and Herceptin as per hormone receptor and HER-2 status. Large operable cancers have been described as those > 3 cm in diameter (Dixon et al. 2001).

1.7.3 Chemotherapy

1.7.3.1 Neoadjuvant chemotherapy (NAC)

NAC has gained acceptance over the last two decades. It allows earlier introduction of systemic therapy in patients at high risk of distant metastasis. Breast cancers which respond well to NAC can be successfully down-staged. However, in the case of resistant cancers, neoadjuvant chemotherapy can delay administering surgical treatment. NSABP B-18 randomised patients with breast cancer (T1, T2, T3, N0, N1, M0), to receive NAC (doxorubicin and cyclophosphamide) or the same agents postoperatively. A decrease in size was reported in 80% of cancers, while 36% showed complete clinical response; of the latter 26% had achieved a complete pathological response. The NAC group had more BCS than the adjuvant chemotherapy group, especially in the subgroup of patients with tumours ≥50mm (Fisher et al. 1997). NAC can thus decrease the mastectomy rates in patients with LABC. However, after 9 years of follow up, there was no significant difference in the OS or DSF between the NAC group and the adjuvant group (Fisher et al. 2002b). Analysis of 400 patients who received NAC + BCT at the M.D. Anderson Centre, found that residual tumour size >20 mm was predictive of loco-regional recurrence rates (Chen et al. 2004). Patients in NSABP B-18 achieving a complete pathological response showed better DFS and OS than those with no or partial pathological response (Fisher et al. 2002b).

1.7.3.2 Adjuvant chemotherapy

Adjuvant chemotherapy aims to eradicate micro-metastatic disease, and is usually reserved for use in Stage II and III cancers, in addition to high risk Stage I disease. These decisions are based on benefit assessment with regards to recurrence and

mortality, and are taken by the MDT. Other factors such as patients' age and physical status are considered and affect the final benefit assessments. An overview examining 15-year follow up data from three meta-analyses examining adjuvant chemotherapy, involving more than 36,000 patients of breast cancer was undertaken by the Early Breast Cancer Trialists Collaborative Group) (EBCTCG 2005). The report included both node positive and node negative patients. Poly-chemotherapy was found to be beneficial when compared to no chemotherapy in terms of mortality and recurrence. The benefit affected all age groups, though it was three times greater for women < 50 years. More benefit was obtained in ER (oestrogen receptor) negative patients than those with positive ER status.

Anthracycline based chemotherapeutic agents (epirubicin and doxorubicin-adriamycin-given as part of FEC or FAC therapy respectively), were found to decrease the annual mortality from breast cancer by 20-40%. This effect is significantly better than CMF (cyclophosphamide, methotrexate, 5-fluorouracil) regimens, which were more commonly used previously. When compared to CMF, anthracycline based regimens resulted in improved DFS and OS – both $p<0.0001$ (EBCTCG 2005). Taxanes are newer agents, whose role is well established in the treatment of metastatic breast cancer, however less so in early disease. The addition of taxanes to anthracycline based adjuvant chemotherapy was examined in a meta-analysis of thirteen randomised trials, and was found to improve both the DFS and OS (De et al. 2008). A Cochrane review published in 2007 (edited with no change to conclusions in 2010) examined randomised trials comparing taxane based versus non-taxane based chemotherapy in the treatment of early breast cancer, with nearly 20,000 women included (Ferguson et al. 2007). After a median follow up of 5 years, taxane-based regimens produced

significant benefit in terms of DFS and OS. Longer term results are still awaited.

1.7.4 Hormone therapy

1.7.4.1 Oestrogen receptors

Oestrogens are well documented regulators of breast development, and the development and pathology of breast cancer. This is reflected in the recognised roles of oopherectomy, adrenalectomy and oestrogen modulating drugs in the treatment of oestrogen dependant early and advanced breast cancer. Circulating oestrogens bind to nuclear protein receptors, and the resulting changes can alter gene transcription and proliferation of breast cancer cells. There are two types of oestrogen receptors; ER α and ER β. These are genetically and structurally different (Osborne and Schiff 2005). Several protein kinases in the growth factor pathways can also activate ER α (Osborne & Schiff 2005). Multiple signalling pathways through which ERs act have been suggested. The classical description is that activation of ERs leads to protein phosphorylation and conformational changes which expose DNA binding sites resulting in gene transcription. The resultant proteins induce changes in cell proliferation, apoptosis and invasion; they include VEGF (vascular endothelial growth factor), IGFR1 (insulin-like growth factor receptor) and TGF α (tumour growth factor). There is evidence that ERs also exist in the cytoplasm and/or cytoplasmic membrane (Li et al. 2003), in endothelial cells, bone and uterine tissue. The effects of these receptors are independent of gene transcription and are known as membrane initiated steroid signalling. They result in activation of growth factor receptors and protein kinases such as MAPK (Mitogen Activated Protein Kinase) and PI3K (Phosphatidylinositol 3-kinase) (Filardo 2002;Osborne & Schiff 2005). In addition, oestrogen metabolites have been

shown to initiate cancer by causing DNA base deletions, thus resulting in point mutations (Yue et al. 2003). The progression of cancer is also dependent on oestrogen; it has been shown to increase transcription of proto-oncogenes such as myc-1 (Subramanian et al. 2008).

1.7.4.2 Tamoxifen

ER is expressed in 50-75% of breast cancers (Bernoux et al. 1998), and these cancers benefit from hormone therapy. Tamoxifen acts as a selective oestrogen receptor modulator (SERM), having oestrogenic and anti-oestrogenic effects which are tissue specific (Osborne et al. 2000). It blocks the ER pathway in mammary cells, thus its use in the treatment of breast cancer, while having oestrogen-like effects in uterine tissue and bone. Tamoxifen competes with oestrogen for the ER binding sites. It thus decreases the transcriptional changes normally produced by oestrogen resulting in G1 cell cycle block and decreased cancer cell proliferation (Osborne 1998b). It has been suggested that Tamoxifen may also exert its anti-oestrogenic effects through activating apoptosis (Ellis et al. 1997).

Tamoxifen confers significant benefits in ER positive tumours. A randomised controlled trial NSABP B-14 reported results of Tamoxifen vs. placebo in ER positive, node negative breast cancers (Mamounas 2003). In women of all ages, after a 10 year follow up period, Tamoxifen resulted in a DFS benefit of 69% vs. 57% (p<0.0001), and a decrease in contralateral breast cancer development from 5.8% to 4% in the Tamoxifen group (p=0.007). In node positive patients, the addition of chemotherapy to Tamoxifen produced superior results. ER positive, node positive postmenopausal women were randomised to receive adjuvant Tamoxifen alone or Tamoxifen plus chemotherapy (FAC with or followed by Tamoxifen). Ten year DFS was significantly

better in the chemotherapy groups (60% and 53%) vs. 48% (Albain et al. 2009). Trial B-16 of the NSABP similarly compared Tamoxifen alone or combined with chemotherapy and reported improve DFS of 84% vs. 67% at 3 years (p=0.0004) (Fisher et al. 1990). However, recently published 20 year follow up data from the GROCTA 01-trial suggested that the benefit of adding chemotherapy decreased after the first 12 years (Boccardo et al. 2011).

1.7.4.3 Aromatase inhibitors

Tamoxifen has been the drug of choice for more than three decades; however it has been superseded by aromatase inhibitors in postmenopausal women. The ATAC trial, (Anastrozole, Tamoxifen, Alone or in Combination) randomised more than 6000 women to receive tamoxifen alone, anastrozole alone, or a combination of the two, as adjuvant treatment. Early interim analysis reported the combination arm to have similar efficacy to Tamoxifen alone; that arm was therefore terminated early. Eight year follow up data has confirmed superior benefit obtained from adjuvant anastrozole. The recurrence rate was significantly lower with anastrozole, HR 0.75 (0.61-0.94), p=0.01. There was, however, no significant difference in OS; 472 women in the anastrozole group *vs.* 477 in the tamoxifen group (Forbes et al. 2008). Tamoxifen remains the drug of choice for ER-positive pre menopausal women.

1.7.5 Molecular therapy

Human epidermal growth factor receptor-2 (HER-2) is a transmembrane receptor tyrosine kinase encoded by the proto-oncogene HER2/neu. It has an important role in cell growth and differentiation (Yarden and Sliwkowski 2001). HER-2 has no identifiable ligand, but is activated through receptor dimerization, i.e. interaction with another

receptor of a similar structure when that receptor is activated through its ligand e.g. EGFR (Epidermal Growth Factor Receptor), also known as HER-1 (Ross et al. 2003a). Spontaneous dimerization and phosphorylation/activation of downstream signalling pathways can occur in cells over- expressing HER-2 (Reese and Slamon 1997). HER-2 activation results in cell proliferation, migration and angiogenesis through multiple signalling pathways, the most studied of which are MAP kinase, PLC-γ (Phospholipase C) and PI3K (Meric-Bernstam and Hung 2006).

HER-2 gene amplification and/or high protein expression were found to occur in 20 - 30% of invasive breast cancers (Slamon et al. 1987;Slamon et al. 1989). More recent studies have reported lower prevalence rates of 12-20% (Tapia et al. 2007;Cronin et al. 2010;Chan and McGregor 2012). Up to 70% of DCIS lesions can over express HER-2 (Tsuda and Hirohashi 1998). Traztuzumab (Herceptin) is a humanized, monoclonal antibody which recognizes the extracellular domain of HER-2, and thus inhibits the proliferation of tumour cells that overexpress HER-2. Herceptin is now an integral component in the adjuvant setting, in combination with chemotherapy, for patients with HER-2 positive cancers. The HERA (HERceptin Adjuvant) trial randomised patients following completion of adjuvant chemotherapy to one year treatment with traztuzumab or observation alone (Piccart-Gebhart et al. 2005). Both early and updated results confirm significant survival benefits. The hazard ratio for the risk of death was 0.66 (95% CI 0.47-0.91; p=0.0115), thus an absolute OS benefit of 3% (92% vs. 89%). Similarly, there was a significant benefit in DFS of 7% at 3 years (Smith et al. 2007).

1.8 Breast conserving therapy

1.8.1 BCT *versus* mastectomy in early stage breast cancer

The current accepted treatment of patients with early stage breast cancer is BCT. BCT constitutes breast conserving surgery with clear margins followed by adjuvant whole breast external beam radiotherapy (WBRT). This is based on long term results of seven key prospective randomized controlled trials which have demonstrated similar survival rates with regards to mastectomy and BCT (Arriagada et al. 1996;Blichert-Toft et al. 2008;Fisher et al. 2002a;Poggi et al. 2003;van Dongen et al. 2000;Veronesi et al. 2002). All these trials started recruiting between 1973 and 1983.

- NSABP trial B-06 randomised 1851 patients with early stage breast cancer to lumpectomy alone, lumpectomy with RT (BCT), or total mastectomy (Fisher et al. 2002a). All patients underwent axillary dissection, and all lesions were ≤40 mm in diameter. There was no difference in the DFS or OS between BCT and mastectomy groups (OS being 51% and 50% respectively), with the hazard ratio for death in the first group being 1.05, 95%CI 0.9-1.23, p=0.51.
- Veronesi et al reported 20 year median follow up data of the Milan trial (Veronesi et al. 2002). This randomised 701 patients into BCT *vs.* radical mastectomy. The authors used quadrentectomy instead of lumpectomy as the surgical option in BCT. Lesions had a maximum diameter of 20mm. There was a significant difference in the local recurrence rate (8.5% in BCT vs. 2% in mastectomy group), but there was no difference in the OS, contralateral or second primary cancers. Survival was 26% in BCT vs. 24% in mastectomy group (p=0.8).
- EORTC trial 10801 randomised 868 patients into BCT and modified radical mastectomy. They did not have a maximum lesion diameter cut off, however their

results showed that 80% had tumours between 2.1 and 5 cm (van Dongen et al. 2000). BCT patients had a radiotherapy boost to tumour bed. After a median 13 year follow up, the OS was similar in the two groups; 65% *vs.* 66% respectively. There was a significant difference in LR; 20% *vs.* 12%, p=0.01. This difference could be due to the fact that 48% of patients receiving BCT had positive resection margins on microscopy. However, this difference did not have an effect on long term OS. A further update was published recently reporting results after a 22 year median follow up; there was no significant difference in terms of OS or time to distant metastasis (Litiere et al. 2012).

- The American National Cancer Institute carried out a similar trial randomising 237 patients with tumours ≤5cm into BCT or modified radical mastectomy. After a median follow up of 18 years, the OS was similar; 54% *vs*. 58% respectively (Poggi et al. 2003).
- The Danish Breast Cancer group trial 82TM followed patients for a median duration of 19 years (Blichert-Toft et al. 2008). There was no cut off limit for maximum tumour diameter; however, 70% were less than 2 cm. They reported OS of 24% in BCT and 21% in modified mastectomy group, out of a total of 731 randomised patients.
- A similar study from the Institut Gustave-Roussy randomised 179 patients into BCT (88 patients) or modified radical mastectomy (91 patients). All patients underwent axillary dissection. The OS and LRR rates were similar with no significant differences (Arriagada et al. 1996).
- One of the first trials to examine the role of conservative breast surgery was the Guy's wide excision study (Fentiman 2000). Patients with clinically negative lymph nodes were randomised to mastectomy and axillary RT or BCS and breast RT (35

Gy). After 25 years, both local recurrence and breast cancer mortality were significantly higher in the BCS group; 54% *vs.* 18%, and 57% *vs.* 44% respectively. The high failure rates can be explained by the lower doses of RT used, and failure to stage and treat the axilla.

A pooled analysis of long term data from these key trials has confirmed that there is a significantly lower risk of local recurrence after mastectomy compared with BCT; odds ratio 1.56, 95% CI 1.28-1.89, $p<0.001$ (Jatoi and Proschan 2005). However, it has also confirmed that there is no significant difference in overall survival between the two groups, pooled odds ratio of 1.07, 95% CI, 0.93 - 1.22, $p=0.33$. BCT has the advantages of organ preservation, in addition to psychological and cosmetic benefits.

1.8.2 Radiotherapy as part of BCT

1.8.2.1 The benefit of adjuvant RT in BCT

Radiotherapy is crucial following BCS, and its advantages were proven by similarly well recognised key trials. NSABP trial B-06 (section 1.8.1) had a third arm comprising patients who received lumpectomy alone. Comparison between this group and patients who had lumpectomy and radiotherapy had showed that a total dose of 50 Gy radiotherapy can significantly decrease local recurrence (LR) rates by 25%, from 39% to 14%, $p<0.001$ (Fisher et al. 2002a). However there was no significant difference in the development of distant metastases, or OS. Further similar trials have confirmed the local control benefit of RT (Table 1.4). The Ontario Clinical Oncology Group randomised node negative patients with tumours ≤4 cm to lumpectomy alone or lumpectomy followed by RT (40 Gy followed by tumour bed boost of 12.5Gy) (Clark et al. 1996). After a median follow up of 8 years, they reported a decrease of LR of 24% (from 35%

to 11%), with a relative risk in the non-irradiated group of 4, 95% CI 2.83 – 5.65, p<0.0001. RT had no survival advantage, having reduced mortality by 3% (24% *vs.* 21%). A similar study at the Milan Cancer Institute randomised 567 patients (Veronesi et al. 1994). Patients who received surgery alone, underwent more extensive surgery in the form of quadrentectomy. The addition of RT decreased LR rates by nearly 9%, to 0.3%. This ultra low LR incidence can be explained by a shorter follow up, and the use of quadrentectomy for surgical excision as opposed to lumpectomy in NSABP B-06 and Ontario trials. The Swedish Breast Cancer Study Group also used a more extensive local resection, which they referred to as 'sectoral resection'. They randomised 381 node negative patients with Stage I breast cancer (tumours ≤2 cm) into lumpectomy with or without radiotherapy (total dose of 54 Gy) (Liljegren et al. 1999), and reported 10 year follow up results. The LR was decreased by 15% (from 24% to 9%), p=0.0001. RT had no effect on the OS (which remained the same at 78%), or the distant recurrence rate. The Scottish Cancer Trials Group randomised 464 patients with tumours ≤4cm into lumpectomy with or without RT (Forrest et al. 1996). All patients had a total radiation dose of 50Gy plus a boost of 10-15 Gy. All patients within this study had adjuvant chemotherapy, compared to only node positive patients in the other studies described in this section. Patients were followed up for a median of 5.7 years. Radiation decreased LR rates from 24% to 7%. They also confirmed that RT had no effect on distant recurrences or OS. Renton et al conducted a British trial in which they randomised 418 patients of BCS to receive RT or not (Renton et al. 1996). Those who had RT demonstrated a 22% lower LR than those who had no RT at 5 years (13% and 35% respectively, p<0.005). A recent meta-analysis by the EBCTCG has examined data from 10,800 patients (17 RCTs) who were randomised to BCS *vs.* BCT (Darby et al. 2011). This study has further confirmed that RT decreases the 10 year overall first

recurrence (whether local, locoregional or distant) from 35% to 19%. However, it has also reported that RT can decrease the death from breast cancer by 3.8% (from 25% to 21%), 2p=0.00005. This finding has not previously been reported by the individual trials, and suggests for the first time that RT may have an effect, not only on the local control of cancer, but also on the systemic control and distant disease recurrence.

Table 1.4 Local recurrence rates following breast conserving surgery with or without adjuvant radiotherapy. Six key randomised clinical trials demonstrating the significant benefit of adjuvant RT in decreasing LR rates following breast conserving surgery BCS. WLE: wide local excision. Quadrant.: quadrantectomy (Adapted from Abeloff et al 2004).

Trial reference	Number of patients	Tumour size (cm)	Surgery	LR – **Excision no RT**	LR – **Excision & RT**
Veronesi et al 1994	567	2.5	Quadrant.	**9%**	**0.3%**
Fisher et al 2002a	1262	4	WLE	**39%**	**14%**
Clark et al 1996	837	4	WLE	**35%**	**11%**
Liljegren et al. 1999	381	2	Quadrant.	**24%**	**9%**
Forrest et al 1996	585	4	WLE	**24%**	**7%**
Renton et al 1996	418	5	WLE	**35%**	**13%**

Radiotherapy, like surgery, addresses local control. Surgery aims to eradicate all macroscopic disease, with a margin of macroscopically normal breast tissue. This margin aims to remove possible microscopic disease not detected by the surgeon or radiologically. Adjuvant RT aims to eradicate possible microscopic residual disease, therefore reducing the risk of LR. Studies have shown that occult cancers occur in >60% of mastectomy specimens, and can be situated in a different quadrant to the index tumour in up to 79% of cases (Vaidya et al. 1996). However, it is known that more than 90% of LR can occur within the index quadrant. Therefore it has being argued that the tumour microenvironment might play a role in stimulating cancer

growth; and furthermore, that RT to the tumour bed could affect the microenvironment surrounding the tumour bed (Belletti et al. 2008). Belletti et al have shown that following BCS, the wound fluid can stimulate cytokine release and activation of tumour cellular growth and migration. Furthermore, they have demonstrated that RT can decrease this effect in vitro, and may therefore contribute to decreased LR through alterations in the mammary microenvironment.

RT may also incur benefit through its action on circulating tumour cells. The "tumour self seeding" concept has been proposed following in vitro studies on breast cancer cell lines (Kim et al. 2009). Cytokines produced by the original load of breast cancer tissue or residual cancer tissue will attract circulating tumour cells, which then leave the circulation to lodge in their original microenvironment as another site of "metastasis". These recruited cells may later be killed by adjuvant RT.

1.8.2.2 Radiotherapy; dose and fractionation

A radiation dose is calculated in total at the start of treatment; this is usually achieved during the first appointment with the oncologist following BCS. The treatment is delivered in fractions with known intervals.

The standard radiation dose to the breast is 45-50 Gy administered over 3-5 weeks. A rare study from the Institut Gustave-Roussy examining 463 breast cancer patients treated with RT alone (no surgery) was conducted in the 1980s. Patients had contraindications to surgery and received RT alone. It was reported that increasing the RT dose by 15Gy can decrease the risk of LR by 2 fold. The dose delivered to the tumour was an independent predictor of LR on multivariate analysis (Arriagada et al. 1985).

The calculated dose may be followed by a boost dose of 10-20 Gy directed to the tumour bed. Boosts are delivered using electron beams from linear accelerators (Abeloff et al. 2004). Administration of the boost dose will depend upon existing prognostic and risk factors. Boost doses have been shown to decrease LR rates in all age groups. More than 5000 patients in EORCT trial 22881-10882 were randomised to receive a 16Gy boost or not following BCT – 50Gy standard dosage (Antonini et al. 2007;Bartelink et al. 2001). The LR rate in the boost group was 4.9% *vs.* 8.8% in the no boost group. However, a subgroup analysis which stratified patients according to age, found that this benefit was significant in those of a younger age. The decrease in LR between boost and no boost groups was 10% in those less than 40 years, but only 2% in those older than 60 years.

Fractionation decreases toxicity on normal tissues by allowing them time to repair. Cancer cells are less efficient in DNA repair. Fractionation allows repetitive attacks on the tumour cells so that they are captured in different phases, including the RT-sensitive phases, of the cell cycle. The fractionation regimen will allow for:

a) Sub-lethal damage repair: fractionation allows cells to attempt DNA repair in-between doses. For this to occur, a minimum of 6 hours is required as an interval (Abeloff et al. 2004). Damage repair in tumour cells can be disadvantageous, while it allows for decreased toxicity effects in normal surrounding tissue.
b) Re-population: undamaged cells continue to proliferate and divide. A longer interval between fractions will allow for extensive re-population of tumour cells thus increasing the total RT requirement and contributing to resistance, while re-populating normal cells maintain the tissue integrity.

c) Re-oxygenation: fractionation allows time for revascularization and oxygen delivery to previously hypoxic cells. Oxygen availability will enhance the cytotoxic effects of radiotherapy, as oxygen is known to fix DNA damage and prevent repair processes.

There are currently many ongoing trials examining the effects of hypofractionation (Tutt and Yarnold 2006). The UK START B trial randomised women who had BCS to standard RT regimen (25 fractions of 2Gy over 5 weeks) or hypofractionation regimen (15 fractions of 2.7Gy over 3 weeks). The 5 year LR rate was similar in the two groups; 3.3% in the standard RT group, and 2.2% in the hypofractiontion group (Bentzen et al. 2008).

1.8.2.3 Administration of RT:

Breast RT is administered using modern linear accelerators, with the patient in the supine position, less commonly prone or in the decubitus position (Kiltie 2005). Nowadays, computed tomography (CT) is used for accurate planning, and the field is demarcated using skin tattoos. The planning aims to include the gross tumour, with normal surrounding tissue to allow for tissue and patient movement during multiple sessions (planning target volume - PTV). With accurate application, the PTV should receive 95% of the treatment dose (Kiltie 2005). This allows for minimal irradiation of the underlying lung, and heart in left sided cancers, and contributes to decreasing pulmonary and cardiac side effects.

Partial accelerated breast irradiation (PABI) is increasingly used in some institutions. Its use is mainly based upon the argument that true cancer recurrences occur in the close vicinity of the original disease, and that tumours occurring elsewhere in the breast are new primary cancers (section 1.9.5). PABI can be administered using interstitial

catheters, balloon catheter technique (mammosite), 3-D conformal external beam radiation or intra-operative radiotherapy (IORT). IORT provides a highly precise application to the tumour bed immediately following removal of the tumour.

PABI can be time saving and convenient. It can produce a better cosmetic result, as a large part of the breast is spared the effects of high dose irradiation; however this is not proven. Occult foci of cancer, distant from the index quadrant, will not be irradiated. It is known that up to 80% of occult tumours within mastectomy specimens exist beyond the affected quadrants (Vaidya et al. 1996), an argument against partial irradiation. On the other hand, it is known that up to 90% of local recurrences occur within the index. PABI is not recommended as routine in any defined group of patients. Well recognised institutions in Europe and the USA have adopted a conservative approach; recommending large scale trials and long follow-up results before routine implementation of PABI (Nag et al. 2001;Sauer et al. 2007). A consensus statement from the American Society of Radiation Oncology has defined three groups of patients based on their clinical and pathological risk factors for local recurrence; PABI "suitable", "cautionary" and "unsuitable" (Smith et al. 2009a). Suitability for PABI included older patients > 60 years, with no known genetic mutations, unifocal, invasive, T1 disease with clear margins of >2mm, and negative lymph node status. The European Society for Therapeutic Radiology and Oncology has published similar recommendations with three categories; low, intermediate and high risk; the latter defining patients in whom PABI is contraindicated and includes patients <40 years, with multicentric disease or tumours >3 mm, or the presence of LVI (LymphoVascular Invasion) and/or EIC (Extensive Intraductal Component) (Polgar et al. 2010). The results of the multicentre TARGIT-A trial were published following this, and provide long

awaited evidence (Vaidya et al. 2010). TARGIT-A trial randomised 2200 patients who had BCS to the usual external beam adjuvant RT or a single dose of IORT (20Gy). At 4 years median follow up the difference in LR between the two groups was <1%, *p*=0.4. Major radiation toxicity (defined by the authors as skin breakdown, delayed wound healing or radiation oncology group grade 3 and 4 toxicity (Cox et al. 1995)), was significantly less in the IORT group; 0.5% *vs.* 2.1% (*p*=0.002) in those receiving external RT (Vaidya et al. 2010). A phase III randomised controlled trial is currently ongoing that compares the usual external beam adjuvant RT with external beam partial breast irradiation (34 - 38.5 Gy in 10 fractions over 5 days) for women with early stage breast cancer (NSABP trial B-39 and RTOG trial 0413 – Radiation Therapy Oncology Group (http://www.rtog.org/members/protocols/0413/0413.pdf). Another trial examining the role of partial external beam RT is IMPORT Low (Intensity Modulated Partial Organ RadioTherapy). This trial has randomised women with early stage breast cancer at low risk of LR into three arms; a) standard WBRT 40 Gy in 15 fractions b) partial breast radiotherapy 40 Gy in 15 fractions c) partial breast RT 40 Gy in 15 fractions plus WBRT at a lower dose of 36 Gy in 15 fractions. This trial has completed recruitment and results are awaited.

1.8.3 Other indications for RT in breast cancer

Apart from the adjuvant setting in the treatment of invasive breast cancer, RT can be used in the management of DCIS. The incidence of DCIS has increased in recent years following the introduction of the screening programme. Depending upon the extent of DCIS, treatment options include conservation surgery and mastectomy. Following BCS for DCIS, RT significantly decreases the rate of LR, both of invasive disease and further DCIS (Fisher et al. 1998). A recent Cochrane review has confirmed the benefit and

reported no significant long term toxicity from the RT (Goodwin et al. 2009). Post mastectomy RT is indicated in high risk patients. The exact criteria for selection vary between different institutions. However, it is accepted that high risk patients are those with more than four positive lymph nodes, T3 and T4 tumours, or inadequate surgical margins (Vilarino-Varela et al. 2009). Less solid criteria include young patients < 40 years, grade 3 tumours, lymphovascular invasion, extranodal spread, and patients with less than four involved lymph nodes. The Danish trial 82b randomised high risk premenopausal patients to post mastectomy RT plus CMF, or CMF alone. High risk was defined as involved lymph nodes and/or T3+ tumours. After a median follow up of 9 years, post mastectomy RT combined with adjuvant CMF therapy resulted in significant improvement in OS and decrease in LR rates (Overgaard et al. 1997). Postmenopausal women were similarly reported to have significant survival and LR control benefits from post mastectomy RT; the Danish 82c study (Overgaard et al. 1999). RT can also be used in the palliative setting for symptomatic relief from bony metastases.

1.8.4 Side effects of radiotherapy:

The severity of radiation side effects has decreased since the introduction of modern equipment. Damage to tissue outside the intended field of radiation is less common with improved planning and application techniques.

Universal grading systems exist, which have made comparisons more feasible, a widely used system is the National Institute for Health toxicity criteria (NCI 2012). Side effects continue to cause significant morbidity. This affects patients' quality of life and the continuation, and therefore the efficacy, of therapy itself. Side effects occur because of the action of RT on normal cells. The occurrence and degree of RT complications is significantly associated with the RT dose (Lilla et al. 2007), in addition to the

fractionation regimen and concomitant chemotherapy. However, even when these are constant, individual patients respond differently, perhaps secondary to an inherent difference in radiosensitivity. This has been linked to the occurrence of polymorphisms in DNA repair genes (Chang-Claude et al. 2005). Radiation side effects can be early or late.

Early or acute complications: these are due to direct tissue damage, and occur within the first three months of treatment (Abeloff et al. 2004). Distinguishing local side effects of RT from local recurrence can be challenging and often breast imaging and tissue biopsy are required to make the diagnosis.

- Skin erythema and moist desquamation.
- Systemic effects manifesting as generalized fatigue.

Late complications: arise secondary to vascular damage leading to tissue fibrosis and possibly organ failure. Different body organs have different radiation sensitivities, and therefore direct damage must be involved in addition to the vascular aetiology. They can occur years after the RT treatment.

- Breast tissue and skin atrophy and fibrosis.
- Telengectasia
- Cardiovascular damage; micro and macro circulatory damage leading to ischaemia, myocardial fibrosis and ultimately cardiac dysfunction. This may be increased with adjuvant anthracycline therapy, which is known to cause cardiac toxicity. The risk, though decreased by current RT techniques, still stands, with doses as low as 1-4 Gy reported to cause damage (Taylor et al. 2006).

- Pulmonary complications: these are usually localized to the areas affected by the radiation, and range from early radiation pneumonitis to late lung fibrosis (Senkus-Konefka and Jassem 2006).
- Second malignancies: Mutations resulting from RT induced DNA damage can give rise to secondary cancers; these have a reported 15 year cumulative incidence of 16-19% following RT for the treatment of breast cancer (Senkus-Konefka & Jassem 2006). However the data are difficult to interpret as reported cancers included contralateral breast cancers and ovarian cancers, which could possibly be related to a genetic predisposition. A large number of tumours have been reported in the literature. Soft tissue sarcomas and angiosarcomas within the irradiated breast area and the ipsilateral arm show a particularly high incidence, usually occurring after a period of 10 years (Senkus-Konefka & Jassem 2006).
- Shoulder and arm complications: Shoulder pain and stiffness are very common and mostly precipitated by fibrosis to subcutaneous tissue, muscle and joint apparatus. Lymphoedema can occur in up to 40% of breast cancer patients following RT (Senkus-Konefka & Jassem 2006). The incidence is greatly increased by combined RT and axillary surgery, and possibly chemotherapy.

1.9 Local recurrence following BCT

1.9.1 The incidence of local recurrence

The incidence of LR following BCS without RT has been reported as 35%-40% (Fisher et al. 2001b). Post operative WBRT significantly decreases the risk to 10-20% within 10 years (Clemons et al. 2001;Fisher et al. 1995;Haffty et al. 1996b), approximately 1-1.5% per year. A more recent meta-analysis of 10 studies examining the addition of RT to BCS has reported an absolute reduction in LR of 19% within the first 5 years post

operatively (Clarke et al. 2005b). Twenty year follow-up results from NSABP trial B-06 has reported a significant decrease in LR rates from 39% (lumpectomy alone) to 14% in those receiving RT after lumpectomy (Fisher et al. 2002a). The START B trial reported LR rate of 2.2% after BCT and a median follow up of 6 years. The first 5 years constitute the peak risk time for LRR with an incidence of 5-10% (Recht et al. 1988). This forms the basis of the common practice of a 5 - year clinical and radiological follow up for cancer patients. The British Association of Surgical Oncology advises that LR rates following BCT should not exceed 5% at 5 years, and further recommends a target of <3% at 5 years (Anon 2009).

1.9.2 The effects of local recurrence

In addition to causing significant morbidity at the time of presentation, LR after BCT is recognised as an independent predictor of distant metastases (Fisher et al. 1991). Reports from NSABP B-06 have suggested that after a 9 year follow up, the risk of distant recurrence was three times higher in patients who had previously developed LR, when compared to patients who had no LR (Fisher et al. 1992). This would affect management decisions, as it would indicate an expected benefit in administering systemic therapy to treat LR.

A study has analysed 900 patients who received BCT who had locoregional recurrence (LRR) of 4.4% at a median follow up duration of 53 months (Lee et al. 2011). The recurrences were categorised into early (less than three years), late (more than 3 years), local (breast) and regional (regional lymph nodes). Of the patients who had LRR, 33% developed distant disease, compared to 7% of the patients who had no LRR. Multivariate analysis reported that early LRR was an independent predictor of the occurrence of distant metastasis; early LR; HR 4.76, 95% CI 1.65 – 13.34, p=0.003.

Patients who had late LR displayed similar distant recurrence rates to those patients who had no LR. Local recurrence has been reported to affect disease specific survival, probably through its association with distant metastases. Vicini et al managed 1169 patients with BCT, and reported an 11% LR rate after 12 years follow up (Vicini et al. 2003). Disease specific mortality was significantly higher in patients who suffered LR (31%), compared with those who had not (12%), $p<0.001$. LR was an independent predictor of cancer mortality using multiple Cox regression analysis; hazard ratio 2.69, $p<0.001$. Similarly, Kemperman studied 1026 patients, and reported LR as an independent predictor of disease specific survival following BCT in multivariate analysis (Kemperman et al. 1995); hazard ratio of 8.8, 95% CI 4.6-16.8.

Even in the absence of distant metastases, up to 35% of patients with LRR may develop further LR (van et al. 1999). Isolated axillary recurrences are less common than isolated LR (Touboul et al. 1999), and up to 50% can progress to develop distant metastases (Newman et al. 2000). Trials EORTC 10801 and DBCG-82TM jointly concluded that patients suffering LRR after BCT have a similar prognosis to those with recurrences after mastectomy, with 5 year survival rates reported at 58% and 59% respectively (van et al. 1999). Survival may however be affected by the different salvage treatments offered to these patients.

The interval to first LR is perhaps the most well established factor associated with a poor prognosis (Kemperman et al. 1995). Others include tumour size, LVI and lymph node stage (van et al. 1999). LR may be an aetiological factor contributing to distant metastasis, or it may be merely a marker of the occurrence of distant disease. Vicini et al found that patients who developed early LR (less than 3 years) had a significantly shorter time to distant metastases, p=0.001, compared with those who had no LR. This

however, was not significant when considering all LR, both early and late (more than 3 years) (Vicini et al. 2003). They therefore suggested that LR directly causes distant spread. Some have suggested that LR is a significant predictor of the risk of distant disease, but not a direct aetiological factor (Fisher et al. 1991).

1.9.3 Risk factors for local recurrence

The risk factors for local and regional recurrence following BCT can be divided into clinical and pathological factors. These include age, menopausal status (Neri et al. 2007), tumour size (Goldhirsch et al. 1998), lymph node status, presence of EIC, LVI (Neri et al. 2007), positive resection margins, and multifocal disease (Marret et al. 2001;Neri et al. 2007). Some are widely accepted e.g. age, EIC and resection margins, while some are still debatable and have shown conflicting results in different studies. This is possibly due to the heterogeneity of patient groups and inclusion criteria, e.g. tumour size and different cut-offs utilised (see part "e" below). In terms of molecular factors, ER status and HER-2 gene amplification and protein over-expression, are the two most commonly known factors. Other markers currently under investigation include: Bcl2, EGFR, VEGF and Cyclin D1.

Clinical risk factors:

a) Age. Young age has been identified as an independent prognostic factor for LRR (Antonini et al. 2007;Mirza et al. 2002). Different age cut-offs have been used; mostly 35-40, but as high as 50 in some studies; this latter is probably compounded by the effect of the menopause. Young age is associated with biologically more aggressive tumours and multiple poor prognostic factors such as negative ER status, positive HER-2 status and high tumour grade (EA et al. 2011;Sidoni et al.

2003). Similarly, a study comparing pre and post menopausal women with similar histological grades found that the latter group showed higher rates of ER positive tumours and lower proliferation rates (Talley et al. 2002). The EORTC trial 22881/10882 examined more than 5000 patients who underwent BCT. The 5 year LR was 18% in those ≤35 years of age, and 3% in those ≥60 years. Although the younger patients demonstrated higher rates of ER negative and larger tumours with more frequent re-excisions, using multivariate analysis, age was found to be an independent predictor of LR (Vrieling et al. 2003). In addition, one can argue that as the risk of disease recurrence continues throughout life, it may contribute to higher cumulative recurrence rates in younger patients.

Pathological factors include:

a) Positive surgical margin. This has been reported as an independent predictor of LR on multivariate analysis; a study of nearly 500 patients who received BCT reported that the LR for those with positive excision margin was 12% compared to 3% in those with negative margins (Leong et al. 2004). A positive margin is defined as the presence of tumour cells at the inked resection margin. There is however no exact definition as to what constitutes a close or a clear surgical margin following BCS (Luini et al. 2009), and values differ between institutions and trials from 1-5 mm. The Milan trial has demonstrated that quadrantectomy was associated with lower recurrence rates than lumpectomy (Veronesi et al. 1994) (Table 1.4). Quadrantectomy is excision of a whole breast quadrant. In most centres in Europe and the USA (and therefore all corresponding trials involving BCS), lumpectomy, or WLE, is practiced. This involves the excision of the tumour with a macroscopic margin of 1-2 cm. The necessary margin will

also differ between invasive and in situ disease, with a wider margin generally required for DCIS. Positive margins are managed by further WLE and RT, or mastectomy.

b) Extensive intraductal component (EIC).This is defined as in situ disease involving >25% of the primary tumour area, with existing foci separate from the main tumour. The exact extent of in situ disease can be difficult to characterise histologically, as it can exist within and/or beyond the tumour and can be multicentric. In addition, different histological definitions exist; some authors have defined EIC with regards to the number of involved ducts; more than 10 would constitute EIC (Sinn et al. 1998). EIC is associated with residual in situ disease, which would progress to invasive cancer. However, even after achieving complete excision, EIC remains a predictor of LR in early stage breast cancer (Vicini et al. 1991). Pooled analysis of two RCT of BCT involving >800 women reported a 10 year LR of 10%, and found that EIC was a predictor of LR; hazard ratio of 2.5, 95% CI, 1.3-5 (Voogd et al. 2001). The different histologic interpretations, and lack of clear definitions, may explain the conflicting results reported by some studies which found no association between EIC and LR rates (Neri et al. 2007).

c) Axillary lymph node status: The status of the axilla is regarded as one of the most important prognostic factors of survival in early stage breast cancer (Soerjomataram et al. 2008). Survival decreases with increasing number of positive lymph nodes. Examining more than 600 patients with stage I and II breast cancer, NSABP found that positive lymph node status was an independent predictor of poor survival at 15 years (Fisher et al. 1993;Fisher el al 2001b). Moreover, compared with node negative disease, the relative risk of

mortality doubled with involvement of 1-3 nodes, and increased by nearly five times with involvement of ≥10 nodes (Fisher et al. 1993). In patients with early stage breast cancer who had BCT and axillary dissection, positive node status was found to be an independent predictor of LRR on multivariate analysis (Mirza et al. 2002;Neri et al. 2007). ANC is not routinely performed nowadays, and SLNB is the gold standard practice (section 1.7.1.1). Positive SLNB is an indication for adjuvant therapy. Indeed, some studies have suggested that positive axillary nodes were not indicative of LR, possibly secondary to the administration of local and systemic therapy in this patient group (Voogd et al. 2001).

d) Lymphovascular invasion (LVI): LVI is defined as the presence of tumour emboli within endothelial lined spaces, and is associated with other negative prognostic factors such as larger tumour size and higher nuclear grade (Lee et al. 2006). In a series of 80 patients, LVI was found to increase with increasing grade; 0% LVI in grade I tumours, 38% in grade II and 77% in grade III disease (Gurleyik et al. 2007). However, examining a series of 500 breast cancer patients, LVI was reported in 23% of cases, and was found to be an independent risk factor for LR at 10 years (Neri et al. 2007). A pooled analysis of two European studies (EORTC 10801 and DBCG-82TM) reported a 10 year LR of 10% following BCT, and identified LVI as an independent predictor of LR, with a hazard ratio of 2.3, 95% CI 1.3 – 4 in the LVI group (Voogd et al. 2001).

e) Tumour size: Tumour size, relative to the size of the breast, is utilised to allocate patients to receive either BCS or mastectomy. Increasing tumour size is a well documented poor prognostic factor for DFS and OS. This is independent of lymph node status. In lymph node negative patients, the ten-year OS was

shown to decrease by 13% when comparing T2 tumours to those < 1cm in diameter (Chia et al. 2004). Tumour size is also traditionally believed to influence local control and is an important factor in deciding between BCT and mastectomy. A retrospective analysis of more than 1100 patients who had BCT reported an 11% LRR at a median follow up of 9 years. Larger tumours were significantly associated with LRR (Mirza et al. 2002). However, this is disputed. A review by Asgeirsson et al examined 9 RCTs and 7 retrospective trials examining the roles of BCT, mastectomy and adjuvant RT, with follow up ranging between 3-20 years (Asgeirsson et al. 2003). Tumour size was found to be a risk factor in two RCTs and one retrospective study. Similar to the age cut off, different sizes have been quoted as the cut off for significance; from 2 to 5cm. Insufficient evidence exists regarding tumours of 5 cm or more.

f) Multifocal disease: Occult multifocality is thought to occur with larger tumours (Asgeirsson et al. 2003), and to be responsible for local recurrences, especially before the introduction of adjuvant RT. Macmillan et al obtained cavity shavings from 300 patients who had BCT (Macmillan et al. 1997). Positive foci (invasive cancer or insitu disease) were found in 40% of patients; however these were not related to tumour size. In a retrospective study of 500 BCS patients, multifocal disease was detected in 7% of specimens, and proved an independent predictor of LR on multivariate analysis with a relative risk of 2.9; CI 95%, 1.09 to 8.04 ; $p<0.05$ (Neri et al. 2007). Similarly, data obtained from EORTC trial 10854, reported a relative risk of LR of 3.34; 95% CI, 1.27 to 8.77 with multifocal disease (Elkhuizen et al. 2000). LR could be associated with residual un-resected disease, radiologically occult or microscopic disease foci. Moreover, patients with multifocal disease were found to have more than four

times the risk of developing cutaneous LR than patients with unifocal disease. Cutaneous recurrences were associated with a significantly decreased probability of survival at 5 years following the recurrence when compared with parenchymatous recurrences; 25% vs. 75% (Marret et al. 2001).

g) ER status: ER-positive cancers usually show lower proliferation rates and better tumour differentiation (Abeloff et al. 2004). Positive ER status in breast cancer is a recognized predictor of a good response to hormone therapy. NABP B-14 trial found that ER +ve patients who were given 5 years of adjuvant Tamoxifen demonstrated a 12% improvement in DFS after 10 years of follow up, compared with those who received a placebo; p<0.0001 (Fisher et al. 1996). On the other hand, the prognostic role of ER receptors has been debated, and is more difficult to demonstrate as all recent trials involve patients who receive other forms of systemic therapy. NSABP B-06 which examined more than 1000 patients who received surgery alone, reported that the 5 year DFS was significantly better in ER+ve patients; 74% *vs.* 66% in ER-ve patients (Fisher et al. 1988). However, this difference decreased after longer follow up. Pichon et al similarly suggested that the prognostic value decreased by 20% each year (Pichon et al. 1996). The role of ER as a marker of LR is even more heavily debated. Neri retrospectively examined 500 patients after a median follow up of 9 years, 69% of whom had ER+ve status. The LR free survival was 90% in ER – ve patients *vs.* 80% in ER +ve patients, $p<0.05$ (Neri et al. 2007). ER negativity was an independent predictor of LR in multivariate analysis with a RR of 3.23 95% CI 1.50-6.95, $p<0.01$. However, multiple similar studies, with similar follow up periods have reported no significant association (Horiguchi et al. 2006;Mirza et al. 2002); a study of more than 4000 breast cancer patients followed up for a

median of 20 years found that ER had minimal value as a prognostic marker of LR with a hazard ratio of 0.9, 95% CI 0.8 to 1.1, *p*=0.4 (Habibi et al. 2008). This would support the argument raised by some authors that ER positivity is associated with lower recurrence rates in the early post operative period but after long follow up. As rates steadily increase with time, the overall prognostic significance decreases (Hilsenbeck et al. 1998;Osborne 1998a). Hilsenbeck et al examined a pool of 2800 patients who had BCT or mastectomy. They reported that at 3 years follow up, ER status provided a significant prognostic information, with a hazard ratio of risk of relapse of 0.87 95% CI 0.78-0.96, *p*=0.007. At 120 months, however, its effect was not significant with a hazard ratio of 0.96 95% CI 0.88-1.05, *p*=0.4 (Hilsenbeck et al. 1998).

h) HER-2: Positive HER-2 predicts the response to Herceptin treatment. As first reported by Slamon et al, HER-2/neu amplification is significantly associated with a shorter DFS and OS (Slamon et al. 1987). In a study of 628 patients with T1-T3, N1 tumours who had mastectomy, HER2 positive patients had a 15% improved OS, and a 7% improved DFS at 10 years when compared to their HER2 negative counterparts, *p*< 0.001 and p=0.03% respectively (Pritchard et al. 2006). A concise review by Ross et al examined 81 studies of HER2-neu with more than 27,000 patients (Ross et al. 2003b). 52 studies reported outcomes of multivariate analyses, and found HER2 to be associated with negative prognosis; either associated with other negative factors (high grade, +ve nodal status) or negative outcomes; overall and progression free survival. Following the publication of the HERA trial results in 2005 (Piccart-Gebhart et al. 2005), HER2 testing and trials of Herceptin treatment became available for early stage breast cancer. In a cohort study of 600 patients with early stage breast cancer

receiving BCT, HER-2 over-expression was not found to be associated with higher LR (Harris et al. 2006). Luminal subtype breast cancers demonstrate lower risk of LR than HER-2+ve/ER-ve and basal types. A study of luminal subtypes in 800 patients and a median follow up of 6 years reported a significantly higher cumulative incidence of LR in the basal subtype and the HER+ve/ER-ve groups (Nguyen et al. 2008). Similarly, Voduc et al underwent molecular studies on tissue from nearly 3000 patients with early breast cancer who were followed up for 12 years. HER-2+ve/ER-ve cancers had the worst 10-year DFS at 79%, compared to 92% in Luminal A tumours (Voduc et al. 2010). In a small case control study, the index group who had local recurrence following BCT alone, had a 56% rate of over-expressing HER2-neu, compared to 18% in the control group; p=0.03 (Haffty et al. 1996a). None of these studies was a randomised controlled trial, or had long term follow up, and further evidence is required to ascertain the role of HER-2 in LR.

i) High nuclear grade: This has been identified as an independent prognostic factor for LRR in some studies, but not in others. Nearly 500 patients with early stage breast cancer who received BCT were followed up for a median duration 6 years. High histologic grade, along with EIC, was an independent factor for LR (Kurtz et al. 1990). Voogd et al, in their pooled analysis of EORTC 10801 and DBCG-82TM (879 patients receiving BCT), found that high grade was a predictor of distant metastasis, but not LRR (Voogd et al. 2001). Mirza retrospectively analysed more than 1000 patients with BCT; LRR was reported in 6% of patients after a median follow up of 9 years, but high grade was not a predictive factor (Mirza et al. 2002). Ki-67 is a protein which is used to assess tumour proliferation; it can be detected in all proliferating cells, but not those in G0

phase. In a study of nearly 2000 patients, Ki-67 was found to be significantly associated with the presence of a higher tumour grade and negative ER status. In addition, Ki-67 was an independent prognostic factor of poor DFS with a hazard ratio of 1.6, 95% CI1.26 to 2.03, p<0.001 (Viale et al. 2008).

j) Adjuvant therapy: Chemotherapy and hormone therapy are systemic treatments aimed at systemic control and the prevention of distant disease recurrence. Their effect on local control is not clearly defined. They were found to be beneficial is some studies. NSABP trial B-21 randomised node negative patients into BCS+RT+tamoxifen (a), BCS+RT (b) or BCS+tamoxifen (c). The 8 year cumulative incidence of LR as lowest in group a (2.8%), followed by group b at 9.3%, and finally group c at 16.5%; tamoxifen and RT resulted in significantly better DFS compared to RT alone (Mamounas 2003). NSABP trial B-20 has shown that the addition of chemotherapy to tamoxifen in node negative patients can significantly decrease the LR rates; 84% vs. 77%. p= 0.001 (Fisher et al. 2001a;Mamounas 2003). Current taxane based chemotherapy is expected to result in more benefit, however long term follow up results are not yet available. Patients with early stage breast cancer who underwent BCT and received chemotherapy or hormone therapy were less likely to suffer LRR than those who received none; 17% vs. 83%, and 9% vs. 91% respectively (Mirza et al. 2002).

1.9.4 Possible origins of local recurrence:

LR is recognized as the re-emergence of the cancer following completion of treatment with a curative intent. It can arise from occult microscopic foci of invasive disease not evident on imaging or during surgery. Since the minimum accepted clearance margin in

BCS is 1 mm (section 1.9.3), it is possible for a small separate focus to be left behind. Residual cancer cells which survive adjuvant RT and continue to proliferate can be labelled as being resistant to radiation. Similarly, an occult focus of DCIS at the time of surgery, can later present as a recurrence of invasive cancer. As discussed, RT has a clear role in the local control of breast cancer; a proportion of LR after BCS can therefore be attributed to RT failure or resistance. Resistance can be innate or can constitute an acquired characteristic. The mechanism of radioresistance (RR) however, remains largely unidentified. Resistance to RT is further discussed in chapter III.

On the other hand, the "recurrence" can be a new primary cancer. A previous diagnosis of breast cancer places patients at an increased risk of a second cancer in the ipsilateral or the contralateral breast. A population based study of 10,000 patients with a history of breast cancer and a median follow up of 5 years, reported an increased risk of a second cancer irrespective of the treatment type; the standardised incidence ratio (comparison against the expected incidence in general population) was 3.5 (Soerjomataram et al. 2005). This could have no association with the RT treatment to the breast. RT however, is known to cause second cancers due to mutations resulting from RT induced DNA damage.

Some studies have demonstrated that histologically normal tissue adjacent to the resected cancer shows evidence of molecular abnormalities which can predispose to the development of cancer; namely loss of heterozygosity (LOH) leading to loss of tumour suppressor function. LOH of chromosomes 3p24, 17p13.1 and 11p15.5, has been detected in both cancer cells and the histologically normal lobular cells surrounding the tumour (Deng et al. 1996). 17p13.1 is the location of the p53 gene. The 3p34 region is known to contain tumour suppressor genes which were suggested

to be associated with head and neck, and breast cancers (Maestro et al. 1993). The 11p15.5 chromosomal region contains many tumour suppressor genes associated with tumours such as rhabdomyosarcoma and Wilms tumour (Anderson et al. 1999). Li et al examined the surrounding tissue of 48 tumours and reported LOH in the surrounding tissue in 25% of cases, the most frequent was 3p24.3 (Li et al. 2002). 55% of the patients who suffered LR had LOH, compared to 27% of those who had no LR. A longer time to LR in patients with LOH (mean of 5 years), compared to 3 years in those with no evidence of LOH, suggests the development of new cancers in the first group (Li, Moore, Meng, Ljung, Gray, & Dairkee 2002).

1.9.5 Time to local recurrence

There is no concrete definition of the time frame for recurrences. It has been suggested that true cancer recurrences occur during the earlier years following completion of treatment. The collective term "local recurrence" has been described as two separate entities; *true recurrence (TR)* being the re-growth of existing malignant cells not killed by adjuvant RT, and *new primary (NP),* malignant cells arising from normal residual breast tissue (Veronesi et al. 1995). It is important to distinguish between these two. Two similarly designed retrospective studies attempted to make this distinction. Huang examined 139 patients with LR; 12 years median follow up (Huang et al. 2002a). Smith et al similarly had 136 patients with LR, mean follow up of 14 years (Smith et al. 2000). These tumours were classified into TR and NP based on histology and location. Those displaying the same histology as the original disease and occurring within the same quadrant and close to original location were labelled as TR. They found that tumours labelled as TR had a mean time to recurrence of 3.7-5.6 years compared with 7.3 years in the NP group. This was statistically significant in one study

(Smith et al. 2000). Both NP groups had better survival rates. Fowble et al examined 1000 patients and noted that of 65 patients developing LR within 5 years, 65% were in the same quadrant. However, 54% of recurrences occurring after 5 years were in a different quadrant (Fowble et al. 1990). This would suggest that recurrences occurring within the first 3-5 years are more likely true recurrences, and could represent a worse prognostic group.

1.9.6 Management of loco-regional recurrence

Following BCT, patients are usually reviewed in combined surgical/oncology clinics. Follow up times vary between institutions but last an average of 5 years, with the intervals increasing with time. Patients usually receive routine clinical examinations and yearly mammography. Suspicious lesions are examined histologically (fine needle aspiration or core tissue biopsy). Additional investigations such as US scans, whole body bone scans and CT scans are carried out if there is clinical suspicion of local or distant metastases.

In the presence of local or regional recurrence, distant disease needs to be excluded before considering treatment options. This is usually in the form of staging imaging investigation; CT thorax/ abdomen/pelvis and whole body bone scan.

The two main treatment options for LR after BCT include mastectomy or a further re-excision plus or minus the administration of RT. Mastectomy is the recognised treatment for LR after BCT. Mastectomy will preclude further parenchymal LR of the same breast. The second option to manage LR after BCT is further conservation surgery. This is less practiced. In a cohort of 50 patients who had salvage conservation surgery, 32% suffered further recurrences at a median follow up of 51 months (Kurtz et

al. 1991). This group was highly selected with small breast recurrences. Previous RT as part of initial BCT would usually preclude the re-use of radiation. Axillary recurrences are managed by axillary clearance.

The role of systemic therapy in the management of local recurrence is less clear. These patients are usually managed on individual basis and following discussions between MDT members. The individual risk for systemic failure and metastases will have to be considered. A Cochrane systematic review (updated 2008), did not find enough evidence to recommend guidelines, and suggested that more patients with LR should participate in clinical trials (Rauschecker et al. 2001).

CHAPTER II

Radiotherapy;

Mechanism of action

Radiotherapy; Mechanism of action

2.1 Types and sources of irradiation

RT is delivered through ionizing radiation. This constitutes electromagnetic fields which have enough energy to displace an electron from a molecule therefore causing it to be "ionized". Clinically, radiotherapy can be applied as:

2.1.1 External radiation:

a) External beam RT: this is the most common type of RT and is usually produced by modern linear accelerators. Electrons are produced and are accelerated to increase their energy thus allowing for more tissue penetration whilst minimizing superficial tissue toxicities (Kiltie 2005). They strike a metal target and this produces photons (x-rays). Photons are then moulded to form an x-ray beam which is directed to the patient. The photons react with cellular molecules to displace an electron, and this act causes DNA damage either directly or via water molecules (Kiltie 2005). Alternatively, the original emission of electrons is scattered and then moulded to produce an electron beam.
b) Teletherapy: this denotes γ-rays, produced by natural decay of the radioactive substance cobalt-60. It was the mainstay of external RT before linear accelerators. Nowadays, its applications are limited.

2.1.2 Internal radiation: α+, β+ and β- (electrons) rays produced from decaying nuclei.

a) Brachytherapy: locally implanted sources of radiation. This is used in cervical cancer and breast cancer.

b) Systemic radionuclide therapy: the most common example is the treatment of thyroid cancer using ingested radionuclide iodine-131.

2.2 Biologic effects of radiation

RT causes cell damage through DNA damage, with the ultimate aim of causing cell death. RT can cause multiple defects in DNA, the most important being DNA strand breaks; consisting of single strand (SSB) or double strand (DSB) breaks. Radiation is measured using the unit gray (Gy). One gray equals 1 joule of energy absorbed per kilogram of mass, and is equivalent to 100 rad. It is estimated that 40 double DSBs can occur following the absorption of 1 Gy radiation, and one DSB can result in significant cell damage (Tutt & Yarnold 2006). Cells respond to DNA damage by activating DNA damage response pathways. Normal cells are more capable of DNA repair than cancer cells. Inability to repair damages will result in programmed cell death (apoptosis), which is the desired effect in RT.

DNA damage can be direct or indirect. Direct damage occurs when DNA strands absorb radiation, acquire an electron and become ionized. Indirect damage occurs through the ionization of the surrounding water molecules. As nearly 80% of the cell consists of water, indirect damage is more common than direct damage. Ionization of water molecules results in the formation of oxygen free radicals such as superoxide (O_2-), hydroxyl ions (OH-) and hydrogen peroxide (H_20_2). Free radicals are highly unstable; they react with DNA causing either SSB or DSB (Abeloff et al. 2004)

DNA damage response (DDR) is a term used to describe the multiple signalling pathways that are triggered following DNA damage, for example as a result of radiation; these are very closely related, and share many proteins as components.

There are three pathways by which the body defends against lasting DNA damage, including that precipitated by radiation; cell cycle check points arrest, DNA repair mechanisms, and apoptosis.

2.3 Cell cycle arrest

These are the mechanisms which sense DNA damage, initiate signal transduction and subsequently activate the downstream proteins, which act on components of the cell cycle to cause cell cycle arrest (Li et al. 2001). Check points exist to protect cells against replicating in the presence of damaged DNA; following the arrest, the two possible outcomes are DNA repair and cycle progression, or else apoptosis.

2.3.1 Cell Cycle

The cell cycle is known to consist of four phases that last for a total of 24 hours (Figure 2.1), and terminates when two daughter cells are produced, each to enter its own new cell cycle. The G1 phase lasts for 12 hours, and precedes the S phase, during which DNA replication and synthesis occur (lasting for 6 hours). Six hours of the G2 phase then precede mitosis (M-phase), which lasts for 30 minutes. The G (gap) phases are required for cell growth, to enable the cell to reach sufficient mass before division (Kierszenbaum 2007). The G0 phase is a resting phase, in which non replicating cells are found.

2.3.1.1 Cell cycle check points

Check points are present throughout the cell cycle to detect irregularities that may affect the final gene product; they can delay progress or abort it altogether. Cancer can develop when there is loss of cell cycle control and uninhibited proliferation, usually secondary to defects in check point mechanisms. The G1-S checkpoint is activated

when DNA damage is detected and functions to prevent a cell with abnormal DNA from engaging in DNA synthesis. The G2-M checkpoint prevents cells with abnormal DNA from undergoing mitosis, thus arresting the expansion of damaged genetic material. The S phase checkpoint is able to detect incomplete DNA synthesis and prevent progress at this stage.

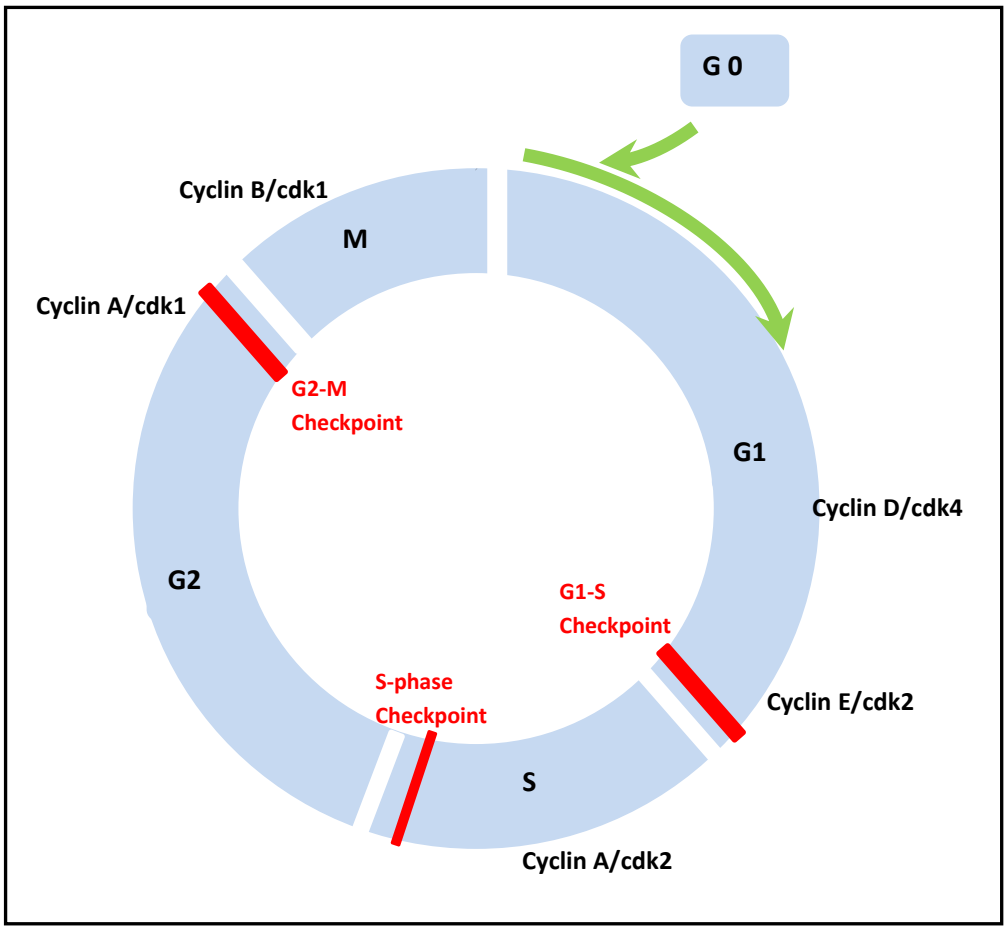

Figure 2.1 The cell cycle. The cycle starts when cells in the resting G0 phase enter the G1 phase. Thereafter follows the S phase during which DNA replication takes place. A period of growth and increase in mass during the G2 phase is followed by cell mitosis; the production of two daughter cells, during the M phase. Two main checkpoints exist to control entry into the S phase, and into the M phase. In addition, S phase delay or arrest is possible through a third check point. Progress through the cell cycle is controlled through kinases known as cyclin dependent kinases (cdk's). These in turn are activated through binding to cyclin proteins (cyclin A-E), and inhibited by cdk inhibitory proteins such as p21, p16 and p27.

A restriction R point exists just before the G1-S checkpoint. This R point separates the "growth" part of the G1 phase where the cell is acquiring mass and is dependent on growth hormones, and the last part where it no longer has these requirements and is committed to entering the S phase and DNA replication (Norbury J 2005). If the cell has not achieved sufficient growth, it is prevented from committing to replication.

2.3.1.2 Cell cycle control

The cell cycle is controlled through proteins known as cyclin dependent kinases (cdk's). Through their catalytic activity, cdk's activate target proteins which result in cell cycle activation and progression (Cordon-Cardo 1995).

Cdk's are regulated by two different types of proteins: cyclins and cdK inhibitory proteins (Cordon-Cardo 1995). These, in turn, are controlled by the ubiquitin proteasome pathway (section 2.3.1.4). There are five major classes of cyclins; A-E. Cyclins C, D and E regulate the cdk's responsible for G1-S transition, while cyclins A and B regulate G2-M transition. The cdk inhibitory proteins inhibit the cdk's and include p21, p15, p17 p18 and p27/kip1 (Sherr and Roberts 1999). The 26S proteasome is responsible for the proteolysis of many regulatory proteins including cyclins and cdk inhibitory proteins and thus plays an important role in cell cycle regulation. The cyclinD/cdk4 complex is important for R point transition. Initiation and promotion of DNA synthesis is a function of cyclins A and E and cdk2 (Norbury 2005). During the G2 phase, A and B cyclins, and cdk1 accumulate, and they later initiate all the processes responsible for mitosis. Cyclin A/B/cdk1 complexes activate proteins responsible for nuclear envelope breakdown, chromosome condensation and spindle formation.

2.3.1.3 Activation of the cell cycle check points

Cells with normal p53 activity usually arrest in G1 phase. DSBs and the resultant RPA coated single strand DNA activate ATM (ataxia telengectasia mutated, a member of the PI-3 kinase family) kinase and ATR (ATM and Rad-3 related) kinase (Jackson and Bartek 2009). ATM kinase causes phosphorylation, and therefore activation of p53. Thereafter follows the activation of p21 which in turn inhibits cdk resulting in failure to progress from G1 to S phase; checkpoint 1 (Bolderson et al. 2009), (Figure 2.2). ATR can initiate S-phase delay in response to DNA damage. It has been suggested that BRCA-1 functions as part of a super complex of proteins to cause S-phase check point delay and DNA repair (Norbury 2005). This complex includes ATM and Rad50.

In the absence of functional p53, p21 can be activated through a mechanism involving ATM, c-ABL and p73 (Fei and El-Deiry 2003). Arrest prevents the propagation of abnormal genetic material, as well as allowing time for repair mechanisms to work (Li et al. 2001). ATM kinase mutation leads to the syndrome of ataxia telengectasia, which causes increased susceptibility to malignancy.

ATM and ATR activate check point proteins 1 and 2 (Chk1 and Chk2) and BRCA1 (Bolderson et al. 2009). BRCA1 is phosphorylated by ATM, and is thought to be important in the activation of the Chk proteins (Yarden et al. 2002). Chk1 and Chk2 inhibit cdk's, leading to slowing down or arrest of the cell cycle at G1-S phase, intra-S and G2-M phases (Jackson & Bartek 2009). The G2 arrest is common with cells that have lost, or have a mutant form of p53 (Abeloff et al. 2004). However, cells over-expressing p53 may also arrest in G2 (Fei & El-Deiry 2003). Cells in the late stages of G2 are most sensitive to RT (Abeloff et al. 2004), i.e. RT will cause delay/arrest in G2 more than G1 or S phases.

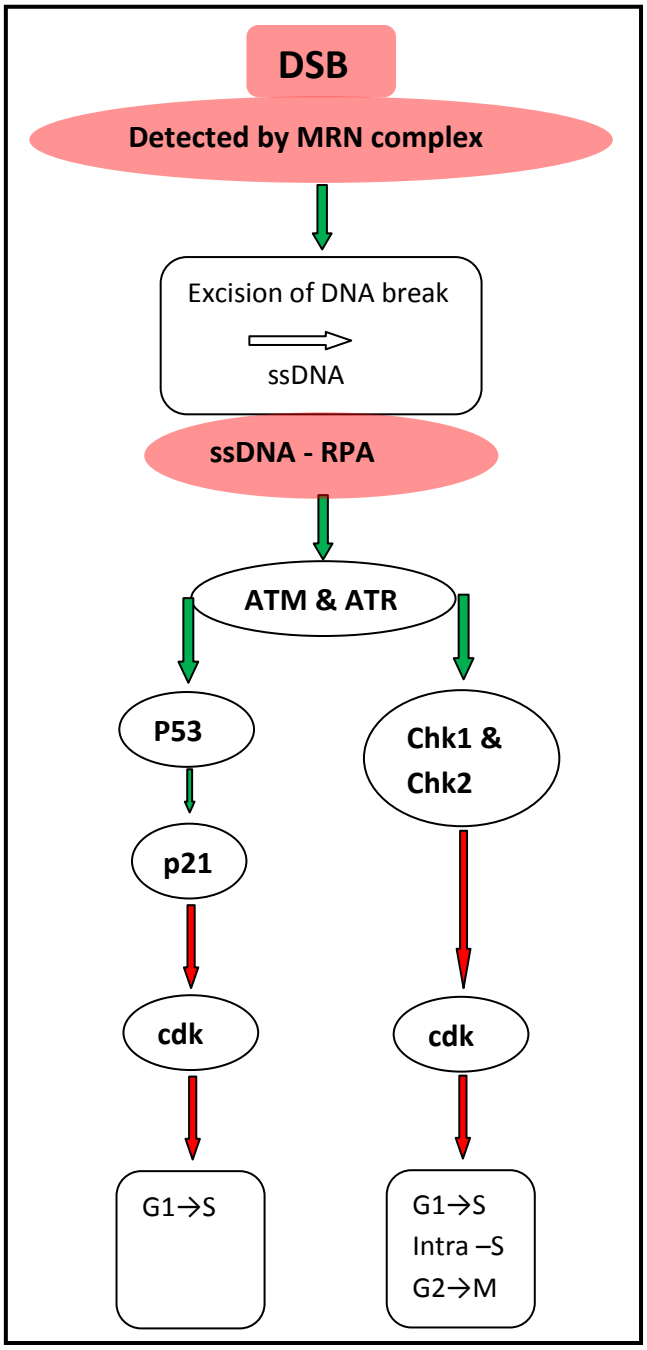

Figure 2.2 Activation of check points pathways following DNA damage. Double strand DNA damage will cause activation of ATM and ATR molecules. These are the main driving forces in the DDR pathway, and in turn inhibit cyclin dependent kinases through cdk inhibitory proteins, p53 and the chk1 and chk2 proteins.
MRN: Meiotic recombination 11, Rad50, NBS1. Green arrows denote "activation", and red arrows denote

RT induced S-phase delay can be especially associated with higher doses of irradiation (Bernhard et al. 1995). DNA synthesis occurs at a much slower rate. It is thought that ATR and chk1 are important for delayed entry into M-phase when DNA synthesis is incomplete and the cell is therefore not ready, while ATM and chk2 are important for G2→M arrest following RT damage during G2 phase (Norbury 2005).

2.3.1.4 Ubiquitin proteasome pathway

The ubiquitin-proteasome pathway (UPP) is one of the two main pathways responsible for protein degradation, the other one being the lysosome. Ubiquitin is a small protein modifier (Bedford et al. 2010), that attaches to proteins destined for degradation thus making them recognisable by the 26S proteasome. Such modification – ubiquitination - acts as a recognition signal for degradation. Initial binding of ubiquitin to a protein substrate is followed by polyubiquitylation. The 26S proteasome is a multi-protein complex normally located in both the nucleus and the cytoplasm. The active 26S proteasome consists of two main parts (Gallastegui and Groll 2010): a) a regulatory molecule known as the 19S cap particle; this is responsible for recognising proteins which are attached to ubiquitin molecules, and are scheduled for proteolysis. b) A core particle, known as the 20S, which is responsible for the actual catalytic process. The 20S core particle is composed of 7 alpha subunits (PSMA 1-7), and 7 beta subunits (PSMB 1-7). The poly-ubiquitinated protein is recognised by the 19S cap particle which is responsible for protein unfolding and de-ubiquitination. This will allow the 20S core particle to accommodate the protein and start proteolysis. The 26S proteasome is thus responsible for controlling the level of multiple proteins through degrading them. Some of the known proteins associated with this system include p53, p27, BcL-2, Bax (apoptosis proteins) and DNA-Pks (DNA repair pathways) (Pervan et al. 2001). It has also been implicated in the degradation of the caspase enzymes of apoptosis (Suzuki et al. 2001). Inhibition of the 26S proteasome or under expression may thus be responsible for increased apoptosis in DNA damaged cells. The 26S proteasome has been shown to be involved in the degradation of cyclin B; cyclin B-cdk2 complex is known as M-phase promoting factor (Norbury 2005). Decreased 26S proteasome

expression and decreased degradation of cyclin B may thus encourage cell cycle progression and proliferation. It is also responsible for the degradation of p21 and p27/kip1, cdk inhibitory proteins. The 26S proteasome is responsible for the degradation of another pro-apoptotic factor; I-κB. NF-κB is a transcription factor nuclear factor that inhibits apoptosis. I-κB is an inhibitory protein against NF-κB. An inhibitor of the 26S proteasome can therefore be expected to increase apoptosis, and indeed such an inhibitor has been approved by the FDA as an adjunct in the management of multiple myeloma (Ludwig et al. 2005).

Vlashi et al have demonstrated that glioma and breast cancer stem cells express low activity of the 26S proteasome (Vlashi et al. 2009). This was achieved by measuring the level of a fluorescent protein that accumulates in cells with a low activity of 26S proteasome. Furthermore, exposure to fractionated RT was reported to increase the cells exhibiting low proteasome activity (Lagadec et al. 2010). This could be explained by increased replication of the cancer stem cells; it had previously been reported that RT can cause cancer stem cells to move from the G0 phase and into the cell cycle (Lagadec et al. 2010).

The 26S proteasome can thus affect the response to radiation, depending on which effector proteins are affected by increased or decreased proteolysis. For example, an increase in the level of caspase enzymes and p53 may encourage cancer cell apoptosis. However, an increase in the cyclin/cdk complexes may encourage cellular progression through the cell cycle and increased proliferation.

2.4 DNA repair mechanisms.

DNA damage can manifest either as SSBs or DSBs. The mechanism of SSBs repair is dependent on the type of injury. Base excision repair is when damaged bases are excised and the damaged segment is replaced with newly synthesized DNA. Enzymes involved in this pathway are poly ADP polymerase 1 and 2 (PARP1 and PARP2), and XRCC1 (Lord and Ashworth 2012). If longer segments are damaged, the SSBs are repaired through nucleotide excision repair (NER), which is activated by the enzymes excision repair cross complementing protein 1 and 4 (ERCC1 and ERCC4). DSBs can be more lethal, however repair is possible through homologous or non homologous repair (Haber 2000). ATM and ATR activated by the DSBs activate DNA repair proteins through initiating transcription, and/or activating modifications such as phosphorylation (Jackson & Bartek 2009).

2.4.1 Homologous repair (HR)

HR is the function of repair enzymes such as exonucleases, helicases and endonucleases, which depend on information provided by an intact chromatid or a homologous chromosome acting as a template to produce missing DNA information. It is mainly active during the late S and G2 phases (Bolderson et al. 2009). Important components of the HR pathway are MRN (Meiotic recombination 11, Rad50, NBS1) complex, ATM, BRCA1, BRCA2, Rad51 and replication protein A (RPA). It is thought that the MRN complex detects the DNA damage and facilitates excision of the DNA break, leaving lengths of ssDNA. After the break is excised, the now single strand DNA is protected against degradation by the binding of RPA, a process facilitated by BRCA1 (Zhang and Powell 2005). Rad51 recombinase protein is responsible for completion of the process and DNA synthesis. Its positioning on the DNA is dependent on BRCA2

(Zhang & Powell 2005). BRCA1 is also involved in non homologous end-joining, S phase, and G2 check points.

2.4.2 Non-homologous end-joining (NHEJ)

During NHEJ, the exposed ends are recognized by repair proteins which bring them together; this mechanism is thus otherwise known as “direct joining”. Being template-independent, NHEJ may result in a mutant product. Most of irradiation induced DSBs are repaired with NHEJ, mainly in the G0 and G1 phases (Bolderson et al. 2009). Being template independent, NHEJ can however occur at any cell cycle phase (Lord & Ashworth 2012). The effecter proteins Ku70 and Ku80 recognise the DNA break, bind to the DNA ends and activate DNA-dependent protein kinase (DNA-PK). The latter activates the XRCC4-ligase IV responsible for the ligation of the two ends. An alternative NHEJ pathway has been described involving PARP1 and XRCC1 (Shaheen et al. 2011). Absence of these proteins may thus result in increased radiosensitivity.

There is a difference between how well normal cells and cells with mutant repair genes can undergo DNA repair. Normal cells are capable of using more than one method to undergo DNA repair, e.g. HR, NHEJ and their respective alternative pathways. In addition, they can promptly repair SSBs before they progress into DSBs. They therefore have strong machinery with multiple options and “spare parts”, should these be required. Cells with a mutation affecting one or more of their repair pathways are much weaker, as they are often reliant on a single pathway that they cannot deviate from. Any affliction to that pathway, e.g. radiotherapy, will result in cell death. Cells with mutant BRCA gene cannot undergo repair of their DNA DSBs. It has been suggested that these cells rely on PARP pathways (SSB repair and the alternative NHEJ pathway) for effective DNA repair (Aly and Ganesan 2011). Inhibition of PARP can

therefore detrimentally affect their survival, and can be used as a form of cancer therapy in BRCA deficient individuals.

Should DNA repair be completed, DDR is deactivated, and the cell may progress and proliferate. In the event of repair failure, chronic signalling may cause the abnormal cell to arrest, undergo terminal differentiation and cell death, or else undergo apoptosis.

2.5 Apoptosis

Apoptosis is programmed cell death. It is a physiological process that aims to counteract proliferation. Two of the defining characteristics of neoplasia are uncontrolled proliferation and deregulation of apoptosis. Apoptosis can also occur in response to cell damage or stress. It can be triggered through one of two pathways; the intrinsic and extrinsic pathways. The intrinsic pathway is associated with radiation damage.

2.5.1 Intrinsic pathway

The intrinsic pathway is activated through damage and signalling occurring within the cell. The exact mechanism of triggering the intrinsic pathway is still being studied. RT, DNA damage, hypoxia and oncogenes can all directly activate the intrinsic pathway. p53 is a main triggering factor. p53 is activated by the DDR pathway proteins ATM kinase and ATR kinase (Figure 2.3), and acts to stimulate the transcription of pro-apoptotic factors, and decreases the transcription of anti-apoptotic factors (Fulda and Debatin 2006). Pro-apoptotic members of the BcL-2 family (BAK and BAX) increase the permeability of the mitochondrial membrane and the release of cytochrome-c and

Smac into the cytoplasm (Fulda & Debatin 2006). This step can be blocked by the anti-apoptotic BcL-2 family proteins (BcL-2 and BcL-xL), which are inhibited by the p53.

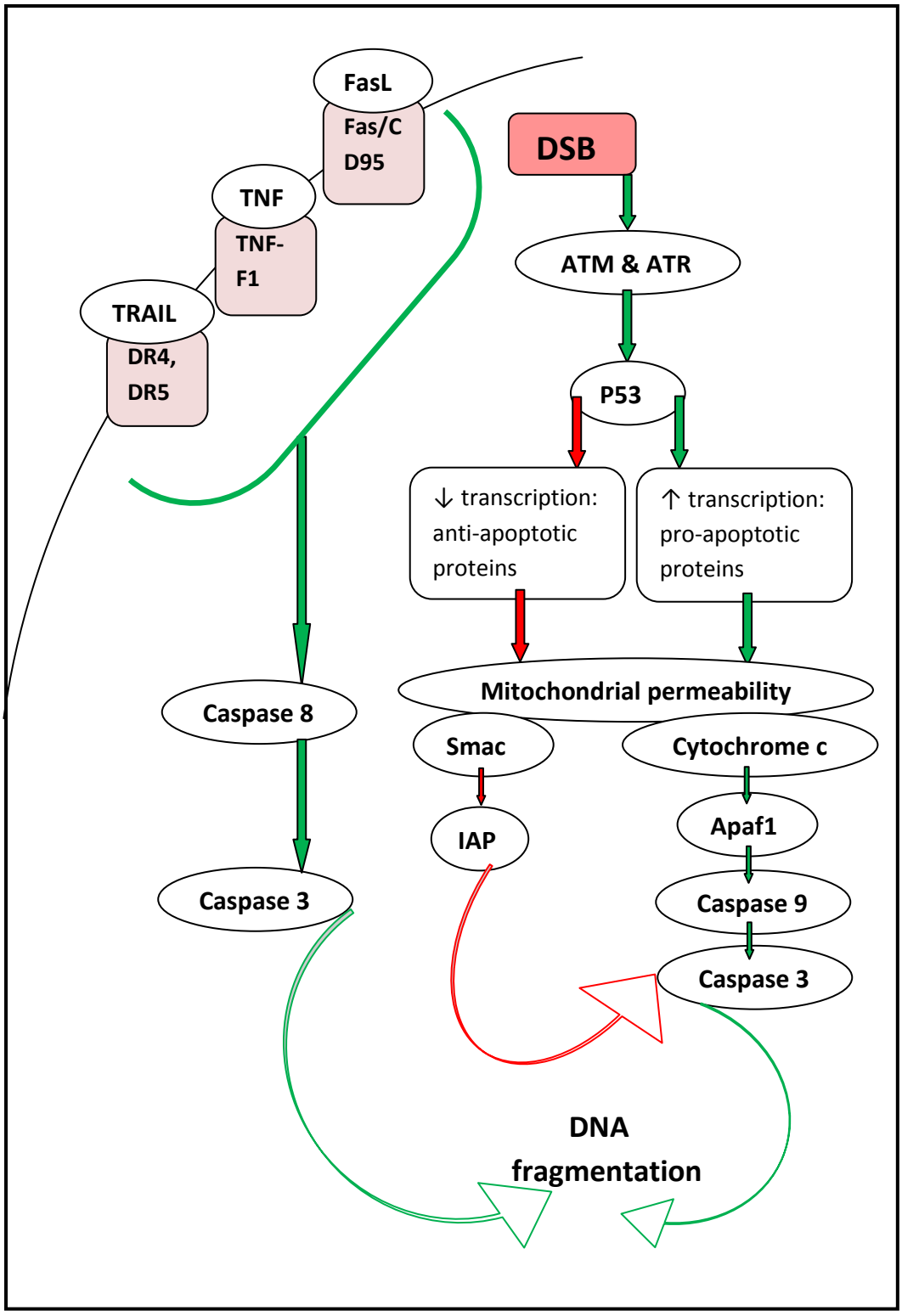

Figure 2.3 Extrinsic and intrinsic apoptosis pathways. Intrinsic pathway is triggered by DSB which can result from RT damage. ATM, ATR and p53 are the initiating proteins for this pathway. Extrinsic pathway is triggered through ligands binding to transmembrane receptors. Caspase 8 can activate pro-apoptotic proteins thus affecting the intrinsic pathway. Green arrows denote "activation" and red arrows denote "inhibition".

In the cytoplasm, cytochrome c binds to cytoplasmic Apaf-1 causing the recruitment and activation of pro-caspase 9 into caspase 9 (Krakstad and Chekenya 2010). This step in turn causes the recruitment and activation of pro-caspase 3; caspase 3 in turn causes DNA cleavage. Two positive feedback loops exist: a) the caspases cause increased mitochondrial permeability and further release of cytochrome-c; b) Smac protein deactivates inhibitors of caspase enzymes (also known as inhibitor of apoptotic proteins - IAPs), thereby increasing apoptosis (Du et al. 2000). Many endonucleases have been identified and studied as the final effectors. A repeatedly identified enzyme is Caspase dependent DNAse (also known as DNA fragmentation factor 40) (Nagata et al. 2003). DNA fragmentation is followed by membrane blebbing and eventual phagocytosis by scavenger cells. Both apoptosis and DNA repair (through DDR) share the same original triggering factors (ATM/ATR, p53), and it still remains unclear how the cell recognises when to increase either pathway over the other.

2.5.2 Extrinsic pathway

The extrinsic pathway is activated by stimuli produced outside the cell membrane. Ligands activate ligand-specific transmembrane receptors through binding to their extracellular domains (Figure 2.3). TRAIL (tumour-necrosis-factor related apoptosis inducing ligand) activates death receptors DR4 and DR5; tumour necrosis factor (TNFα) activates TNF-R1 and FasL activates Fas/CD95 (Krakstad & Chekenya 2010). Upon activation, the intracellular domains (death domains), activate the caspase pathway (Fulda & Debatin 2006). Pro-caspase 8 is recruited and activated, and in turn activates pro-caspase 3 which results in DNA fragmentation and apoptosis as discussed above. Caspase 8 can also activate the intrinsic pathway through activating pro-apoptotic proteins (Krakstad & Chekenya 2010). TRAIL is a known initiator of p53-independent

apoptosis especially in cancer cells, which often express mutant p53, as compared to normal cells (Chinnaiyan et al. 2000). A synergistic apoptotic effect has been reported to exist between TRAIL and RT in breast cancer MCF-7 cell lines (Chinnaiyan et al. 2000); this was associated with DR5 but not DR4 increased expression. Recombinant TRAIL is currently being developed as a cancer treatment. Conversely, based on immunohistochemistry (IHC) studies in cervical cancer tissue, the absence of TRAIL has been significantly associated with a better response to RT (Maduro et al. 2009).

CHAPTER III

Resistance to Radiotherapy

Resistance to radiotherapy

3.1 Radioresistance; possible mechanisms

The exact mechanisms responsible for RR remain relatively unknown. The response to RT, radiosensitivity or RR, can be affected by multiple factors.

1. The type of cell

   Some cancers are known to be highly RR, e.g. melanoma and renal cell carcinoma. RT therefore rarely forms part of the treatment for these cancers. Lymphomas are known to be highly radiosensitive, consequently requiring low doses of radiation. Breast cancers are generally radiosensitive, but require higher doses. Radiosensitivity in malignant cells can manifest as a good response to treatment, while in normal cells it can produce unwanted side effects. Indeed the occurrence and degree of side effects following RT has been used as an objective measure of radiosensitivity.

2. Dose and fractionation; radioadaptive response

   Small repetitive doses of irradiation can lead to acquired RR as demonstrated by the *in vitro* creation of RR cancer cell lines. This is probably through re-population of cells showing changes in the molecular composition and pathways. It has also been proposed that cancer stem cells displaying RR properties may increase following RT doses, thus contributing to a resistant sub-population following the start of treatment (Phillips et al. 2006).

3. Re-population.

   Re-population of tumour cells and the resultant tumour burden is another important factor determining the outcome of RT. Hypo-fractionation and decreasing the total treatment time can limit re-population, but at the same time

increasing side effects. It has been suggested that by assessing the proliferation rate of tumours using markers such as KI-67, fractionation can be individualized to achieve the most benefit.

4. Cancer stem cells.

   Cancer stem cells have been implicated in the aetiology of RR. Adult stem cells have the ability to differentiate into specific cell types and maintain proliferation. Cancer stem cells, also known as cancer initiating cells, share these properties and can produce and maintain the cell population that comprises a specific tumour. Breast cancer stem cells have been identified. Philips et al utilised MCF-7 and MDA-MB-231 cell lines to obtain and separate cells according to their surface markers (Phillips et al. 2006). $CD24^{-/low}/CD44^{+}$ cells demonstrated an increase in the percentage of cancer stem cells compared to non-stem cell cancer cells. Al-Hajj et al implanted cancer tissue obtained from nine human breast cancer patients into soft tissue and pleural cavities of mice (Al-Hajj et al. 2003). Heterogeneity of surface cell markers was observed, and flow cytometry was used to separate the cells. $CD24^{-/low}/CD44^{+}$ cells formed larger sized colonies when compared with $CD24^{+}/CD44^{-}$ cells, suggesting increased tumourogenecity. Glioblastoma tumours are known for their very poor response to RT. $CD133^{+}$ cells have been identified as glioma cancer stem cells (Bao et al. 2006a). Following the application of RT, the cancer colonies showed up to 4 fold increases in the percentage of $CD133^{+}$ group of cells compared with $CD133^{-}$. The authors suggested that the relatively improved survival may be secondary to more efficient DNA repair mechanisms (Rich 2007). Applying a Chk1 inhibitor resulted in a decrease in the survival, further suggesting that the mechanism of improved cancer survival is secondary to altered DDR and more efficient DNA repair (Bao et al. 2006a;Lord & Ashworth 2012).

5. Tumour hypoxia

   Hypoxic regions exist within tumours secondary to uncontrolled growth and abnormal microcirculation. Oxygen is known to increase the cytotoxic effect of RT resulting from free-radical DNA damage (Abeloff et al. 2004). It forms irreversible bonds with the damaged DNA molecules thus preventing repair and subsequent propagation of the genotype. Hypoxic cells within a tumour may escape RT damage. However, re-oxygenation occurring between fractions will facilitate the death of further cell populations in subsequent doses. Hypoxia is thus recognized as a marker of tumour aggressiveness and resistance to RT, while re-oxygenation is a major factor contributing to the success of RT through the process of fractionation. Oxygen is thus regarded as a radiosensitising agent.

6. Inherent tumour characteristics

   Different patients display different responses to the same RT regimen. This can be explained by alterations in the RT response pathways such as DNA repair mechanisms, cell cycle check points and apoptotic mechanisms. Radiosensitivity can also be altered during the course of treatment, as suggested by invitro studies creating RR cell lines. This is also likely to be secondary to alterations in the signalling pathways. Considering different signalling pathways and possible variations in protein interactions, a large number of proteins may be involved in the response to RT (Scaife et al. 2011). Some of these have been investigated such as cyclins and caspase enzymes.

3.2. Radiosensitisers:

Detailed understanding of all the above mentioned mechanisms implicated in RR can be utilised in altering the cellular functions thus rendering tumour cells more radiosensitive.

3.2.1 Chemotherapeutic agents

Different chemotherapeutic agents are used concurrently with RT. Combination treatment is now recognized to improve outcomes, not only through action on distant metastases but also through improving loco-regional control. Taxanes are widely used agents that have been reported to have radiosensitising effects (Nabell and Spencer 2003). Taxanes arrest the cells in the G2/M phase of the cell cycle, during which cells are most sensitive to RT. Cisplatin, used along with RT in the treatment of lung cancer, has also been implicated as a radiosensitiser. Cisplatin reacts with DNA bases thus causing changes in DNA morphology and activation of DDR pathways; possibly directly stimulating ATM, ATR and Chk2 (Wilson et al. 2006). As cancer cells are less likely to undergo DNA repair, apoptosis ensues. RT increases the cell uptake of cisplatin, in addition to preventing DNA repair. The later is achieved through “fixing” the DNA damage, and inhibiting repair machinery, namely decreasing the efficiency of Ku80 protein and NHEJ through its binding to DNA (Wilson et al. 2006). Another radiosensitiser is 5-flurouracil, given in combination with RT in the treatment of rectal adenocarcinoma and head & neck cancer. Though the exact mechanism is not well defined, it is thought that 5-flurouracil attacks the cells during the S-phase, therefore complementing RT which is effective in other cell cycle phases (Ojima et al. 2006). Other suggested, but poorly defined mechanisms, include increased drug delivery

caused by increased blood flow, and decrease in the time available for DNA repair machinery (Wilson et al. 2006).

3.2.2 Molecular agents

Molecular therapeutic agents have also been successfully used as synergistic agents. Cetuximab, a monoclonal antibody against EGFR has shown beneficial effects when used in combination with RT, as opposed to RT alone; a survival advantage of 20 months (p=0.03), and a progression free survival advantage of 10 months (p=0.006) (Bonner et al. 2006). Another reported radiosensitiser is celecoxib; a selective inhibitor of COX-2 (Cyclooxygenase 2). COX-1 and COX-2 are enzymes which convert arachidonic acid into prostaglandin (PG). PGE_2 has been reported to increase cell proliferation and angiogenesis. Involved signalling pathways include activation of the PI3K survival pathway, and inhibiting apoptosis, possibly through increasing BcL-2 (anti-apoptotic) proteins (Aoudjit et al. 2006;Tuo et al. 2007). Radiation can increase the levels of COX-2 and PGE_2. An *in vivo* study on head and neck squamous cell carcinoma (SCC) xenografts demonstrated that celecoxib has radiosensitising effects through an antiangiogenic mechanism; contrast MRI has demonstrated increased vasculature permeability (Davis et al. 2004).

3.2.3 Oxygen mimetics

Nimorazole is an oxygen mimetic. In comparison with RT alone, the combination of nimorazole and RT in the treatment of head and neck cancer has shown significant positive correlation with improved loco-regional control (Overgaard et al. 1998). Hyperbaric and normobaric oxygen therapy has also shown some benefit. A Cochrane systematic review reported that hyperbaric oxygen produces significant improvement

in LRR and survival in head & neck cancers (Bennett et al. 2005). However, it was associated with significant severe radiation toxicity. In addition, the effects were not predictable and seemed to vary with different fractional regimens.

3.2.4 Other modulators of the cell cycle as radiosensitisers

Individuals naturally defective in ATM protein kinase (ataxia telangiectasia) show hypersensitivity to RT (Figure 3.1). *In vitro* tests utilizing specific ATM inhibitors have demonstrated similar defects in the ATM downstream phosphorylation pathways, and increased sensitivity to RT (Rainey et al. 2008). Cancer cells that are able to repair DNA damage can resist being killed by RT. Suppressing the genes, or the resultant protein products, that play roles in DNA repair may improve radiosensitivity (Figure 3.1).

The role of DNA-PK in NHEJ DNA repair is well documented (section 2.4.2). A peptide inhibiting the interaction between DNA-PK and the Ku complex has resulted in decreased DNA repair following RT on breast cancer cell lines (Kim et al. 2002). *In vitro* suppression of the Ku70 protein expression through DNA transfection has shown resultant increased radiosensitivity in lung SCC cell lines (Omori et al. 2002). Components of the DNA HR pathway have also been investigated. Conflicting reports exist as to the prognostic significance of RAD51. Decreased expression and increased expression of RAD51 have both been associated with significantly higher rates of LRR in breast cancer patients (Le et al. 2010;Soderlund et al. 2007). Reduced expression correlates positively with a good response to RT (Soderlund et al. 2007). Indeed, suppressing the expression of RAD51 in animal models, when coupled with RT, has resulted in increased survival in comparison with RT alone, suggesting a possible radiosensitising effect (Ohnishi et al. 1998).

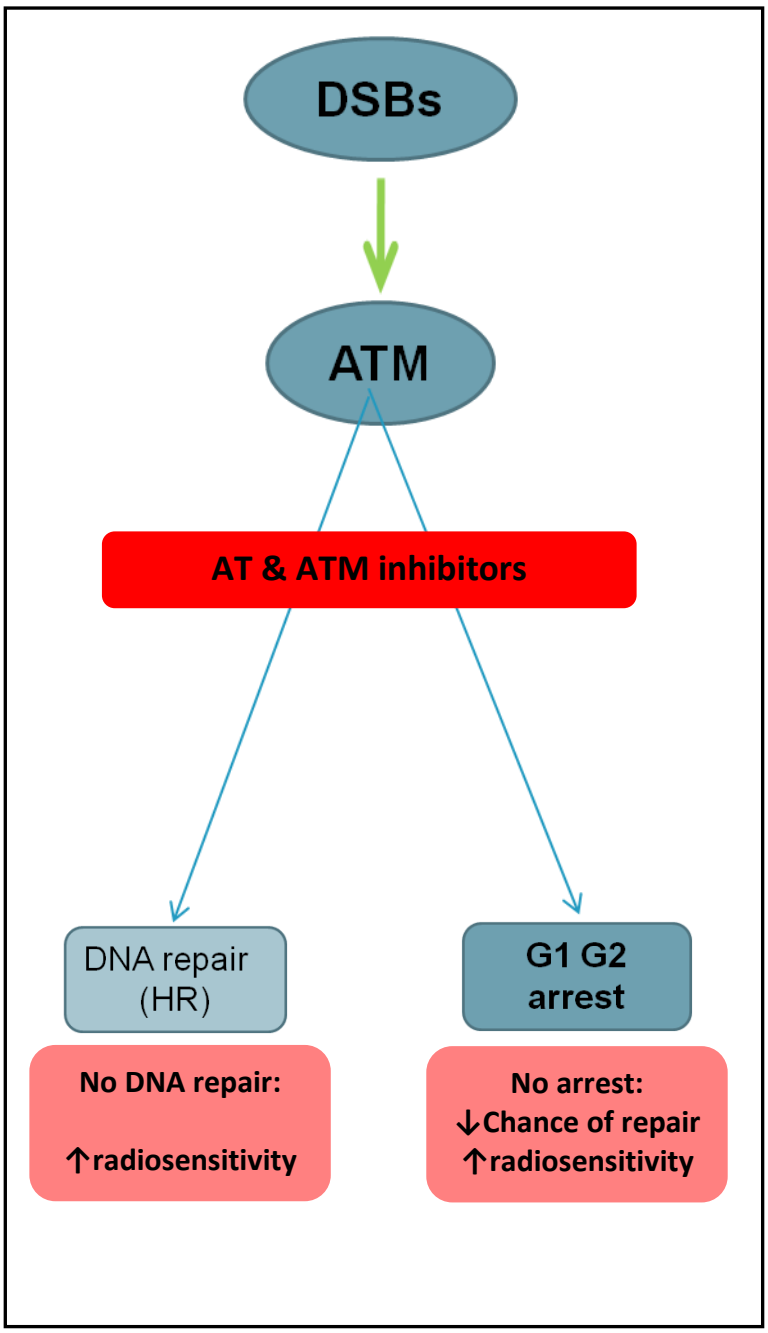

Figure 3.1 Modulation of the cell cycle by ATM inhibition. Ataxia talengiectasia (AT), and synthetic ATM inhibitors can prevent cell cycle arrest and DNA repair, thus resulting in more cells undergoing apoptosis.

Delay in the cell cycle phases, e.g. arrest in the G2 phase, has been associated with RR (Bernhard et al. 1995). The delay will allow more time for effective DNA repair, and therefore survival of the cancer cells. Over expression of Chk1, an important effecter in G2 arrest has been associated with RR in rat embryo cells (Hu et al. 2001). Extensive

research into RR has been carried out in glioblastomas as they are known to be highly resistant to RT. Experimental evidence exists in which RR cells expressing a marker for cancer stem cells, were shown to increasingly activate check points and DNA repair mechanisms (Bao et al. 2006b;Ropolo et al. 2009). Furthermore, inhibiting Chk1 and Chk2 can decrease this effect, thus resulting in increased radiosensitivity (Bao et al. 2006b).

Some studies have suggested patient factors such as age and smoking as predictive of the response to RT (Lilla et al. 2007). Normal genetic variations dictating different protein compositions of the mechanism involved in DNA repair and cellular response to damage could be involved.

It would seem that an imbalance in the cellular response to RT induced DNA damage will determine the degree of sensitivity or resistance i.e. DNA repair and survival or apoptosis. This is true assuming that other external factors, such as radiation dose, remain constant. However, as outlined in the above suggested mechanisms and pathways, much of our understanding of RR remains elementary, probably due to the fact that the mechanism of action of RT itself is not fully understood.

3.3. Molecular markers

3.3.1 Molecular markers of cancer

Molecular markers can be:

1. Prognostic markers: prognostic of the outcome independent of therapy.
2. Predictive markers: predictive of the response to specific therapy, whether sensitivity or resistance. Different cancers and different individuals vary in their

response to anticancer treatment. Therefore these markers can direct clinicians in the decision making process.

3. Both prognostic and predictive: ERs provide an important example, having both prognostic and predictive values. Their value in predicting the response to hormone therapy is well established; patients with positive status will respond to hormone therapy. An even better response can be observed in tumours that are both ER and PR positive (Abeloff et al. 2004). ER can be used as a positive prognostic factor, especially in the first few years after treatment; this however has been debated as previously discussed (section 1.9.3, part "g").
4. Therapeutic targets: molecules identified as effectors in the response to RT can be targeted. Antibodies can act to attach to cellular proteins and block their action, or act to block other triggering factors from binding. Therapeutic agents can also be used to alter the protein synthesis, up-regulate or down-regulate its expression, therefore altering the response to radiation.

3.3.2. Molecular markers of radioresistance

There have been major recent advances in the treatment of breast cancer, mainly in the fields of chemotherapy and hormone therapy. WBRT following BCS has been the mainstay of treatment for many decades, with recent advances including PABI and IORT to the tumour bed. RR constitutes an obstacle to long term DFS. Resistance can be an inherent characteristic of the cancer cells. It can also be acquired through molecular changes secondary to the treatment itself. Several proteins have been associated with RR. It is postulated that several interacting mechanisms are involved, though the exact manner remains largely undefined.

The study of molecular markers promises many clinical benefits. Pre-treatment identification of patients at risk of local treatment failure and disease recurrence allows planning of alternative therapy options. For example, the identification of a certain molecular marker labelled as predictive of RT failure in the serum or tissue of a patient, labelled as low risk by clinical and pathological indicators, allows more aggressive treatments to be offered, which would not have been indicated otherwise. Molecular markers associated with breast cancer have long been the focus of research, and some were identified as potentially linked to resistance to radiotherapy. These will be discussed below.

3.3.2.1 Epidermal growth factor receptor (EGFR) – (HER-1)

EGFR has been extensively studied, and its association with cancer, and more recently with resistance to RT, has culminated in therapeutic agents used in the clinical setting. The detailed available information about structure and function has contributed to better understanding of its role in disease processes and its utilization in disease management.

EGFR activates multiple signalling pathways resulting in cellular proliferation, maturation, survival and migration (Wells et al. 1998;Wells 1999; Scaltriti and Baselga 2006), (Figure 3.2).

a) EGFR activates Ras, which results in the activation of Raf-1. This latter phosphorylates MAPK1 and MAPK2. These are transferred into the nucleus where they phosphorylate transcription factors that increase cell proliferation. Ras also stimulates the PI3K/ Akt pathway (Grana et al. 2003). Grana et al suggested that Ras contributes to RR through three different pathways; the Ras-Raf pathway, Ras-

PI3K pathway, and through an autocrine pathway phosphorylating and thus activating EGFR (Grana et al. 2003).

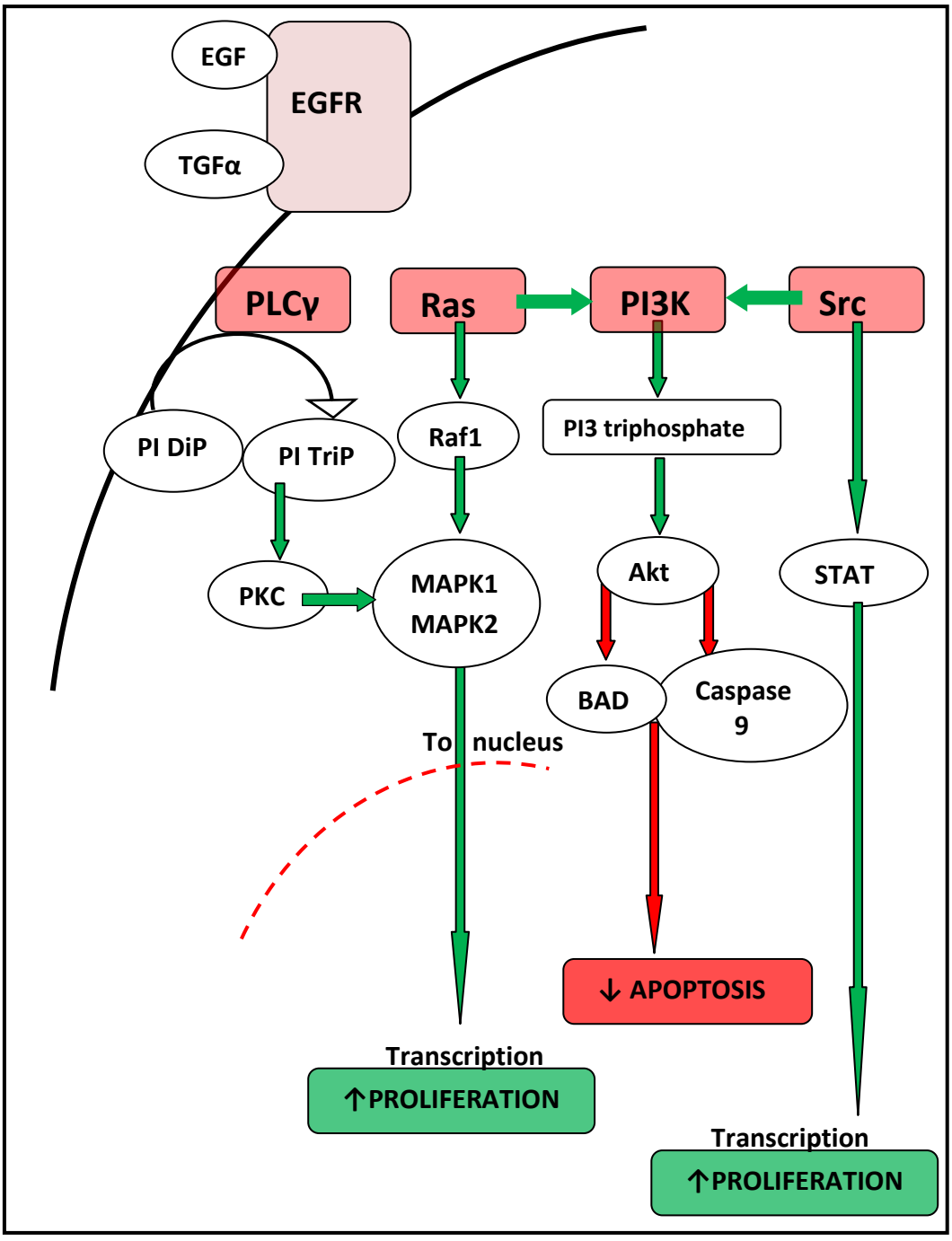

Figure 3.2 EGFR downstream signalling pathway. The transmembrane receptor is activated through extracellular ligands, and causes the activation of multiple downstream proteins which contribute to increased cellular proliferation and inhibition of apoptosis. Green arrows denote "activation", and red arrows denote "inhibition".

b) Activation of EGFR, and especially HER-3, directly activates PI3K, which through activating PI 3,4,5 triphosphate, activates Akt. Phosphorylated Akt inhibits apoptosis, possibly through deactivating BAD (a pro-apoptotic protein), pro-caspase 9 and survival transcription factors (Chang et al. 2003;Gupta et al. 2002a). RT induced EGFR activation may thus cause RR through this pathway.

c) PLCγ reacts with EGFR to hydrolyse PI 4,5 diphosphate producing PI 1,3,5 triphosphate. This increases intracellular calcium and activates protein kinase-C (PKC), and thereafter MAPK.

d) Activation of STAT (signal transducer and activator of transcription) leads to increased proliferation through activation of transcription.

e) Src activation leads to activation of PI3K and STAT pathway. Src may also become active independent of EGFR.

EGFR link to the DDR pathways and RT is still a subject of research. Ligand independent phosphorylation and activation of EGFR can occur following RT, and is followed by EGFR transfer into the nucleus. DNA binding to DNA-PK is an important step in NHEJ DNA repair. The protein kinase must disengage from the DNA to allow progression of repair. EGFR has been reported to react with Ku70 and DNA-PK to increase the efficiency of this process, thus allowing DNA repair (Chen and Nirodi 2007;Dittmann et al. 2005). In addition, PI3K and Akt produced in response to EGFR activation block apoptosis pathways, and thus may decrease the effect of RT (Chen & Nirodi 2007).

EGFR is over-expressed in up to 80% of non-small-cell-lung cancer (NSCLC), in head and neck (SCC) and several other cancers including: oesophageal, gastric, bladder, ovarian, endometrial and cervical (Gullick 1991;Salomon et al. 1995;Veale et al. 1993). A positive correlation has been suggested between the level of expression and DFS & OS

in head and neck SCC (Scambia et al. 1991), and breast cancer (Koenders et al. 1993). EGFR is over-expressed in breast cancer cell lines and breast cancer tissue. An analysis of more than 40 studies examining the role of EGFR in breast cancer has reported a positive correlation with tumour grade, and an inverse relationship with steroid receptor status, DFS and OS (Klijn et al. 1992).

Molecular inhibition along with RT may enhance radiation induced apoptosis, and act as a radiosensitiser (Harari and Huang 2001). Survival assays of murine ovarian cancer cells transfected with EGFR vector and exposed to irradiation demonstrated increased resistance to RT. This was reversed following transfection with C225, a monoclonal antibody against EGFR, thus further proving an association with RR (Liang et al. 2003a). A trial of 400 patients with advanced head and neck SCC randomised patients into RT alone or RT and C225 (cetuximab) (Bonner et al. 2006). After a median follow up duration of 54 months, the second group had a survival advantage of 20 months (50 months vs 30 months in the control group, p=0.03), and a progression free survival of 10 months (p=0.006). Cetuximab has received FDA approval in 2006 for the treatment of locally and regionally advanced head and neck SCC – in combination with RT, and as a single agent in metastatic cases in which platinum-based therapy has failed. In the UK, NICE has recommended cetuximab for certain patients with metastatic head and neck SCC.

3.3.2.2 HER-2

Over-expression of HER-2 is associated with poor prognosis, high recurrence risk, and poor response to hormonal and chemotherapeutic agents. HER-2 is however, a good predictor of the response to trastuzumab. HER-2 shares the same downstream effectors as EGFR. These have been associated with RR (Liang et al. 2003b); breast

cancer MCF-7 cells transfected with phosphorylated Ras, or Akt, were exposed to radiation. Survival was measured using clonogenic assays, while apoptosis was assessed using ELISA measurement of DNA fragments. They both showed increased survival when compared with controls. A study on HER-2 over-expressing MCF-7 cells exposed to RT, showed increased phosphorylation of MAPK and Akt when compared to cells with lower levels of HER-2; thus leading to increased cell survival (clonogenic assay), and decreased apoptosis (ELISA) (Liang et al. 2003c). The addition of trastuzumab resulted in decreased levels of phosphorylated MAPK and Akt, with consequent improved cell kill. Inhibiting Akt, but not MAPK, similarly resulted in improved cell death and radiosensitivity.

3.3.2.3 Cyclin D1

Cyclins are a group of proteins expressed in all proliferating cells and function in controlling the cell cycle through cdk's. Cyclin-D specifically controls the G1 phase and progression to DNA synthesis (S phase). Cyclin-D levels were found to be low during G0, increase progressively during G1, and decrease markedly at S phase (Baldin et al. 1993). Inhibition would thus result in cell cycle arrest in G1 phase, and up-regulation can result in increased proliferation. Cyclin-D1 can be over-expressed in head and neck SCC (Izzo et al. 1998;Lai et al. 2002). Cyclin-D1 gene amplification or protein over-expression can occur in up to 15% and >50% of human breast cancers respectively (Dickson et al. 1995; Dickson et al. 1995;Gillett et al. 1994;McIntosh et al. 1995). Over-expression has largely been linked with worse survival figures and aggressive biological behaviour (Tomazic et al. 2004). However, associations with better OS and DFS in human breast cancers have been reported (Gillett et al. 1996).

Yu et al suggested that cyclin-D1 was essential for the oncogenic effect of the Ras pathway (Yu et al. 2001). Conflicting results exist with regards to irradiation; cyclin-D1 has been linked to both increased apoptosis following RT (Pardo et al. 1996), and reduced sensitivity to RT (Milas et al. 2002). Rat embryo cells transfected with cyclin-D1 and exposed to radiation showed significantly increased apoptosis when compared to non transfected cells (Pardo et al. 1996). Conversely, a study conducted in mice reported that cancer cells expressing low levels of cyclin-D1, and exposed to RT, showed increased apoptosis (Milas et al. 2002). A study of 64 patients treated with RT for SCC and followed up for 10 years reported a significant association between cyclin-D1 and LR. 68% of patients over-expressing cyclin-D1 had LR in less than 5 years compared with 10% of those not over-expressing cyclin-D1 (Lai et al. 2002). Immunohistochemical analysis of 125 patient samples following RT treatment for oral cancer revealed that cyclin-D1 over-expression was associated with a poor DFS (Jayasurya et al. 2004).

3.3.2.4 p53

p53 is a nuclear protein. It has been extensively studied, and its role as a tumour suppressor has emerged following the observation that it was found mutated in many human cancers. Wild-type p53 can be activated secondary to hypoxia, DNA damage and RT. p53 is regulated by proteins such as ATM, ATR, Chk1 and Chk2 (Fei & El-Deiry 2003). p53 has well documented roles in apoptosis activation (Figure 2.3), through the activation of the pro-apoptotic proteins, inhibition of anti-apoptotic proteins, activation of the cascade pathway and cell arrest in G1 phase. p53 also acts as a transcription modulator; it can bind to certain genes and activate or inhibit transcription (Elledge and Allred 1998). Its role in DNA repair is less well understood.

Through its role in cell cycle arrest, it allows time for repair mechanisms to function. However, it has been suggested that p53 has a direct role in both HR and NHEJ (Fei & El-Deiry 2003), possibly through binding to ssDNA and promoting repair. It has also been reported to have an exonuclease function. There is more than one possible response or pathway following p53 activation; which one is chosen and why will depend on the type of cell involved and the degree of damage, among other factors, and is not well understood (Tutt & Yarnold 2006).

Mutations in p53 can be found in 30% of breast cancers, and can assume different characteristics (Borresen-Dale 2003). Mutations may allow the damaged cells to escape the regulatory mechanisms and therefore the propagation of abnormal DNA (Figure 3.3).

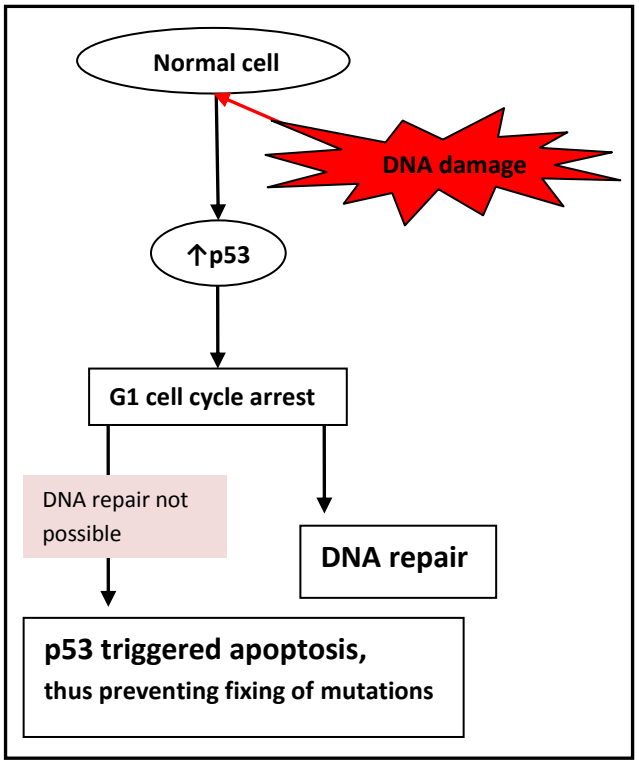

Figure 3.3 Diagrammatic representation of p53 function and association with RT. p53 mutations in cancer cells will prevent G1 cell cycle arrest, thus allowing damaged cells (e.g. by RT) to progress through the cell cycle and proliferate. Apoptosis may not progress, and damaged cells will escape death, thus achieving resistance to radiation

Mutant p53 has been shown to be an independent prognostic factor of LR in breast cancer (Borresen-Dale 2003). McIlwrath et al used 12 human cancer cell lines to demonstrate this (McIlwrath et al. 1994). The cell lines had intrinsic varying radiosensitivities e.g. a radiosensitive teratoma line and a radioresistant glioma cell line. Eight hours after exposure to RT, the radiosensitive but not the radioresistant lines showed a significant increase in p53 levels.

Radiosensitivity had a significant correlation with successful G1 arrest. In addition, an ovarian cell line (radiosensitive) was transfected with mutant p53, and displayed more RR than a control transfected with vector alone. Similarly, an *in vitro* study in which rat embryo fibroblasts were transfected with mutant p53 reported increased RR associated with its expression. Simultaneous transfection with Ras vector resulted in further augmentation of the RR response (Bristow et al. 1994).

p53 was found to be an independent prognostic factor for poor local control in T1 pre-treatment laryngeal cancer patients (Narayana et al. 2000), and head and neck SCC (Couture et al. 2002). Contradicting results were however reported where p53 over-expression as detected by IHC, did not show any prognostic value following curative RT treatment in head and neck SCC (Awwad et al. 1996;Hirvikoski et al. 1999).

P53 mutations occur in 20-40% of human breast cancers, and can be associated with a worse prognosis (Bergh et al. 1995;Marchetti et al. 2003;Thorlacius et al. 1993). However, genetic mutations do not always correlate with over-expression of the protein (Mineta et al. 1998), and indeed the two can have opposite effects, or have no correlation to survival data (Neri et al. 2006). Elledge et al reviewed studies reporting on p53 and effects on prognosis in breast cancer. The study designs were very heterogeneous; however 36 studies reported a significant correlation to a worse

prognosis, while 14 studies failed to show such as relationship (Elledge & Allred 1998). It is known that wild type p53 has a short half life and is difficult to detect by immunohistochemistry (Banks et al. 1986); genetic mutations can result in over-expression of a more stable p53. A synthetic compound known as PRIMA-1 (p53 reactivation and induction of massive apoptosis) has been shown to convert mutant p53 to wild type p53 in breast cancer cell lines. T47-D breast cancer cells (mutant p53), and MCF-7 breast cancer cells (wild type p53) were both treated with PRIMA-1. T47D cells showed cellular death, while no effect was demonstrated in MCF-7 cells (Liang et al. 2009). The authors went on to test this on mice xenografts using two breast cell lines with mutant p53 (BT-474 and HCC1428) (Liang et al. 2011). Immunofluorescence detected conversion of mutant to wild type p53. Resultant induction of apoptosis was reported by increased levels of fragmented DNA, p21 and caspase. Such a compound can act in combination with RT to cause more efficient killing of cancer cells.

3.3.2.5 Bcl-2 family

This is a family of proteins which have different functions in apoptosis; Bcl2 and Bcl-xL inhibit apoptosis, while BAX, BIM, BID and BAD are pro-apoptotic proteins (through inhibiting or activating the release of cytochrome-c respectively) (Reed 1998). Bcl-2 is the most studied member of this family. It can be over-expressed in breast cancer, theoretically incurring a worse prognosis through preventing cancer cell death (Neri et al. 2006). However, Bcl-2 has been previously linked to positive ER status, which is a known good prognostic indicator (Berardo et al. 1998;Linjawi et al. 2004), and indeed reports exist of Bcl-2 being a positive prognostic indicator with regards to both DFS and OS (Berardo et al. 1998;Neri et al. 2006).

Nix et al conducted a retrospective analysis of 124 patients treated with primary curative intent RT for T1-T2 laryngeal SCC (Nix et al. 2005). Pre-treatment biopsies (suggesting an inherent tumour characteristic) were tested for the expression of apoptotic proteins using IHC. The recurrent group of cancers were associated with significant increased expression of Bcl-2 and Bcl-xL, and under-expression of BAX when compared with disease free samples (Nix et al. 2005). RR could contribute to the local failure. A similar study examined breast cancer microarrays from 500 patients following BCT (Yang et al. 2009). BcL-2 was expressed in 28% of the cases, and was an independent predictor of LR in multivariate analysis.

3.3.2.6 Insulin like growth factor receptor (IGF-IR)

This is a tyrosine kinase transmembrane receptor that promotes cellular proliferation, adhesion and has a role in migration and the invasive potential of cancer cells (Peretz et al. 2002;Surmacz 2000). Effector molecules, IGF-I and IGF-II, act through p38 and PI-3K, Akt and PKC (Bartucci et al. 2001;Surmacz 2000). IGF-IR is over-expressed in breast cancer, and is significantly associated with LR after BCT, also suggesting an association with RR (Turner et al. 1997). Research has also suggested that the growth effects of IGF-IR are more pronounced in ER-ve MDA-MB-231 breast cancer cell lines, when compared with ER +ve MCF-7 cells (Bartucci et al. 2001). As discussed previously, ER-ve tumours may be associated with higher LR, especially in the first years following BCT (section 1.9.3).

3.3.2.7 Vascular endothelial growth factor

VEGF is secreted secondary to hypoxic conditions, and is an important regulator of both physiologic and tumour angiogenesis (Banai et al. 1994). VEGF is reported to

inhibit endothelial apoptosis, possibly through alterations in the Bcl-2 family of proteins (Ge et al. 2009). However, a recent study has suggested that VEGF may possibly induce apoptosis as a preliminary step required for the induction of angiogenesis by TGF-β1 (Ferrari et al. 2009).

Preclinical and clinical studies have culminated in the approval of the drug bevacizumab; a humanized monoclonal antibody that binds to all isoforms of VEGF, thus preventing attachment to its receptors. A phase III trial has reported improved progression free survival in patients with metastatic breast cancer when using bevacizumab plus paclitaxel as opposed to paclitaxel alone (Miller et al. 2007). However, there was no difference in the OS. There is no current evidence to support its use in the adjuvant setting.

A study by Gupta et al suggested that VEGF expression may be associated with acquired but not inherent RR (Gupta et al. 2002b). Two rat fibrosarcoma cell lines; VEGF+ve and VEGF-ve; were exposed to RT, and demonstrated similar radiosensitivities as measured by clonogenic assays. They were then implanted into mice, and further exposed to RT. The tumour volume was used as a measure of the response to RT; VEGF+ve xenografts showed more RR as demonstrated by higher tumour doubling time (Gupta et al. 2002b). RT was reported to induce the expression of VEGF in human cancer cell lines (Gorski et al. 1999), and this could explain the change in radioresponsiveness. Indeed, a pilot study on 17 patients with different brain tumours compared serum VEGF levels pre and post RT, and reported an increase in VEGF, along with other proliferation factors such as EGF and TGF-β (Gridley et al. 1998). This suggests that RT may activate VEGF secretion leading to tumour progression and therefore RR. Anti-angiogenic factors may therefore have

radiosensitising effects. This is in contradistinction to existing concerns that hypoxic conditions precipitated by anti-angiogenic agents may contribute to RR, as hypoxia is a known factor associated with radioresistance.

3.3.2.8 Hypoxia markers

The role of hypoxia in RR has been discussed (sections 3.1, 3.2.3). Hypoxia can be objectively assessed using intra-tumour electrodes which measure the partial pressure of oxygen. Molecular markers of hypoxia exist, and can be utilised as predictors of the resistance to therapy. These include intrinsic factors such as hypoxia inducible factor-1 (HIF-1) and carbonic-anhydrase IX (CA IX), and extrinsic factors such as the nitroimidazole compounds. Under hypoxic conditions, nitroimidazoles are reduced into metabolites which can bind to intracellular proteins (Mees et al. 2009). They therefore accumulate in hypoxic regions such as malignant tissue (Hoogsteen et al. 2007). They are usually labelled before being injected into patients to enable detection by PET scans (Mees et al. 2009). Immunohistochemical detection of the extrinsic marker pimonidazole has also been associated with significantly worse loco-regional control in patients with head and neck cancer suggesting a relation with RR (Kaanders et al. 2002). HIF-1 is a transcription factor thought to trigger a cascade of reactions in response to hypoxia that allow the cell to survive its unfavourable environment, and subsequently produce a resistant genotype (Mees et al. 2009). Genes regulated by HIF-1 include CA IX, VEGF, IGF, GLUT1 and GLUT3 (Hoogsteen et al. 2007). HIF-1 has been associated with poor prognosis after RT; it was measured in pre treatment samples from 67 patients receiving primary RT with a curative intent for cervical cancer (Bachtiary et al. 2003). Patients received daily fractionated external beam RT to a total dose of 40-50 Gy. IHC revealed HIF-1 expression in 72% of patients to varying degrees.

Strong expression was associated with partial response to RT, as measured clinically and radiologically, 3 months following treatment, and was found to be an independent predictor of a short time to disease progression (Bachtiary et al. 2003). High levels of CA IX are detected following hypoxic conditions and in necrotic tissue. A study on pre treatment tissue biopsies of 198 patients with head and neck SCC receiving primary RT showed CA IX to be an independent predictor of LR. CA IX was positive in 58% of patients, and was associated with LR after a mean follow up of 5 years; p=0.004 (Koukourakis et al. 2006). Osteopontin is a plasma protein that is secreted by hypoxic cancer cells, and was found to be associated with a poor outcome in head and neck cancer treated with primary RT (Overgaard et al. 2005).

Considering the above mentioned potential markers of RR, EGFR is the only biomarker that has resulted in the production of a therapeutic agent. Cetuximab is a molecular agent that is in the clinical arena as a synergistic factor to RT. It is an example of a molecular biomarker proven to be associated with RR, EGFR, which had been utilised for the development of a treatment modality.

p53 has been extensively studied, yet no biomarker is in use in clinical practice. Promising results have been reported in pre-clinical studies with regards to conversion of mutant p53 to wt-p53, thereby activating apoptotic pathways. This could potentially be utilised in conjunction with RT, to optimise treatment.

No relevant biomarkers of RR exist in clinical practice, as yet. There still is a need for molecular biomarkers that can be used for the identification of the response to radiotherapy, and possibly for the creation of therapeutic agents.

CHAPTER IV

Mechanisms of

Biomarker identification

Mechanisms of biomarker identification

4.1 Biomarker discovery pipeline

Protein molecules can be specifically associated with particular diseases, or particular disease processes. Identifying them can therefore enable us to gain insight into early diagnosis and optimum management. The term “biomarker discovery pipeline” has been in existence for nearly a decade (Makawita and Diamandis 2010;Rifai et al. 2006). It denotes the different stages involved in identifying molecular markers of disease. Three main stages have been described:

1. Discovery stage: here, high throughput proteomic mechanisms are used to generate a large number of potential markers. Examples include antibody microarray (AbMA) and mass spectrometry techniques. These processes will yield a large number of potential targets, including false positives. Therefore, a detailed assessment of these targets is necessary to choose which ones to carry forward for further confirmatory testing. This latter is known as “mining”, and denotes literature search into the known functions of the protein and its known, or potential, direct and indirect associations with other proteins. Mining aims to reduce the number and improve the quality of target proteins.
2. Confirmation stage: Mechanisms used include western blotting (WB) and ELISA. Western blotting will confirm the differential expression of certain proteins associated with a disease process, in addition to describing the direction of change, i.e. up-regulation or down-regulation. An even smaller number of proteins are then carried forward for validation.
3. Clinical validation: the differential expression of proteins is examined in clinical samples where relevant clinical details are used to construct inclusion and

exclusion criteria. Immunohistochemistry (IHC) is a tool for clinical validation using tissue samples. Adequately validated biomarkers can then be further examined in large scale clinical trials, with the ultimate aim of using them in daily clinical practice.

All three stages; microarray, WB and IHC techniques, are dependent upon the availability of antibodies against specific proteins.

4.2 Mechanism of protein biomarker discovery

Proteomics, as compared to genomics or transcriptomics, could reflect a more accurate molecular makeup of an organism. The level of mRNA had been previously used to reflect the level of corresponding protein. However, changes can occur to mRNA before translation, and changes can occur in protein following this. The numbers of identified genes far exceed the numbers of proteins. The human genome project has estimated that there are between 20,000 – 25,000 protein coding genes (International human genome sequencing consortium 2004). The number of proteins is unknown but is thought to be much higher. A human proteome project is currently under way; launched in 2010 and overseen by the Human Proteome Organisation. The discrepancy in the number between genes and proteins can be secondary to multiple factors. mRNA undergoes alternative splicing, resulting in multiple transcripts from one mRNA (Nilsen and Graveley 2010). In addition, mRNA is known to have a shorter half life than protein. This has been estimated to average about 7 hours in mice stem cells (min 1 hour) (Sharova et al. 2009), and 10 hours in human HepG2 cells (min 2 hours) (Yang et al. 2003). The discrepancy can also be secondary to post- translational modifications in proteins which can change the cellular location, function and interactions of proteins. The most common modification is phosphorylation, which is

responsible for the activation of many enzymes and signalling pathways (Seo and Lee 2004). Other modifications include acetylation, sumoylation and ubiquitination. A comparative study was performed to compare expression levels of proteins and their corresponding mRNA in lung adenocarcinoma tissue (Chen et al. 2002). Gel electrophoresis and mass spectrometry were used to identify 165 proteins, while mRNA levels were determined using oligonucleotide microarrays. A single protein as the product of a single genetic precursor was the case in 69 proteins, while nearly 100 proteins were the product of only 29 genes. Protein and cDNA analyses undertaken on frozen human liver cells and yeast cells have similarly shown a poor correlation between mRNA and protein levels (Anderson and Seilhamer 1997;Gygi et al. 1999). The function of the majority of existing proteins remains unknown. Identifying these functions could provide important information that could guide disease aetiology and treatment.

The most well established and widely used proteomic technique is 2-D polyacrylamide gel electrophoresis (2-DE) coupled with mass spectrometry. This technique has many limitations; high expense, long assay time, bias towards the most abundant proteins, poor sensitivity for membrane proteins, large required sample size and poor reproducibility. This has led to the development and progress in the protein microarray technology (Debernardi et al. 2005).

Protein array technology is based on the same principles as DNA microarrays; the latter having being extensively, and successfully utilised (Sotiriou and Pusztai 2009), and recently introduced into clinical trials (Mook et al. 2007). Protein microarrays can be functional or analytical (Zhu and Snyder 2003). Functional arrays are used to study

protein function and interactions (MacBeath and Schreiber 2000a), while analytical arrays are mainly utilised for protein profiling.

4.2.1 Antibody microarrays

AbMA are a form of analytical arrays which are based on the principle of an antigen antibody reaction. They constitute high throughput screening tools that can be used to study a large number of proteins. These are predetermined prior to an experiment, and their number will depend on the availability of antibodies specific to them. Antibody arrays are based on two main concepts:

4.2.1.1 One-antibody label-based assays

In this assay, antibodies are immobilised on special support surfaces and will act as capture molecules to bind labelled proteins (Haab et al. 2001;Sreekumar et al. 2001). Labelling is usually through fluorescent cyanine based dyes. This technique will allow qualitative comparison between two different samples each labelled with a different colour dye (Figure 4.1). A “test” sample is compared to “reference” sample. Labelled samples are applied to the glass slides, and antigen-antibody reactions will localise proteins to individual spots of antibodies. The degree of fluorescence reflects the concentration of the antigen. Different arrays, containing different combinations of antibodies are available commercially. Previously, robotic arrayers were used by institutions to deliver custom chosen proteins (Chan et al. 2004;Haab et al. 2001;Sreekumar et al. 2001). The AbMA protein profiler utilised in the work presented in this thesis is an example of the 1-antibody label-based arrays.

4.2.1.2 Sandwich (2 antibody assays)

Un-labelled test proteins are captured by immobilised antibodies, and detected by another antibody, which binds a different part of the test protein (Huang et al. 2002b). This technique allows only one sample to be assessed per array, therefore does not allow comparison. It also needs two antibodies to identify a single protein, with consequent improved sensitivity and specificity.

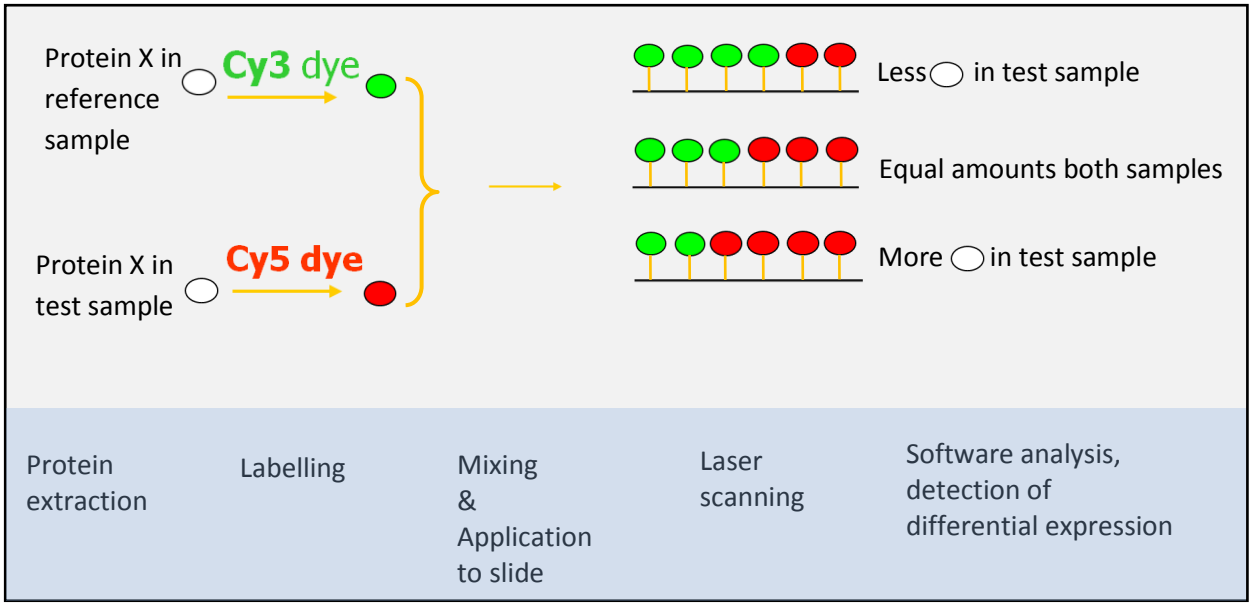

Figure 4.1: Diagrammatic representation of the basic reactions in comparative AbMA technology using cyanine dyes. The experiment compares the protein expression in two samples; a test and a control sample. Each sample is labelled with different coloured dyes such as the cyanine dyes. The relative abundance of labelled protein is detected using the dye intensities to produce a fold change in expression.

4.2.1.3 Principles of antibody arrays

The surface of the slide is commonly made of glass that coated with nitrocellulose or poly-L-lysine to allow optimal binding (Haab et al. 2001). Binding to both these surfaces is through non-covalent interactions (Templin et al. 2002). Antibodies are deposited on the slide surface using robotic contact arrayers. These aim to deposit nanolitre volumes of antibodies to produce uniform sized spots usually of small

diameters calculated in micrometres (Chan et al. 2004). Non-contact arrayers are also available. It is postulated that though small amounts of molecules are present, the resultant high density increases the sensitivity of the assay. Each spot or "feature" contains one antibody that is specific to a named protein. These arrays are usually utilised to compare the difference in a particular protein (or proteins) expression between two different protein lysates. Following protein extraction, the two samples to be compared are labelled with two different fluorescent dyes (e.g. red and green cyanine based dyes) and mixed in equal amounts. They are then applied to the glass slide. Fluorescent labelling is through covalent linkage (Haab et al. 2001); it produces high resolution images, but may affect the functional activity of proteins (Haab 2003). Fluorescent dyes, however, remain of the most efficient labelling tools available because of the quality of resolution produced. Strict adherence to labelling protocols and removal of any excessive dye will aid in reducing error. Cyanine based dyes are the fluorescent dyes used in the AbMA kit utilised in this project. The amount of protein within a sample will determine the amount of dye that binds to it. This in turn will be reflected in the intensity of the fluorescence in each sample, which is detected by laser scanners, and converted to numerical values. Comparing dye intensities between two different samples will determine the level of differential expression of the protein in question (Figure 4.1). The colour intensity is calculated in the two samples using specifically designed software and translated into a fold change (section 5.1.5.6). A fold change of 2 or more is usually accepted as the cut off for significant differential expression between the two samples (section 5.3).

4.2.1.4 Applications of the AbMA technology

The AbMA technology has proved robust, and has been extensively applied in cancer and non-cancer settings. In the field of breast cancer, the AbMA has been applied in comparing differential protein expression in different cancer cell lines (Vazquez-Martin et al. 2007). Comparing breast cancer tissue with adjacent normal tissue using a 378 AbMA has revealed that malignant tissue was associated with overexpression of p53, annexin XI, MAPK and under-expression of 14-3-3e (Hudelist et al. 2004). AbMA has been used to study breast cancer cell lines and the role of IL-8 in tumour invasiveness and angiogenesis (Lin et al. 2004).

The AbMA technology has also been used in the study of proteins associated with adjuvant therapy. A custom made array of 146 antibodies was used to study irradiated LoVo cancer cells. Four hours following RT, there was an up regulation of proteins including p53, DNA fragmentation factor 40, death receptor 5, TRAIL; all factors that are known to increase apoptosis (Sreekumar et al. 2001). The Genepix software was used for data analysis. Indeed, using fluorescent microscopy, there was a 13% increase in apoptosis in the irradiated *vs*. non-irradiated cells. In another study, a doxorubicin-resistant MDA-MB-231 breast cancer cell line was created, and compared to the parent cell line using an array with 224 spotted antibodies (Smith et al. 2006). This array is similar to the one used in this thesis, albeit with fewer antibodies. Cyclin D2, p-ERK, and cyclin B1 were found to be down-regulated in the resistant subline; a finding confirmed by western blotting.

4.2.1.5 The Panorama antibody microarray XPRESS Profiler

The Panorama® Antibody Microarray-XPRESS Profiler725 (Sigma Aldrich) is a recently developed array (Sigma Aldrich 2012). It consists of 725 antibodies (Appendix B) spotted in duplicate onto a nitrocellulose coated slide, and treated with a proprietary blocker to ensure optimal signal-to-noise ratio. These antibodies are representative of different groups of proteins involved in a variety of cellular pathways including cell signalling, proliferation and apoptosis. The slide has the dimensions of a typical microscope slide (25 x 75.6 x 1 mm). It is nitrocellulose coated, with a thickness of ~9µm. Each spot/feature on the slide has a diameter of 300 µm with an inter-spot distance of 500 µm. It has been used by Jain et al in studying the expression of proteins interacting with Tax, a viral oncoprotein implicated in the pathogenesis of T cell leukaemia (Jain et al. 2007). Mohri et al used the array to identify potential biomarkers of gastric cancer; they compared gastric cancer tissue extracts of 17 patients with adjoining normal tissue (Mohri et al. 2009a). Similarly, it has been used to compare normal and cancerous oesophageal tissue from three patients; 58% of the potential markers resulting from the AbMA were confirmed using western blotting, and 37% were confirmed using both western blotting and immunohistochemistry (Uemura et al. 2009a).

This slide was chosen for this project because of the large number of spotted antibodies. These correspond to proteins belonging to a wide array of cellular processes, including apoptosis, cell signalling, cell adhesion and proliferation. The number of antibodies included in the slide is limited by the size of the slide and the minimum size of the spots. In addition, not all known proteins have commercially

available antibodies to enable detection, and indeed not all proteins have been identified as yet.

4.2.2 Western blotting

Antibody microarray techniques will yield a large number of putative biomarkers. Therefore, following the process of mining, a second technique such as WB is required to confirm the differential expression of the chosen biomarkers, and to determine the direction of change.

4.2.2.1 Principles of western blot technique

WB is a widely used and well known technique, performed to identify the presence of a specific protein within a particular sample, and to give a semi-quantitative indication of the difference in expression between different samples. A brief description is provided. Detailed methodology is given in chapter VI. Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins in a homogenous sample based on their polypeptide chain length. Protein is extracted and denatured using a combination of heat and lysis buffer (e.g. Laemmli buffer). This consists of distilled water, Tris-HCl buffer, SDS (an anionic detergent which solubilises proteins, disrupts protein-protein interactions, and coats the proteins in a negative charge), glycerol (weighs down the sample, facilitating loading and running within the gel), and bromophenol blue (to visualise samples). β-mercaptoethanol is added to expose epitopes for protein binding. Enzyme inhibitors are added to the buffer to protect proteins against proteases and phosphatases.

Protein lysates are then loaded onto a polyacrylamide gel in equal amounts. This is ensured through protein quantification to calculate the volume required for loading.

The use of loading controls further ensures equal protein loading, and will detect human loading errors, and unequal protein transfer. The gel is submerged in the running buffer, and electrophoresis takes place with protein separation based on polypeptide chain length. This is followed by protein transfer onto a nitrocellulose membrane. The membrane is then incubated with a blocking agent to prevent non-specific binding, and is thereafter incubated with a primary antibody. A secondary antibody conjugated with HRP (horse radish peroxidase) enzyme is then incubated with the membrane to bind the primary antibody. The protein band is then detected using luminol and hydrogen peroxide through a reaction with HRP producing 3-aminophthalate. This product gradually decays producing light, which is then detected on X-ray films.

Following the incubation of an antibody specific to a loading control protein, a densitometer is used to obtain a ratio for each protein test-control pair. Comparisons are reported as fold change. A significant fold change would indicate that the particular protein is differentially expressed in one sample relative to the other. It would therefore qualify for further clinical validation studies. Data mining and further assessment is also carried out. In practice data is scrutinised before and after each stage to ensure that only biomarkers which have a good chance of success are taken forward.

4.2.3 Immunohistochemistry

Following the verification process, clinical validation is required. It involves further assessing the data produced by the verification techniques, but using clinical subjects, e.g. tissue, with known clinical history. There are two main components to the process of IHC; selecting a clinical sample, and carrying out the experimental procedure.

4.2.3.1 Clinical sample selection

The first stage is selecting a clinical sample which satisfies certain criteria that define the disease process in question. This sample comprises a test and a reference (or control) group. Assessment of the clinical information will serve to narrow down the two groups so that they are as homogenous as possible. Once the clinical series is defined, formalin fixed paraffin embedded tissue is obtained for each case.

4.2.3.2 Principles of IHC

Prompt tissue fixation in formaldehyde (forms covalent crosslinking between proteins) preserves cellular morphology. The fixed tissue is then embedded in paraffin wax to preserve its architecture. Formalin fixed paraffin embedded (FFPE) tissue blocks are sectioned into thin, 4-5µm thick, slices and mounted onto glass slides.

Heat and enzymatic reactions (proteases) are used to expose the antigenic sites. Blocking buffers prevent binding of antibodies to non-specific binding sites on antigens. The addition of hydrogen peroxide eliminates peroxidase activity. Peroxidase enzyme exists in many tissues, and has the potential to react with DAB (3`-diaminobenzidine tetrahydrochloride), thus resulting in non-specific staining.

Multiple methods exist for antigen detection. Indirect methods which involve a secondary labelled antibody result in signal amplification, and are known to be more sensitive than direct methods in which the only reaction is between the antigen and the primary antibody. They can involve an immune-fluorescent or an immune-enzymatic reaction. Here, the labelled StreptAvidin- Biotin method is described. Following incubation of the primary antibody with the tissue sample, the secondary biotinylated antibody is added, followed by streptavidin conjugated to HRP. The

chromogen DAB is added, and reacts with HRP to produce colorimetric precipitates. These allow the visualisation of the antigen-antibody complex. The stained slide is thereafter examined, and the protein expression is compared between members of the test and the control groups.

4.3 Breast cancer, radioresistance and the biomarker discovery pipeline

The most recognised way of identifying protein markers of disease is to follow the biomarker discovery pipeline as previously discussed. This strategy was chosen for this study, to identify biomarkers associated with resistance to radiation. A breast cancer model was selected. AbMA is a relatively new technique (compared to WB and IHC) with promising results, and its high throughput yield makes it ideal for the screening phase. WB and IHC are well tried techniques, and these were chosen for the confirmatory stages of the study.

Aims

4.4 Project objectives

1. Utilise five cancer cell lines and derived radioresistant novel sublines, and the antibody microarray technology as a screening tool, to identify potential target biomarkers of resistance to radiotherapy.
2. Confirm the resulting target biomarkers through western blot technique. Parent and radioresistant cancer cell lines will be used for this purpose.
3. Identify a clinical series of radiosensitive and radioresistant groups, and utilise breast cancer tissue samples obtained from these groups to further validate the data through IHC, and prove or refute any clinical relevance.

Chapter V

Antibody Microarray

Methods & Results

Chapter Aim:

To utilise the antibody microarray technology and cancer cell lines in identifying potential protein markers that are associated with resistance to radiotherapy

Antibody microarray; Methods and Results

5.1 Methods

Antibody microarray

AbMA technology was used as a high throughput screening tool in the first the first stage of the biomarker discovery pipeline. In this project, it was used to identify the differential protein expression between parent radiosensitive (PN) cancer cell lines, and derivative radioresistant (RR) counterparts. RR sublines had been previously created in our institution (section 5.1.2).

5.1.1 Cancer cell lines

Protein lysates were obtained from cancer cell line protein extracts. These breast cancer cell lines were chosen because of their different characteristics in expressing oestrogen receptors and p53, thereby representing different cancer subtypes.

1. *MCF-7:* This invasive ductal carcinoma cell line was originally derived from a pleural effusion in a female patient with metastatic breast cancer, who had previously received RT and hormone therapy (Soule et al. 1973). MCF7 cells are ER and PR positive, in addition to expressing receptors for EGF and IGF. They also express wild type p53 gene.
2. *MDA-MB-231:* This adenocarcinoma cell line was derived from a metastatic pleural effusion in a 51 year old female patient, at the M D Anderson Cancer centre in Houston, Texas in 1973 (Cailleau et al. 1978). This cell line does not express ER, and expresses a mutant p53 gene.

3. *T47D:* This is an invasive ductal carcinoma cell line which was derived from a malignant pleural effusion of a breast cancer patient. These cells are ER positive, and express a mutant p53 gene (Wang et al. 2008). They can lose the ER during long-term oestrogen deprivation *in vitro*.

In addition, two oral cancer cell lines were used in this project. Oral cancer cell lines were chosen as the RR sublines were available (section 5.1.2). In addition, they would provide a satisfactory clinical model should IHC confirmation is attempted in oral cancer tissue. As RT is the main primary modality for treating head and neck cancer, recurrences in these patients would provide an ideal model for testing RR, without the confounding effects of surgery and chemotherapy.

4. *PE/CA-PJ41:* Oral SCC line, obtained from the European Collection of Cell Cultures (Health Protection Agency 2012a). This was originally derived from a 67 year old female patient.
5. *PE/CA-PJ49:* Oral SCC line, obtained from the European Collection of Cell Cultures (Health Protection Agency 2012b). It was originally derived from the tongue of a 57 year old male.

5.1.2 Establishment of the radioresistant breast and oral cancer cell lines.

Three breast cancer lines (MCF7, MDA-MB-231 and T47D) and two cancer lines (PE/CA-PJ41 and PE/CA-PJ49) were used to create derivative RR sublines. This work was performed as part of a PhD thesis by Dr Laura Smith (Smith et al. 2009b), and Dr Justin Murphy. The inherent radiosensitivity of the cell lines was determined using survival assays following their exposure to single fractions of radiation (Smith et al.

2009b). The cells were then rendered RR through exposing them to weekly fractions of radiation, and their RR confirmed using the modified colony counting assay.

5.1.3 Cell culture

For each cell line, all the cell culture steps were simultaneously carried out for the PN and RR populations. The following description applies to each single cell line. Cell culture was performed in class II laminar flow cabinets. These were sterilized at regular intervals, and cleaned prior to and following each session using a disinfectant with 70% alcohol. The hood was also cleaned and left standing for 15 minutes in-between work on PN and RR cell lines to prevent cell contamination. The cells were previously stored in liquid nitrogen or in -80°C freezer in 1 ml microfuge tubes. They were thawed, and transferred into a 30 ml centrifuge tube. Ten ml of supplemented RPMI 1640 media was slowly added to the cells over 3-5 minutes. The cells were centrifuged at 1300 rpm for 3 minutes. The supernatant was decanted and the cells re-suspended in media and transferred into a T-75 culture flask.

The cells were cultured in RPMI 1640 media (from Invitrogen), supplemented with 1% penicillin, 1% fungizone, 1% L-glutamine and 10% foetal calf serum (FCS). Cells were grown in a humidified laboratory incubator at 37°C and 5% CO_2. The media were changed at least every 48 hours. The cells were sub-cultured once, after attaining 80% confluence, using Tryple™ Express (from Invitrogen) for trypsinization. After removal of the growth media, three ml of Tryple were added to the culture flask, and this was incubated at 37°C for 5 minutes. The cells were inspected under the microscope to ensure detachment. Seven ml of RPMI media was added to neutralize the Tryple thus minimizing further cell damage, and the mixture transferred into a 30 ml centrifuge tube. This was centrifuged at 124xg for 3 minutes. The supernatant mixture containing

the Tryple was discarded, and the cells were re-suspended in 20 ml RPMI media, and equally split into four T-75 culture flasks. Subsequently, 10 ml of media was added into each culture flask to make a total of 15 ml. Protein extraction was carried out after the cells achieved cellular confluence of 70-80%.

5.1.4 Antibody Microarray Methods

A standardised protocol utilizing the Panorama® Antibody Microarray-XPRESS Profiler725 kit (#XP725, Sigma-Aldrich) was implemented (Smith et al. 2006).

The array consists of 725 proteins (Appendix B) spotted in duplicate onto a nitrocellulose coated glass slide. It is estimated that there are approximately 100,000 known proteins. The number on the array (725) is comparatively small. However the array represents different cellular processes, and is one of the largest available commercially. The proteins are arranged in 32 sub arrays, each containing 48 spots (24 duplicate items). These spots represent different proteins, one pair of positive controls (positioned at the bottom right hand corner for orientation) and a variable number of negative controls. The arrangement of the array is depicted in Figures 5.6 and 5.7.

The antibody microarray experiment was carried out for five cell lines and their corresponding RR counterparts (MCF-7, MDA-MB-231, T47D, PJ41 and PJ49). Each pair was processed simultaneously. Cells were thawed, cultured and protein extraction performed at the same time. The RR subline was sub-cultured only once to allow the availability of four T-75 (75cm^2) culture flasks. The parent cell line was kept in culture until the radioresistant subline was ready for protein extraction. Protein lysate from three culture flasks was extracted and utilised for each one AbMA experiment.

5.1.4.1 Protein Extraction for AbMA

All steps were performed wearing latex-free gloves and using polypropylene plastic ware as recommended, to avoid protein adsorption. Protein was extracted from three tissue culture flasks (75cm^2), each with a cellular confluence of 70-80%. The cells were washed thrice in cold phosphate buffered saline (PBS), to remove dead cells and other proteins from FCS. PBS contains sodium chloride and sodium phosphate at a pH of 7.3, and is prepared by dissolving two PBS tablets in 500 ml of ionised water. Five ml of PBS was then added to each flask and a plastic mechanical scrapper was used to harvest the cells. They were transferred into a tube container and spun at 124xg for three minutes. The supernatant was discarded, and the cells were re-suspended in 1 ml of PBS and transferred into a previously cooled polypropylene microfuge tube. This was spun at 1300 rpm, and the cells were finally re-suspended in 1 ml of AbMA Buffer A. This latter was prepared by adding 50 µl of Protease inhibitor Cocktail (#P4495, Sigma Aldrich), 100 µl of Phosphatase Inhibitor Cocktail II (# P5726) and 1.2 µl of the Benzonase Working solution (all supplied in the AbMA kit) to 10 ml of Extraction/Labelling buffer (# E0655). The Protease Inhibitor Cocktail was reconstituted in the laboratory by adding 0.3 ml of distilled water to the vial supplied. The Benzonase Working solution was reconstituted by mixing Benzonase ® Ultrapure (# B8309) with Extraction/Labelling buffer in the ratio of 1:9. The cells were incubated in the AbMA Buffer A for 10 minutes at 4°C while maintaining gentle mixing using a rotor. They were then spun at 10,000 xg for 2 minutes. The supernatant was transferred into a fresh cooled microfuge tube and the protein lysate quantified.

5.1.4.2 Protein quantification

The extracted protein lysates were quantified using the Bradford Assay (Bradford Reagent, #B6916, Sigma Aldrich) as recommended by the manufacturer of the AbMA kit. Standard dilutions were prepared to test for protein concentrations between 0.1 mg/ml and 1.4 mg/ml. Eight standards were prepared using Bovine serum albumin (BSA) at a concentration of 2 mg/ml and AbMA Buffer A for dilution; 0.1, 0.2, 0.4, 0.8, 0.6, 1.0, 1.2 and 1.4 mg/ml. For each protein lysate, three replicates of undiluted sample, and three replicates of a sample diluted to 1:5 were used. Two identical replicates of each BSA standard were used. Five µl of each BSA standard and 5 µl of each protein replicate was added to a separate well in a 96 well plate. A reference well was represented by 5 µl of Buffer A. A volume of 250 µl of the Bradford reagent was added to each well, followed by mixing for 30 seconds. The plate was incubated at room temperature for 5 minutes. A spectrophotometer (Multiscan MS plate reader, Labsystems) was used to measure the protein absorbance at a wavelength of 595nm. A curve was then constructed of the absorbance versus the dilution values to calculate the protein concentration using the equation y=mx+c.

5.1.4.3 Protein labelling

The quantified protein lysates were diluted in Buffer A to achieve a concentration of 1 mg/ml. The lysates were labelled using the highly fluorescent, water soluble monofunctional dyes - Cy™3 (#PA23001, Amersham GE Healthcare) and Cy™5 (#PA25001, Amersham GE Healthcare). These dyes were recommended by the Sigma-Aldrich technical bulletin, and previously used in our laboratory (Smith et al. 2006). Protein extracts from untreated parent cell lines were labelled with Cy3 dye and extracts from RR sub lines were labelled with Cy5 dye. All steps following protein

labelling were carried out in the dark, as the dyes are light sensitive. One milligram (1 mg/ml) of each protein lysate was added directly to one dye vial and mixed thoroughly. The dye vials were incubated at room temperature for 30 minutes, with further mixing at three 10 minute intervals. The free un-conjugated dye was removed from the labelled sample by adding 150 µl of the labelled sample to the SigmaSpin™ Post-Reaction Clean Up column (#S0185, Sigma Aldrich), and centrifugation at 750xg for 4 minutes. The eluate, containing the protein-dye conjugate, was retained and re-quantified using the Bradford Assay. Re-quantification is to ensure effective protein labelling. This value was then used to determine dye: protein ratio.

5.1.4.4 Determination of the Dye: Protein Ratio

A dye: protein molar concentration ratio was calculated for each sample. A minimum ratio of 2 is required to proceed to the next experimental steps – this ascertains satisfactory labelling (Smith et al. 2006). Inadequate labelling can result in weak signal intensity. The dye absorbance was measured by means of a spectrophotometer (Libra S11, Biochrom), and utilised to determine the dye concentration. The Cy3 dye absorbance was measured at 552nm excitation wavelength, and the Cy5 dye absorbance at 650nm excitation wavelength. The protein sample was first diluted in Buffer A to 1:10 to allow the spectrophotometer to read the absorbance. The following formulae were used as recommended by the dye manufacturer. The molecular mass 60,000 was used as an average numerical value to account for the presence of variable proteins within a non-homogenous sample.

$$\text{Cy3 dye concentration (µM)} = \frac{\text{Absorbance 552nm} \times 10}{0.15}$$

$$\text{Cy5 dye concentration (µM)} = \frac{\text{Absorbance 650nm} \times 10}{0.25}$$

$$\text{Protein concentration (µM)} = \frac{\text{Labelled protein concentration (mg/ml)} \times 10^6}{60{,}000}$$

$$\text{Dye: protein ratio} = \frac{\text{Dye concentration (µM)}}{\text{Protein concentration (µM)}}$$

The numbers 0.15 and 0.25 represent the µmolar extinction coefficients of Cy3 and Cy5 respectively.

Only samples that achieved a D/P ratio above the minimum recommended ratio of 2 were further analysed. Equal amounts (50 ug) of the labelled protein lysates were added to the Array Incubation Buffer (#A9602, Sigma Aldrich), gently mixed by inversion and then poured onto the quadriPERM® Cell Culture Vessel. The Panorama Antibody slide – XPRESS Profiler725 (#P9499, Sigma Aldrich) was first momentarily immersed in BSA - to minimize nonspecific binding (MacBeath and Schreiber 2000b) - before being incubated in this mixture, in the dark, on an orbital shaker at room temperature for 30 minutes. The slide was handled using forceps, and hand touch was avoided. The slide was then incubated in 5 ml of Washing Buffer (#P3563, Sigma Aldrich) on the orbital shaker for a total of 15 minutes to remove any unbound dye. The washing buffer was supplied with the AbMA kit and reconstituted in the laboratory by adding 1 litre of distilled water to powder PBS, TWEEN 20 and filtering the mixture through a 0.45 nm filter. Finally, the slide was incubated in distilled water for 2 minutes. It was left standing until it was visibly dry. Complete dryness aids in obtaining the maximum possible signal intensity.

5.1.4.5 Scanning and image acquisition

The slide was scanned using the GenePix ® Personal 4100A microarray scanner (Axon Instruments). Preview scans were carried out while manually adjusting the photomultiplier tube (PMT) gains for each dye. A final scan achieving a satisfactory

count ratio of (1 ± 0.1) was saved. This ensures equal visibility of the two dyes, in addition to ensuring a satisfactory signal-noise ratio greater than 10:1. Preview scans were kept to a minimum to avoid excessive exposure of the dyes to laser emissions.

It was sequentially scanned by a red coloured laser at 635nm which excites the Cy5 dye, followed by a green coloured laser at 532nm which excites the Cy3 dye. A final image was then created from these two, and saved in tagged image file format (TIFF). The image was acquired using GenePix™Pro software (version 4.1). The image was gridded and linked to a protein list (#P9624, Sigma Aldrich). This links each spot to a corresponding protein name and number. A feature manipulation tool was used to correctly align and size each spot. For example, if a spot had been missed by the software, it can be manually flagged for detection. Results depicting each feature, its protein name, dye intensities, and the ratio of the dyes were obtained and saved as GenePix Results Format (Gpr) file.

5.1.4.6 Data Analysis

Data were imported into and further analysis carried out using Acuity™ software (version 4.0). The data were first normalized using the non-linear LOWESS (locally weighted scatter plot smoother) normalization method (Smith et al. 2006). This method aims to normalize bias resulting from chemical characteristics of the two dyes causing inherent variation in intensity and fluorescence. It is also known that dyes fluoresce differently at different levels; this is secondary to dye molecules absorbing light from each other when in closer proximity, thus decreasing the emitted signal (Yang et al. 2001). For each spot, the intensity of each dye was measured separately, and the ratio of these was normalized. Lowess log ratios were calculated and utilised to produce a new dataset with the Lowess-corrected ratios of Cy5/Cy3 (635/532).

The median of individual pixels was measured for each spot. The log ratio of the two medians was calculated for each spot (Log ratio 635/532). These intensity ratios were then normalized using the Lowess normalization method (M Lowess log ratio 635/532). A final ratio of the average of the two spots was then calculated.

Predefined inclusion criteria were entered as software commands, and used to identify spots of superior quality (Smith et al. 2006). Only such spots were further analyzed for differential protein expression. These criteria included: spots with a small percentage of saturated pixels (<3%), spots with a relatively uniform intensity and uniform background, and spots that were detected above background.

Manual manipulation was performed to align the spots within the circular indicators. The diameter of the indicators is altered to fit exactly around the feature spot. It is crucial to demarcate the separating line between the feature spot and background. Future analysis calculates the mean and median pixel intensity of the dyes within the boundaries of this indicator. Also, the background intensity is subtracted from the feature intensity to reduce the effect of non-specific fluorescence. This latter could be secondary to slide fluorescence or non-specific binding of the dyes. Changes in excitation or activity levels of the two dyes will then be converted into numerical values. Slides were suitable for analysis only if ≥90% of the substances matched the inclusion criteria. Otherwise they were considered of low quality, and unsuitable for further analysis.

A fold change – increase or decrease – in each protein level was thus calculated. Proteins demonstrating an absolute log ratio (635nm/532nm) value of ≥1.0/-1.0 (representing a ≥2.0-fold change in expression) were deemed of significant differential expression.

5.2 Results

5.2.1 Cell culture

Cell culture was carried out successfully. Figure 5.1 shows MCF-7 cells and their corresponding radioresistant derivative cell line before harvesting. The RR cells showed slower growth, and took a longer period to attain the same quantitiy. The parent, rapidly growing cells were therefore re-cultured to allow simultaneous protein extraction.

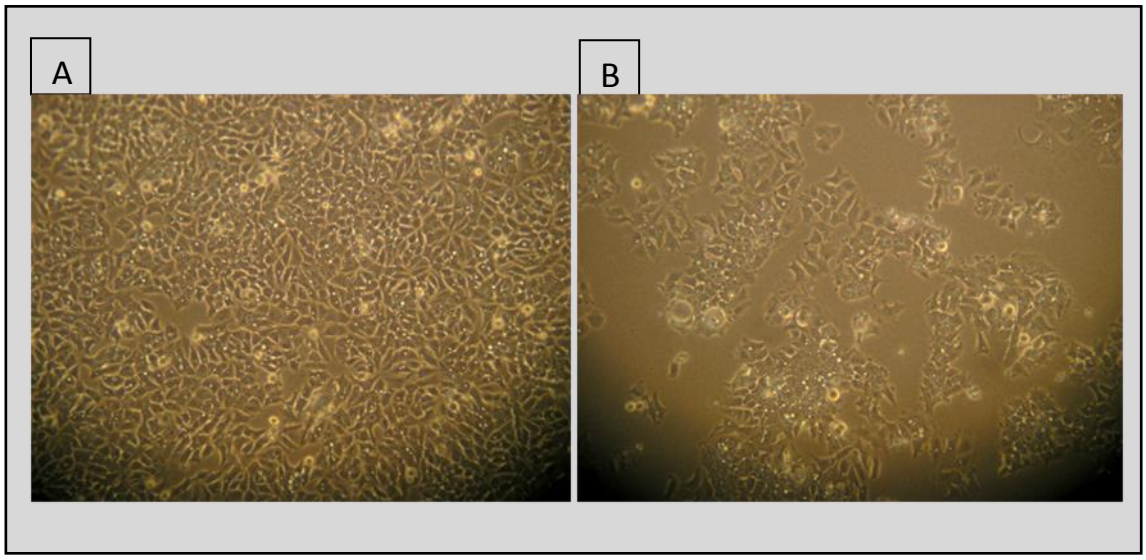

Figure 5.1 Images of MCF7 and MCF7RR cell culture results. A: shows MCF-7 parent cell lines immediately before cell harvest. B: shows the derivative radioresistant MCF-7RR cells, which were growing at a slower rate.

5.2.2 Antibody Microarray

The Panorama AbMA XPRESS Profiler725 Kit protocol was followed as detailed above for five cell lines; MCF-7, MDA--MB-231, T47-D, PJ41 and PJ49. The final quantification results, labelling, dye-protein ratio determination and slide PMT gains of all five cell lines and derivative sublines are shown in Appendix A. MCF7/MCF7RR results are given below as an example.

Determination of dye: protein ratio

The labelled samples were diluted in Buffer A to a 1:10 dilution, and their absorbencies and protein concentrations were measured as previously described. All samples achieved D/P ratios more than 2.

- Absorbance of Cy3-PN = 0.649 (at 552 nm) D/P Ratio = 2.53
- Absorbance of Cy5-RR = 1.486 (at 650 nm) D/P Ratio = 3.079

Slide incubation, scanning and analysis was then performed as previously described in the methods section. PMT gains of 270 (635) and 220 (532) were used with a resultant count ratio of 1. Following manual manipulation as previously described, images were obtained at wavelength 635nm using PMT gain of 270, and wavelength 532nm using PMT gain of 220 (count ratio= 1). The MCF7/MCF7RR slide showed evidence of residual water marks at the bottom left hand corner (Figure 5.2). This however did not interfere with the final analysis, as the final image is produced through higher resolution scanning of the demarcated area (the area within the red box in figure 5.2). All other slides were of satisfactory quality.

The overall arrangement of the AbMA slide is shown in figures 5.3 and 5.4. The slide consists of 32 sub-arrays. The orientation of the slide can be determined using the positive controls, which appear as two yellow spots on the lower right corner of each sub-array. Slide orientation is crucial to the interpretation of protein significance, and linking spots to their respective proteins. Each sub-array consists of 48 spots or features, representing 24 pairs of proteins, positive controls and negative controls.

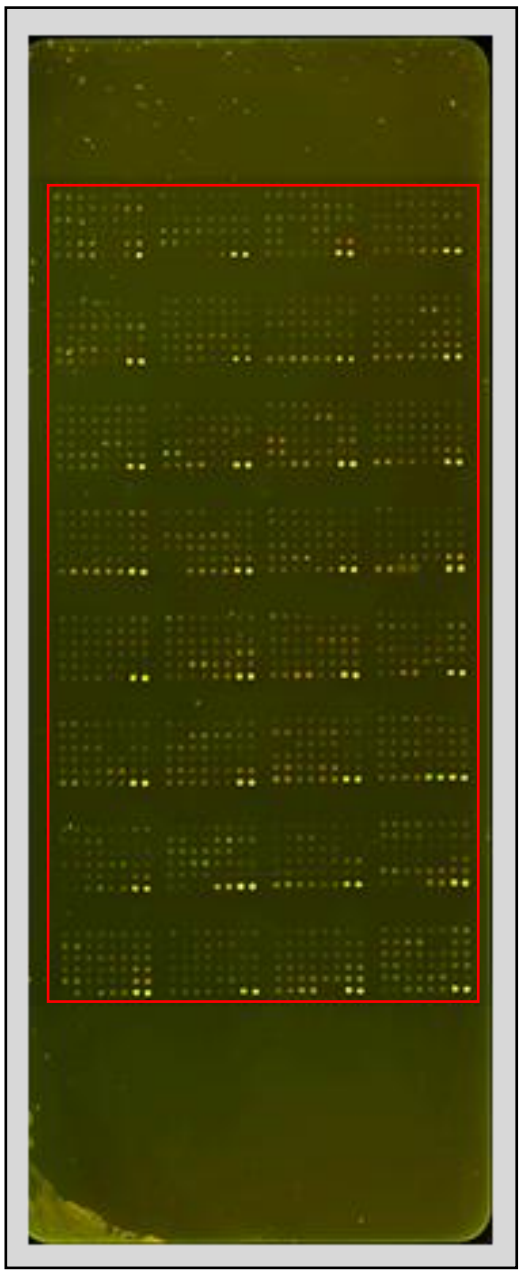

Figure 5.2 MCF7/MCF7RR Antibody microarray slide. The red rectangle demarcates the part of the slide that constituted the final image following high resolution scanning.

The positive controls comprise equal concentrations of monoclonal antibodies to Cy3 dye and Cy5 dye. As these should theoretically bind equal amounts of the two dyes, one would expect equal amounts of red and green giving a resultant yellow colour. They act as positive controls as they bind no proteins from the sample lysate.

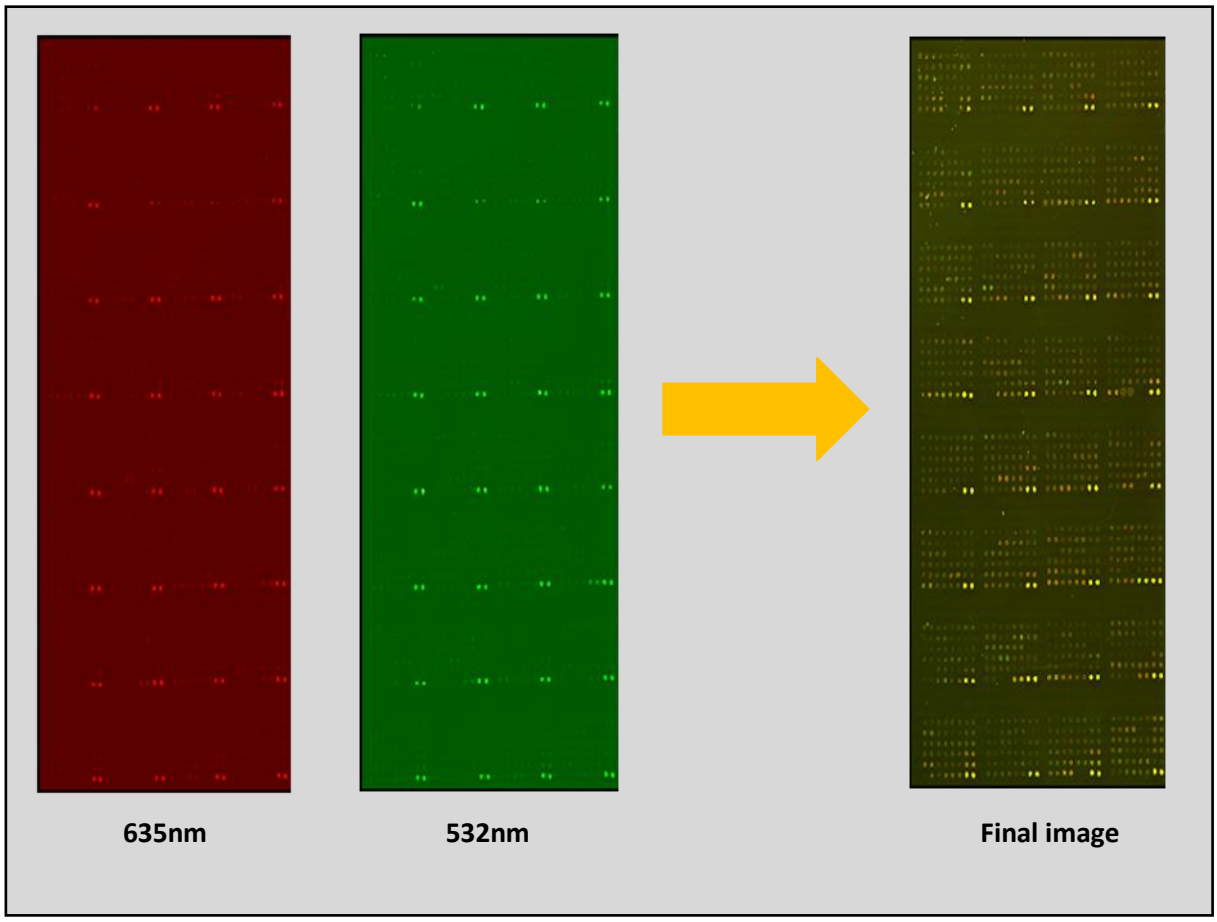

Figure 5.3: The final antibody microarray slide image was created using two preliminary images at two different wavelengths. The slide was scanned using the GenePix ® 4100A scanner. It was first scanned using a red colour laser at 635 nm to excite the Cy5 fluresecent dye. It was sequentially scanned by a green colour laser to excite the Cy3 dye (532nm). The two images were superimposed to create the final image, which was used for analysis. Image acquisition was achieved using GenePix™Pro (version 4.1) software.

The negative controls comprise spotted un-labelled BSA. Therefore, no binding is possible with either dyes or sample proteins, and the final result is a blank colourless spot (Figure 5.4).

The red and green spots reflect the competitive hybridization that is the focal characteristic of the antibody microarray. A protein labelled with the red Cy5 dye and existing in relative abundance in the RR sample will give rise to a red spot, as more red dye creates higher activity. Similarly, if it exists in relative scarcity, a green spot will result.

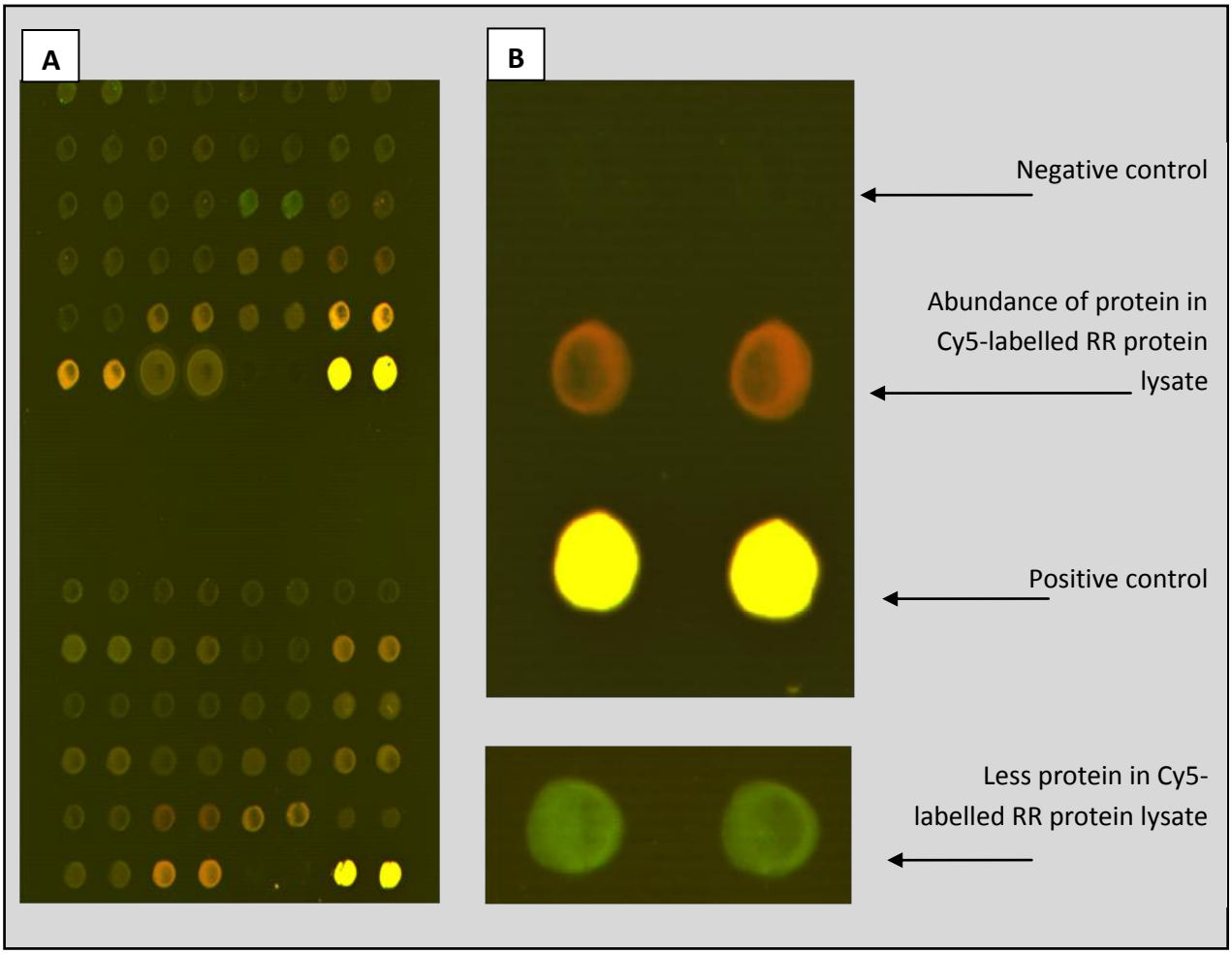

Figure 5.4 The arrangement of the XPRESS Profiler 725 microarray slide. A: Slide and block orientation. Two blocks out of a total of 32 blocks, each with a pair of positive control spots (always on right bottom corner for orientation), a variable number of negative control pairs, and duplicate antibody spots. B: Colour representation. A positive control spot contains equal amounts of anti-Cy3 and anti-Cy5, therefore should be a uniform yellow colour. A negative control spot contains bovine serum albumin, therefore appears blank as there is no binding to protein lysate.

As discussed previously, only spots satisfying certain criteria are included in the analysis. Features with poor qualities are excluded from the analysis (Figure 5.5).

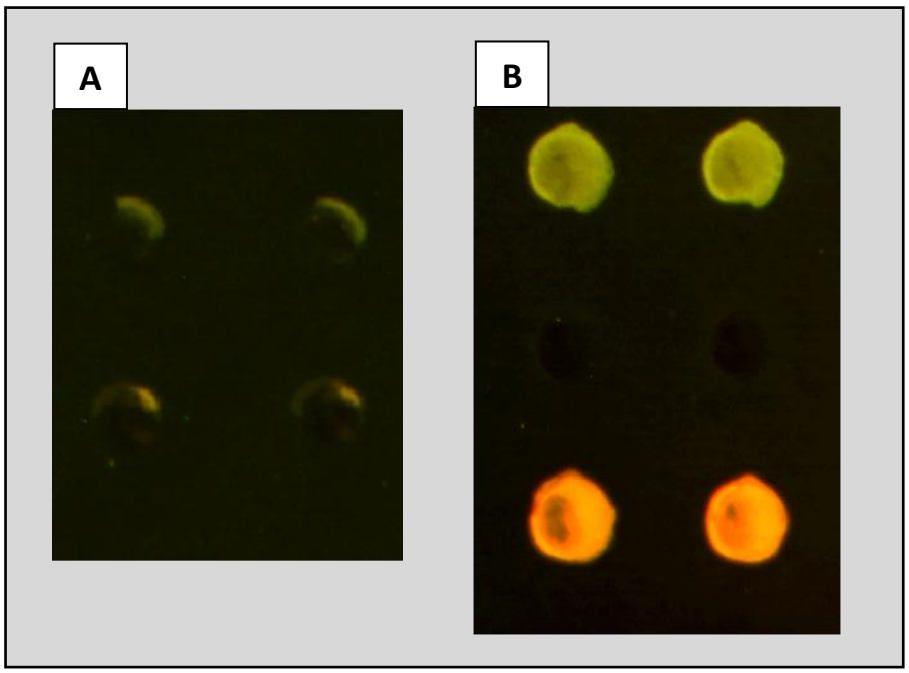

Figure 5.5 A "poor quality spot" on the XPRESS Profiler 725 microarray slide. A: The spots lack uniform dye intensity through the existing area, and the difference between the features and the background signal is minimal. These spots were/were not included in the analysis (check the corresponding numbers). B: Comparison is made with brighter more uniform spots.

The Lowess log ratio of each analysed protein is then calculated. An example, (DR4 protein) is shown in Figure 5.6.

The ratio was converted into a fold change, using the formula: $Y=\log_2 x$. Therefore $x= 2^y$, where y is the log ratio. For example: for a log ratio of 1.719, fold change = $2^{1.719}$ = 3.29. Only log ratios of +1 or -1, corresponding to a ≥2 fold change were considered as significant.

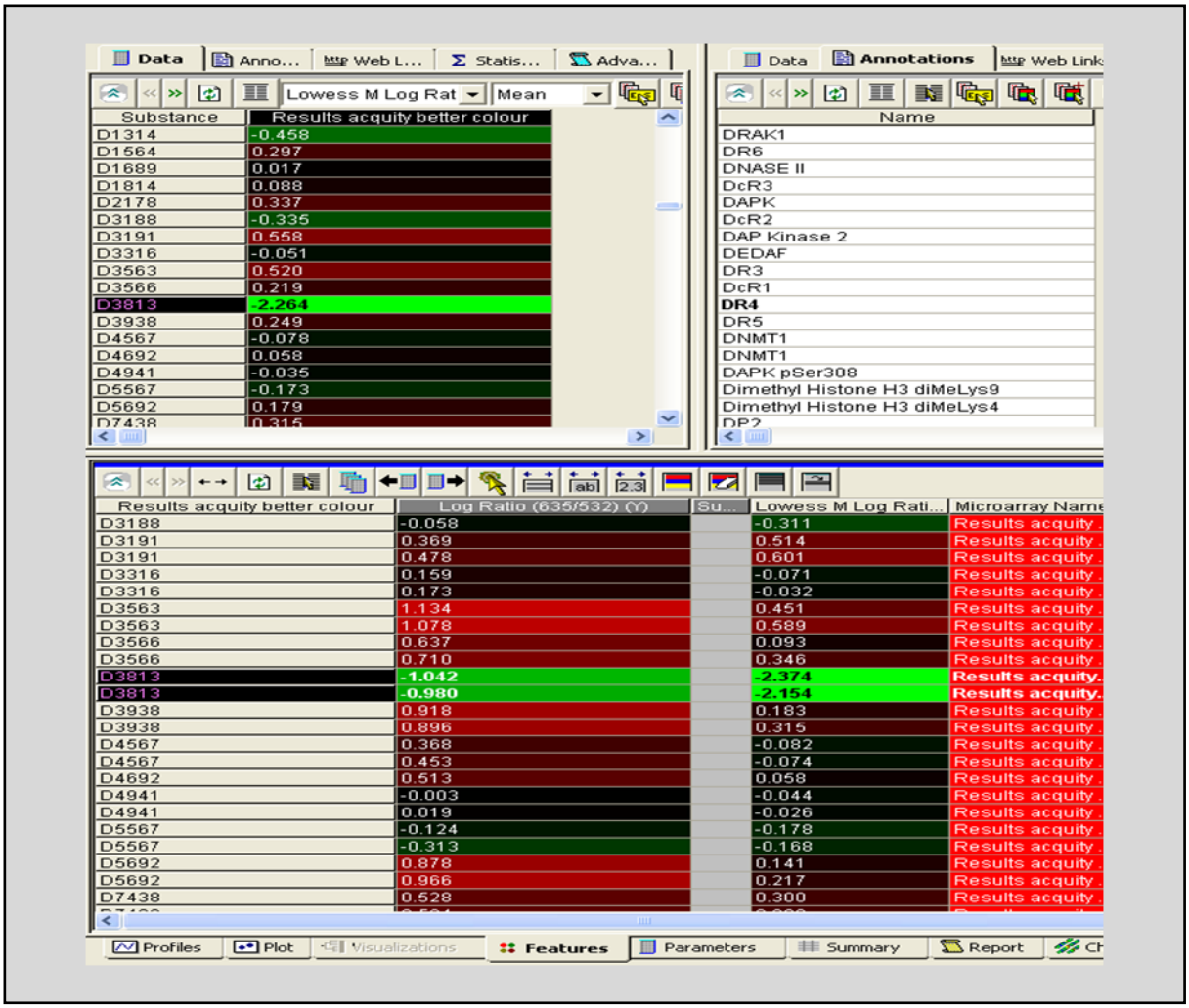

Figure 5.6 An example of the Acquity software data analysis tool. The figure shows part of the data analysis as it appeared on the Acquity software screen. The target protein DR4 (No.D3813) is highlighted and is shown with the two dye intensity log ratios before and after Lowess normalization. The top half of the screen shows the final ratio, which is the average of the two values obtained from two duplicate spots.

5.2.3 AbMA spot images

As discussed previously, the antigen-antibody reactions on the AbMA slide are visualised as spots of red or green colour, with varying intensities. Specialised software will assess those intensities to determine the differential protein expression in the RR sample relative to the radiosensitive or parent sample. Examples of spot images are given in Figure 5.7 and 5.8. A list of all the differentially expressed proteins is provided in Tables 5.1 and 5.2. Importin-α was the most differentially expressed protein, having

shown differential expression across two breast cancer and two oral cancer cell lines. GFI-1 was differentially expressed across all three breast cancer cell lines. The green coloured spots would indicate that these proteins were differentially expressed in the radioresistant sublines. The direction of change was not taken into account at this stage of the experiment. This will be further discussed in section 5.3.

Figure 5.7 shows the protein DR4, in MCF7 and MDA cell lines. It was found to be differentially expressed only in the MCF-7 subline, with a fold change of 4.1. The differences in intensity of the green cyanine dye can be very subtle to the eye, but quite significant when considered through the analysing software.

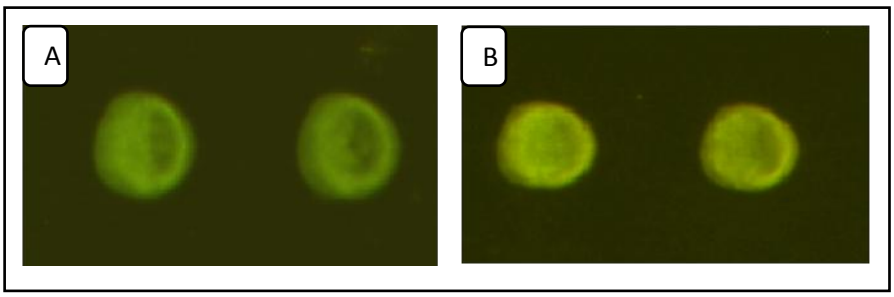

Figure 5.7 DR4 spots in MCF7 and MDA AbMA slides. A: DR4 in MCF7 (fold change 4.1), B: DR4 in MDA (fold change 1.6) The latter did not reach the significant fold change, however showed an upward trend. The visual differences are very subtle, and computer analysis is required to determine significance.

Further examples of significantly differentially expressed proteins are provided in Figure 5.8.

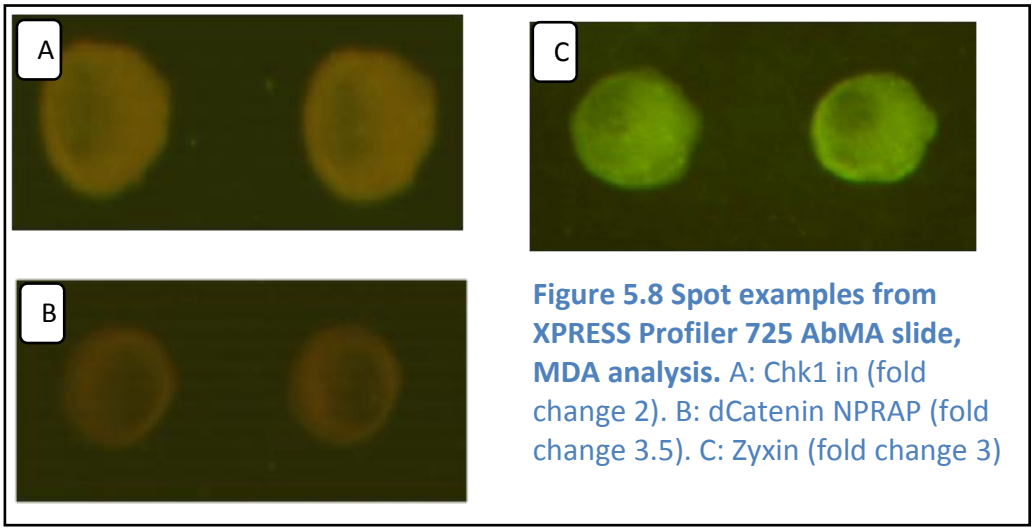

Figure 5.8 Spot examples from XPRESS Profiler 725 AbMA slide, MDA analysis. A: Chk1 in (fold change 2). B: dCatenin NPRAP (fold change 3.5). C: Zyxin (fold change 3)

5.2.4 Differentially expressed proteins in breast cancer radioresistant sublines

Antibody microarray technology was performed to identify proteins that were differentially expressed in, and therefore associated with, RR cancer sublines when compared to their parent cell lines (MCF-7, MDA-MB-231 and T47D). A significant difference in protein expression was defined as a fold change of ≥ 2. A total of 43 proteins showed differential expression in at least one of the three RR breast cancer sublines (Tables 5.1 and 5.2). Two of these proteins, GFI-1 and Zyxin, were differentially expressed in all three RR sublines. Seven proteins were differentially expressed in 2 of the 3 RR sublines (Table 5.1).

Table 5.1: Differentially expressed proteins in radioresistant breast cancer sublines compared to parent cell lines, using AbMA - targets differentially expressed in more than one subline . Nine proteins demonstrated a ≥2 fold differential expression between parent and radioresistant cell lines, across more than one cell line. Green arrows denote down-regulation, while the red arrows indicate up-regulation in the radioresistant protein lysate. ns= non significant fold change <2. MCF-7RR = radioresistant MCF-7 cell line. T47DRR = radioresistant T47D cell line. MDARR = radioresistant MDA-MB-231 cell line.

	Protein name	**Antibody no.**	MCF-7**RR**	T47D**RR**	MDA**RR**
1	**GFI-1**	G667	**2.5 ↓**	**3.6 ↓**	**2.4 ↓**
2	**Zyxin**	Z0377	**3.3 ↓**	**3.5 ↓**	**3.0 ↓**
3	**DR4**	D3813	**4.1 ↓**	**5.1 ↓**	ns
4	**Importin a1**	19658	**3.5 ↓**	**2.1 ↓**	ns
5	**SynCAM**	S4945	**2.2 ↓**	**2.6 ↓**	ns
6	**PIASx**	P9498	ns	**2.7 ↓**	**2.3 ↓**
7	**Spred2**	S7320	**2.1 ↓**	ns	**2.1 ↓**
8	**GRK2**	G7670	**2.3 ↓**	ns	**2.0 ↓**
9	**dCatenin NPRAP**	C4864	**2.3 ↑**	ns	**3.5 ↑**

Table 5.2 Differentially expressed proteins in radioresistant breast cancer sublines compared to parent cell lines, as determined by AbMA - targets differentially expressed in a single subline. 34 proteins showed differential expression in a single radioresistant subline when compared to a parent cell line. Green arrows denote downregulation in the radioresistant cell line derivative, while red arrows indicate upregulation. ns = not significant fold change <2.

	Protein name	Antibody no	MCF7**RR**	T47D**RR**	MDA**RR**
10	hnRNPU	R6278	3.2 ↓	ns	ns
11	CaM Kinase IIa	C6974	2.6 ↓	ns	ns
12	PTEN	P7482	2.2 ↓	ns	ns
13	Tubulin Tyrosine	T9028	2.2 ↓	ns	ns
14	Protein Kinase C PKC	P5704	2.0 ↓	ns	ns
15	MDMX	M0445	2.2 ↑	ns	ns
16	MADD	M5683	2.1 ↑	ns	ns
17	PERP	P5243	2.1 ↑	ns	ns
18	NCadherin	C2542	2.1 ↑	ns	ns
19	bCOP	G6160	2.1 ↑	ns	ns
20	bTubulin	T5201	2.0 ↑	ns	ns
21	Caspase13	C8854	2.0 ↑	ns	ns
22	MyD88	M9934	2.0 ↑	ns	ns
23	ARNO	A4721	ns	2.2 ↓	ns
24	BUB1	B0561	ns	2.3 ↓	ns
25	Bcl10	B7806	ns	2.2 ↓	ns
26	MSH6	M2820	ns	2.1 ↓	ns
27	SKM1	S9568	ns	2.1 ↓	ns
28	SUV39H1 Histone Methyl Transferase	S8316	ns	2.0 ↓	ns
29	gParvin	P5746	ns	2.6 ↓	ns
30	Siah2	S7945	ns	2.2 ↑	ns
31	RAIDD	R9775	ns	2.6 ↑	ns
32	RAIDD	R5275	ns	2.4 ↑	ns

33	Cytokeratin 8 12	C7034	ns	**2.4 ↑**	ns
34	Epidermal growth factor	E2520	ns	**2.0 ↑**	ns
35	MAP Kinase 2 ERK	M7431	ns	**3.3 ↑**	ns
36	Proliferating Cell Protein Ki67	P6834	ns	**2.5 ↑**	ns
37	APRIL	A1851	ns	**2.1 ↑**	ns
38	Melanocortin3 Receptor	M4937	ns	**2.9 ↑**	ns
39	Tryptophane Hydroxylase	T0678	ns	**2.2 ↑**	ns
40	TRAIL	T3067	ns	**2.4 ↑**	ns
41	IAfadin	A0349	ns	ns	**2.2 ↓**
42	ARP3	A5979	ns	ns	**2.1 ↑**
43	Chk1	C9358	ns	ns	**2.0 ↑**

5.2.5 Differentially expressed proteins in oral cancer radioresistant sublines

A significant difference in protein expression was defined as a fold change of ≥ 2. A total of 21 proteins showed differential expression in at least one of the two oral cancer radioresistant sublines when compared with their respective parent cell lines. In addition, Importin α3 , Annexin VII and BID proteins were differentially expressed in both sublines (Table 5.3).

The protein target Importin α has shown differential expression across both breast and oral cancer cell lines (Table 5.4). Importin α1 showed differential expression (down-regulation) in breast cancer cell sublines MCF-7RR and T47DRR. Its isomer, importin α3 has shown differential expression (down-regulation) in the oral cancer sublines PJ49RR and PJ41RR subline.

Table 5.3 Differentially expressed proteins in radioresistant oral cancer sublines compared to parent cell lines, as determined by AbMA. Twenty one proteins demonstrated a ≥2 fold differential expression between parent and radioresistant sublines, three of which were across both sublines. Green arrows denote down-regulation, while the red arrows indicate up-regulation in the radioresistant protein lysate. ns= non significant fold change <2.

	Protein name	Antibody no.	PJ41 RR	PJ49 RR
1	**Annexin VII**	A4475	3.1 ↑	3.3↑
2	**BID**	B3183	2.3 ↑	2.2↑
3	**Importin a3**	19783	2.7 ↓	2.2 ↓
4	BTK	B0686	2.1 ↓	ns
5	BUBR1	B9310	2.2 ↓	ns
6	Chondroitin sulphate	C8035	2.3 ↑	ns
7	DAP kinase 2	D3191	3.9 ↑	ns
8	TWEAK receptor	T9700	2.1 ↓	ns
9	Serine threonine protein phosphatase 2ABg	P8359	2.3 ↓	ns
10	Serine threonine protein phosphatase 1b	P7484	4.1 ↓	ns
11	Serine threonine protein phosphatase 1g1	P7609	2.5 ↓	ns
12	hBRM hSNF2a	H9787	ns	2.0 ↓
13	IKKA	I6139	ns	2.3 ↑
14	MTBP	M3566	ns	2.0 ↑
15	Nitric oxide synthase bNOS	N7155	ns	3.6 ↓
16	Nerve growth factor b		ns	2.3 ↓
17	P14 arf	P2610	ns	2.3 ↑
18	Protein kinase cb2	P3203	ns	2.3 ↑
19	Proliferating cell nuclear antigen	P8825	ns	2.3 ↓
20	Rad 17	R8029	ns	2.2 ↓
21	SGK	85188	ns	2.3 ↑

Table 5.4 Differentially expressed proteins associated with radioresistance in five cancer sublines (MCF7RR, MDARR, T47DRR, PJ41RR and PJ49RR), as detected using AbMA. Combining the breast and oral cancer radioresistant sublines, eleven proteins in total were differentially expressed across more than one cancer subline. Importin alpha was the only protein that showed differential expression across both breast and oral cancer cell lines. ns=non significant difference in protein expression. Green arrows indicate down regulation in the radioresistant sublines, red arrows indicate up-regulation in the radioresistant sublines.

Protein name	Antibody no	PJ41 RR	PJ49 RR	MCF7 RR	T47D RR	MDA RR
Annexin VII	A4475	↑	↑	ns	ns	ns
BID	B3183	↑	↑	ns	ns	ns
Importin α 3	19783	↓	↓	ns	ns	ns
Importin α 1	19658	ns	ns	↓	↓	ns
GFI-1	G667	ns	ns	↓	↓	↓
Zyxin	Z0377	ns	ns	↓	↓	↓
DR4	D3813	ns	ns	↓	↓	ns
SynCAM	S4945	ns	ns	↓	↓	ns
PIASX	P9498	ns	ns	ns	↓	↓
Spred2	S7320	ns	ns	↓	ns	↓
GRK2	G7670	ns	ns	↓	ns	↓
dCateninNPRAP	C4864	ns	ns	↑	ns	↑

5.3 Discussion

The AbMA technology was successfully utilised to detect a number of differentially expressed proteins associated with RR breast and oral cancer sublines. AbMA was used to compare protein expressions between five parent cancer cell lines, and their RR derivatives. A total of 63 proteins were detected across all five cancer cell lines, with some proteins showing differential expression across multiple cell lines.

The technique was utilised while satisfying all the recommended precautions to ensure accurate results, e.g. dye-protein ratio >2, and high percentage of high quality slide spots. Every AbMA experiment is designed to compare two protein lysates, and provide information regarding individual protein differential expression, significance of differential expression and the direction of change, i.e. up-regulation or down-regulation. The significance of differential expression is determined by the cut-off of less, or greater than 2 fold difference. This is the customary cut off value for significance used as standard in cDNA microarrays, and is common for data analysis with commercial packages (Akopyants et al. 2004;Meiklejohn and Townsend 2005). Kanda et al conducted a comparative study between exploratory and statistical analysis of differential gene expression in cDNA microarrays, and concluded that the two-fold threshold produced a satisfactory representation of significance (Kanda K et al. 2012). Pin et al compared Lowess normalization and two fold cut off criterion with two other methods of significance on experimental datasets with prior knowledge of gene expression (Pin and Reuter 2007). Of three recent studies which utilised the Antibody Microarray-XPRESS Profiler725 kit, three different levels of cut off were used; more than 1.5, 2 and 2.5 (Mohri et al. 2009b;Uemura et al. 2009b;Wu et al. 2010). The number of differentially expressed proteins was 17, 24 and 28 respectively.

Information supplied by Sigma-Aldrich suggested that fold changes below 2 may be regarded as significant and confirmatory tests employed, especially if supported by relevant known protein functions and associations. Indeed, Hodgkinson et al compared 13 different AbMA experimental results, and noticed that some of the repeatedly differentially expressed proteins across multiple cell lines/tissues did not reach the 2-fold cut-off (Hodgkinson et al. 2011). They were however very close to that level. A cut-off of 1.8 was proposed, to ensure that proteins which might prove significant following further confirmation were not overlooked in the discovery phase. In this work, results are presented with a 2-fold cut-off as this has provided a satisfactory number of proteins to take forward. Further analysis of the AbMA data base of the five cancer lines using the lower cut-off of 1.8 may produce more differentially expressed proteins.

The AbMA technology is also designed to determine the direction of differential expression; up-regulation *vs.* down-regulation. The direction of change can be confirmed by repeating the experiment using a dye swap technique. This involves the application of the green cyanine dye to the parent sample and the red dye to the radioresistant sample, then repeating the experiment with the two dyes swapped over. Here, the direction of change is presented as reported by a single experiment, and confirmation is performed through western blotting. Repeating the AbMA using the dye-swap has the potential of consuming higher volumes of protein lysates. In addition, it has significant monetary implications; the cost of one AbMA slide runs at £1200-1300.

AbMA is a high throughput screening tool, and further confirmatory tests are required. Some of the proteins were differentially expressed in multiple cell lines, which

strengthen their position to be taken forward for further confirmatory tests. Zyxin and GFI-1 are such proteins, both having shown differential expression along all three breast cancer cell lines. However, the frequency of differential expression needs to be considered carefully. In our previous work, we have reported a number of proteins showing differential expression in AbMA experiments of different and unrelated protein samples; repeatedly identified differentially expressed proteins (RIDEP) (Hodgkinson et al. 2011). These proteins may thus be a reflection of a cellular stress response, and may not indicate a true association with RR. Zyxin was the most frequently identified RIDEP. Conversely, Zyxin has a known function in maintaining cellular adhesion, and loss of adhesion has been suggested as a stimulus for apoptosis. A recent study by Hervy et al has suggested a definite role for Zyxin in promoting apoptosis (Hervy et al. 2010). Wild type fibroblasts and fibroblasts lacking the zyxin gene were exposed to UV-C irradiation as a stimulus for apoptosis. 16% of the wild type cells survived compared to 27% of the second group. Zyxin has been linked to promoting apoptosis through its close association with CARP-1 (cell cycle and apoptosis regulator protein-1). The latter is known as a zyxin binding protein. In addition, CARP-1 is related to DBC-1 (deleted in breast cancer 1), which is important for promoting apoptosis through the intrinsic pathway, and which is known to be a modulator of BRCA1 function (Hiraike et al. 2010). Therefore, down regulation of Zyxin could disrupt the apoptotic process and contribute to RR. We therefore decided to include zyxin in confirmatory tests using WB and IHC.

The second protein showing differential expression in three breast cancer cell lines was GFI-1. GFI-1 has been reported to contribute to increased cellular proliferation and inhibition of apoptosis. Thus down-regulation, as reported in our AbMA results, might

not directly explain the association with RR. Using AbMA alone, the direction of differential expression should ideally be interpreted after using the dye swap technique. However, as discussed above, this will instead be determined using further confirmatory tests; WB and IHC.

Known protein functions were also taken into consideration in selecting proteins for further analysis. DR4 is a known downstream molecule of TRAIL pathway, and plays an important role in the extrinsic apoptosis pathway. DR4 has shown differential expression in two breast cancer cell lines. Down-regulation of this molecule could therefore be implicated in cell survival and radioresistance.

PIASx is less well known, however it has been suggested to play a role in cellular proliferation and survival, in addition to post translational modifications of proteins through sumoylation.

Importin-α has a well documented role in protein transport and haemostasis. It was the only protein that showed differential expression across both breast and oral cancer cell lines, and was therefore considered an attractive candidate for further confirmatory tests (chapters VI and VII).

Chapter VI

Western blot;

Methods & Results

Chapter Aim:

To utilise the western blot technique and cancer cell lines in confirming the association between radioresistance and previously identified potential biomarkers

Western blot; Methods & Results

6.1 Introduction

The AbMA technology is a useful screening tool that identifies differential expression in a large number of putative markers. However, there is a chance of false positives, and further confirmation is required. In addition, as discussed previously, the direction of differential expression of the individual proteins in the RR sublines needs to be ascertained. WB is a widely tested technique that can be used in the second stage of the biomarker discovery pipeline (chapter IV). We utilised the WB technique to further examine data obtained from AbMA assays of three breast cancer cell lines and their RR sublines. Target proteins were chosen for confirmation based on the frequency by which they were differentially expressed across the different cell lines in the AbMA experiments, and secondarily based on possible functions related to radioresponsiveness in signalling pathways (section 6.2). A fold change of ≥2 indicated significant differential expression. In addition, those showing significant differential expression in more than one cell line were considered better candidates for confirmation than proteins which were differentially expressed in one cell line only. Proteins whose known role in cell signalling could clearly explain their role in RR were also considered for confirmation. Proteins chosen for WB confirmation are shown in Table 6.1 along with their AbMA expression results.

Table 6.1 Proteins chosen for western blot analysis. Proteins which showed differential expression in the radioresistant breast and oral cancer sublines (MCF7, MDA, T47D, PJ41 and PJ49) using AbMA technology were identified as potential biomarkers of radioresistance. Following further data examination, zyxin, PIASx, DR4, GFI-1, Spred2 and KPNA2 were chosen for further confirmation using western blot based on the frequency of their differential expression in different cell lines, and their known functions and protein associations.

	AbMA		
	MCF7 **RR**	MDA **RR**	T47D **RR**
KPNA2	↓3.5	Ns	↓2.1
GFI-1	↓2.5	↓2.4	↓3.6
Zyxin	↓3.3	↓3.0	↓3.5
PIASx	ns	↓2.3	↓2.7
DR4	↓4.1	Ns	↓5.1
Spred2	↓2.1	↓2.1	ns

6.2 Proteins selected for western blot confirmation

Importin-α (KPNA2)

Importin-α (KPNA2) is a protein found both in the cytoplasm and in the nucleus. It recognizes NLS (nuclear localization signal) sequence within a protein which is to be transported within the cell (Kohler et al. 1999). It facilitates the interaction between the target protein and importin-β, which then acts in transporting the protein across the nuclear membrane (through nuclear pore complexes). Numerous proteins required for cell cycle regulation are dependent on this process (e.g. cyclins and p53). Importin-α is expressed in most human tissues (Dahl et al. 2006). Expression in normal breast tissue was variable and often absent; from 2% (Dahl et al. 2006) to 12% (Dankof

et al. 2007). IHC studies of breast cancer tissue showed Importin-α in 56% (Dahl et al. 2006), 40% (Gluz et al. 2008) and 30% (Dankof et al. 2007) of cases, predominantly in the nucleus. DCIS was found to express more Importin-α than normal breast tissue (Dankof et al. 2007). It has also been suggested that it is associated with the basal subtype of breast cancer (Gluz et al. 2008). Importin-α was significantly associated with poor prognostic parameters such as higher tumour grade, +ve HER-2 status, -ve ER/PR status, Ki67 (Dahl et al. 2006;Dankof et al. 2007;Gluz et al. 2008). It was shown to be a negative independent predictor of OS and DFS in breast cancer patients (Dahl et al. 2006). Preliminary studies in yeast have suggested that mutations in importin-α were associated with G2 arrest and failure to progress to mitosis (Loeb et al. 1995). More recent evidence suggests an association with G1/ S phase delay (Pulliam et al. 2009). Cell cycle disruption could be secondary to transport failure or yet unidentified mechanisms. It does however confer more time for DNA repair and could therefore contribute to RR. Importin-α was differentially expressed in two breast cancer sublines. In addition it was the only protein to be differentially expressed in the two oral cancer sublines as well. It was therefore felt to be an important contender to be a marker of RR.

GFI-1

GFI-1 (growth factor independent 1) is a proto-oncogene that is known to bind to DNA and inhibit transcription. Expression of GFI-1 has been extensively studied in haematopoietic tissues, especially thymus tissue, neural tissue, gut epithelium, neuroendocrine lung cancer cells, spleen and testis (Jafar-Nejad and Bellen 2004;Wallis et al. 2003). GFI-1promotes cellular proliferation in a number of ways:

a) GFI-1 inhibits apoptosis, possibly through inhibiting transcription of pro-apoptotic factors (Jafar-Nejad & Bellen 2004).

b) Of interest is the documented relationship between GFI-1 and PIAS-3 (further discussed under PIASx below). PIAS-3 exerts an inhibitory effect on STAT3. Phosphorylated STAT3 is known to activate oncogenesis (proliferation, angiogenesis, and invasion). In vitro preliminary studies have linked STAT3 to chemoresistance in multiple myeloma (Bharti et al. 2004), and inherent RR in lymphoma cells (Otero et al. 2006). It has been shown from in vitro studies that GFI-1 interacts with PIAS-3 and inhibits its blocking effect on STAT3, thus increasing STAT3 mediated transcription and possibly proliferation (Rodel et al. 2000).

c) GFI-1 has been shown to interfere with the G1 arrest/checkpoint in T-cells, thus resulting in un-inhibited proliferation (Karsunky et al. 2002). In the context of radiotherapy and resistance, down-regulation of GFI-1 may thus allow G1 arrest and time for DNA repair; with resultant increased cell survival after RT.

Considering its functions in survival and apoptosis, and multiple protein interactions, GFI-1 would constitute a suitable candidate for western blot confirmation.

Zyxin

Zyxin is a phospho-protein which belongs to the LIM domain protein family. It has been shown to exist in both the nucleus and the cytoplasm and to function in signal transduction (Nix and Beckerle 1997). Through its close association with actin filaments and α-actinin, Zyxin has been implicated in the regulation of cell adhesion, motility and mitosis (Crawford et al. 1992). Zyxin has been proposed as an apoptotic promoting protein (section 5.3), and down regulation may thus be associated with an undesired

cancer cell survival. In addition, several proteins belonging to the LIM domain family have been associated with other aspects of carcinogenesis. The over-expression of TRIP6 (thyroid receptor interacting protein 6), another member of the LIM family of proteins, has been associated with cancer invasiveness (Chastre et al. 2009). Though zyxin was identified as a RIDEP, and although its differential expression could therefore reflect a stress response, it had logical and well grounded associations with carcinogenesis that we felt it reasonable to further assess its relationship with RR. It was therefore chosen for WB confirmation.

DR4

DR4 is located on the cell surface and forms part of the receptor mechanism for the TRAIL ligand (Figure 2.3), which is responsible for the extrinsic pathway. This causes activation of caspase 8, caspase 3, and finally resulting in DNA cleavage and cell death. DR4 has been detected in breast cancer tissue samples using IHC (Ganten et al. 2009). In this study DR4 showed positive correlation with positive prognostic factors such as positive ER status, well differentiated tumours and negative lymph node status (Ganten et al. 2009).

A similar study in colon cancer showed DR4 expression in all cases, with 78% of cases showing high expression (Strater et al. 2002). Similarly, higher expression was associated with a better prognosis. Levels of DR4 and DR5 were shown to be increased in osteosarcoma cell lines following 10-20Gy of irradiation (Hori et al. 2009). However, a similar study showed elevation of DR5 but not DR4 following the irradiation of MCF-7 cells with 8Gy; DR4 remained constant throughout (Chinnaiyan et al. 2000). Doses of 40Gy have not previously been tested. Down-regulation of DR4 can decrease stimulation of the extrinsic pathway, leading to survival of cancer cells and therefore

resistance to therapy. DR4 was differentially expressed in two of the three breast cancer cell lines, and considering its well documented role in the extrinsic apoptotic pathway, it was chosen for further confirmatory testing using WB.

PIASx

PIAS; protein inhibitor of activated STAT is a nuclear protein (Arora et al. 2003). There are at least 5 members of the PIAS family (Jackson 2001), one of which is PIASx - alternative name PIAS2 - (Arora et al. 2003). The proposed functions of the PIAS protein family include:

a) Prevention of the binding of STATs to DNA, thus regulating transcription (Figure 6.1). PIASx-α and PIASx-β are known to interact with STAT4 to inhibit gene activation (Arora et al. 2003;Jackson 2001). STAT4 has been mainly linked to inflammatory processes. STAT3 is the most studied member of the STAT family; it has been shown to be activated secondary to growth factor and interleukin activation and to result in triggering processes such as malignant transformation, cellular proliferation, angiogenesis and metastasis (Aggarwal et al. 2009). Activation of STAT3 has been implicated in RR of peritoneal B cells in animal experiments (Otero et al. 2006).
b) Regulation of androgen-receptor activity 6.
c) They act as SUMO (small ubiquitin-related modifier) ligases. Sumoylation is a form of post-translational modification; covalent conjugation of a SUMO protein to a target protein. PIASx members thus regulate the modification of many proteins by SUMO. These include p53 (Kotaja et al. 2002), and BRCA1 (Morris et al. 2009). PIASxβ enhances the sumoylation of p53, and in vitro experiments in HeLa cells

have suggested that co-expression of p53, SUMO and PIASxβ was associated with reduction in p53 activity (Schmidt and Muller 2002).

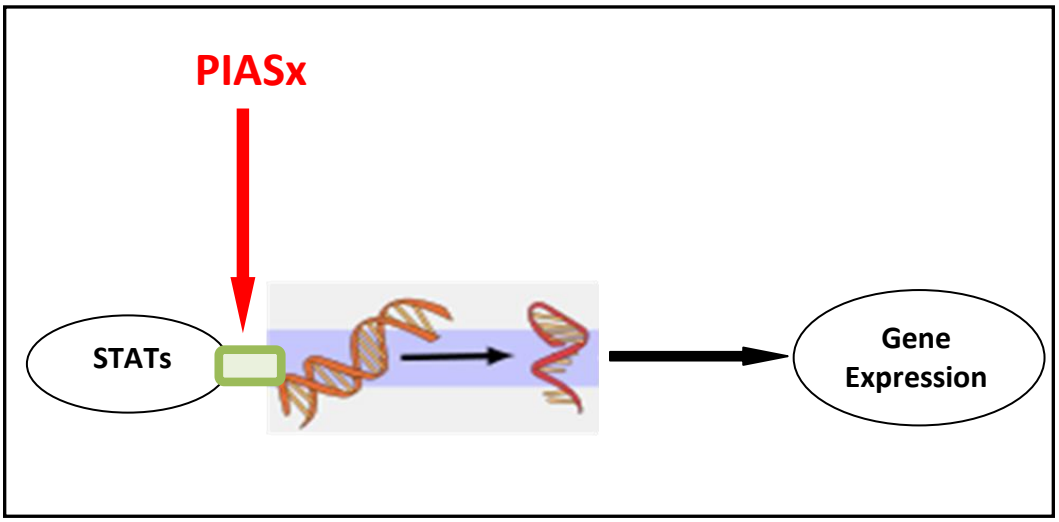

Figure 6.1 STAT related function of PIASx. PIASx inhibits the binding of STAT to DNA, thus inhibiting gene transcription.

Through its effects on sumoylation and mRNA transcription, PIASx can theoretically affect the expression and therefore the function of a large number of proteins, including those involved in cell cycle, signalling and apoptosis.

Spred2

Spred (Sprouty related EVH1 domain) proteins are a family of proteins containing Spred1 and Spred2. They are known to inhibit MAPK and Ras pathways (King et al. 2005). These exist downstream from EGFR pathway, and result in increased transcription and cell proliferation (Figure 3.2). Down-regulation of the inhibitor of these downstream effectors may therefore be a factor in promoting survival of cancer cells. Using AbMA, Spred2 has shown a significant differential expression in the RR sublines, MCF7RR and MDARR.

6.3 WB Methods:

6.3.1 Protein Extraction

For each cell line, cells were simultaneously grown in RPMI 1640 culture media as the cells utilised for the AbMA (sections 5.1.3). One T-75 cm^3 culture flask was sub-cultured into four identical T-75 cm^2 culture flasks, and these were allowed to achieve cellular confluence of 70-80%. Protein was extracted from three flasks for the AbMA experiment, and simultaneously from one flask for WB.

Culture media were removed from the flask, and the cells were washed three times with cold PBS. Five ml of PBS was then added to the flask, and cells were harvested using a mechanical plastic scraper. The suspension was transferred into a 30 ml centrifuge tube. This was centrifuged at 124xg for 3 minutes. The supernatant was discarded. The cell pellet was re-suspended in 1 ml of cold PBS and transferred into a cold microfuge tube. Centrifugation was carried out at 3000 rpm for 3 minutes at 4°C. The supernatant was again discarded and the remaining PBS was completely removed by inverting the microfuge tubes on absorbent paper. This process was then repeated three times. Finally, all PBS was removed and the cells were re-suspended in 150 µl of a mixture of WB extraction buffer – Laemmli buffer – and enzyme inhibitors; protease and phosphatase inhibitors, in addition to β-mercaptoethanol (Appendix C). The following components are added to the extraction buffer on the day:

a. β-mercaptoethanol; this reduces disulphide bonds, thus breaks disrupting quaternary and tertiary protein structures. Combined with heat denaturation, it allows exposure of protein epitopes to facilitate antibody binding.
b. Protease inhibitor enzyme; inhibit protease enzymes, which break down peptide bonds.

c. Phosphatase inhibitor enzyme; inhibit phosphates enzymes which can dephosphorylate phospho-proteins.

The mixture was prepared on the day (Appendix C).The suspension of cells and 150 µl of western buffer were mixed thoroughly using a vortex mixer. 1-5 µl of 10% benzonase (#B8309, Sigma Aldrich) in western buffer was added to the suspension to achieve maximum fluidity and free movement. Benzonase is an endonuclease which can degrade DNA and RNA to reduce sample viscosity. The sample was placed on a vertical 360° slow rotating stand at 4°C for 30 minutes. It was then centrifuged at 14,000 xg for 15 minutes at 4°C to remove cellular debris, followed by transfer into a cold microfuge tube, and storage at -80°C waiting further processing.

6.3.2 Protein Quantification

All protein samples were quantified simultaneously – from three breast cancer cell lines (MCF7, MDA-MB-231 and T47D) and two oral cancer cell lines (PE/CA-PJ41 and PE/CA-PJ49), in addition to their corresponding radioresistant sublines; MCF7RR, MDA-MB-231RR, T47DRR, PE/CA-PJ41RR and PE/CA-PJ49RR. Protein quantification was undertaken using the RC-DC Assay – (Reducing agent Compatible – Detergent Compatible) (#500-0122, Bio-Rad UK). This is a colorimetric assay for protein quantification, which is compatible with the Laemmli buffer (RC-DC protein assay instruction manual). It is based on the original Lowry assay (LOWRY et al. 1951). This is based on two main reactions. The first is coupling of protein and copper tartrate in an alkaline medium. The second is reduction of a Folin phenol reagent by the protein-copper combination. Loss of oxygen atoms causes the solution to change into a characteristic blue colour with absorbencies between 405nm and 750nm. The

magnitude of the colour change depends on the protein concentration, and is detected by a spectrophotometer which determines the absorbance.

Standard dilutions were prepared to test for protein concentration between 0.1 and 2 mg/ml. Six standards were prepared using BSA at a concentration of 2 mg/ml, and distilled water for dilution to produce; 0.1, 0.2, 0.25, 0.75, 1.5 and 2 mg/ml. A volume of 25 µl of each standard was added to a microfuge tube. The protein samples to be quantified were diluted in distilled water to 1:5 and 1:10 dilutions. Three replicates of each dilution were made. A volume of 25 µl of each sample dilution was added into a microfuge tube. RC Reagent I (125 µl), was added to each microfuge tube, a vortex was used for mixing, and the tubes were incubated for 1 minute at room temperature. RC Reagent II (125 µl) was added to each tube, a vortex mixer was used for mixing, and the tubes were centrifuged at 15,000 xg for 5 minutes. The supernatant was discarded, and any residues were removed by inverting the tubes onto dry absorbent paper. Reagent A (127 µl) was added to each tube, and the tubes were mixed by a vortex mixer for 5 minutes. Reagent A was prepared by adding 40 µl of DC Reagent S to 2 ml of DC Reagent A (alkaline copper tartrate solution). One ml of DC Reagent B (dilute Folin reagent) was added to each tube, followed by vortex mix, and incubation for 15 minutes at room temperature. Fifteen minutes is the time required for 90% of maximum colour development to occur (DC protein assay instruction manual). A 96-well plate was used, and 200 µl of each tube (standard and protein sample) were added into a separate well. Distilled water (200 µl) was used as a reference blank. A spectrophotometer (Multiscan plate reader from Labsystems) was used, and the absorbencies were read at wavelength 690nm. A standard curve was plotted of the

absorbance vs. the standards' concentration, and protein concentration was determined (section 6.4 and Figure 6.5).

6.3.3 Gel loading and electrophoresis

The quantified protein samples were kept in ice at all times. The samples were first diluted in a sample buffer to achieve concentrations of 15-20 µg of protein in a volume of 25 µl. The sample buffer consisted of 190 µl of western blot buffer and 10 µl of β-mercaptoethanol. Dilution was undertaken to achieve equal concentrations of the different protein samples in equal loading volumes. For example to obtain a concentration of 20 µg in 25 µl of sample:

$$\frac{20}{\text{Protein concentration (mg/ml)}} = \text{volume of protein sample (µl)}$$

25 (µl) – volume protein sample (µl) = volume of sample buffer (µl)

A vortex mixer was used to thoroughly mix the protein and buffer samples. The tubes were then heated at 95°C in a thermal cycler for 5 minutes to denature the proteins. They were promptly placed back into ice, to prevent reversal of denaturation, before being mixed in a vortex and centrifuged at 15,000xg for 30 seconds. Gels were prepared in the running tank. We used 12% acrylamide Precise Protein Gel (#25222 Thermo-Scientific, UK). This is a fixed concentration, 12 well, 1 mm thick precast gel. The running buffer was prepared from 500 ml of Tris-HEPES-SDS buffer (#28368, Thermo-Scientific, UK) and 9.5L of distilled water. Two gels were run simultaneously. If one gel only was running at a time, a plastic blank was used in place of a second gel. The gel was loaded slowly using gel loading pipettes with fine tips. The protein samples

were loaded in wells 1-10, while well 11 was loaded with Laemmli sample buffer; 20µl in each well.

It is recommended to avoid vacant wells in order to ensure similar electrical resistance across the width of the gel. 10 µl of Precision Plus Protein™ WesternC™ Standards (#161-0376, Biorad, UK) was added to well number 12. This standard can be visualized during gel electrophoresis (to monitor protein migration), following transfer (to assess the efficiency of transfer), and finally after blot development. It consists of ten pre-stained, dual coloured and fluorescent protein bands with a range of molecular weights between 10 – 250kDa. Gel electrophoresis was performed at a voltage of 140 for 60 minutes. Satisfactory running of the gel was ascertained by the appearance of bubbles at the side of the tank, the migrating blue dye front and the coloured bands of the standard protein marker (Figure 6.2).

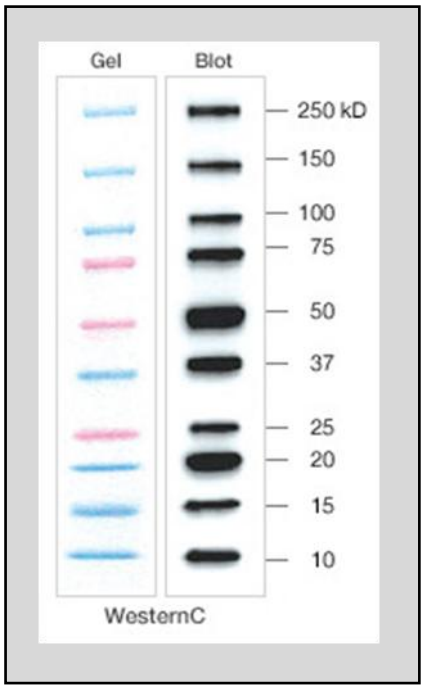

Figure 6.2 Western C precision plus dual colour molecular weight standards (Biorad). This standards is visualized on the running gel, on the membrane (thereby testing efficacy of transfer), and also on the developed x-ray film. It is made up of ten pre-stained proteins with molecular weights ranging from 10 to 250 kDa.

6.3.4 Protein transfer from gel to membrane

The gel was removed from the running tank, and placed in a tray containing cold transfer buffer to remove any residue of the Tris-HEPES-SDS running buffer. The transfer buffer was prepared using 200 ml of ethanol, 800 ml of distilled water, 3.3g of Tris and 14.4g of glycine. Also soaked in the cold transfer buffer were filter paper, foam pads and the nitrocellulose membrane.

The gel was removed from the plastic cast, and the transfer cassettes were prepared in the order depicted in Figure 6.3. The foam pad was placed on the black cover of the cassette, followed by the filter paper, the gel and the nitrocellulose membrane. A cylindrical rod was passed over this stacked arrangement to ensure the absence of any air bubbles which might interfere with the transfer. A second piece of filter paper was placed over the membrane, followed by a foam pad and the translucent cover of the cassette. An ice block was placed next to the cassette within the transfer tank. A small stirrer was placed at the bottom of the transfer tank to ensure equal temperature and ion distribution. The tank was filled with the transfer buffer, ensuring that the gel was completely submerged. Electrophoretic transfer was performed at 400mA for 1 hour, at 4°C.

6.3.5 Antibody probing

Following the transfer, the membrane was removed from the cassette and placed in a Nalgene (polyethylene) box. Blocking milk solution (20ml), was added to the membrane; this was made of 5% Marvel (a milk powder of <1% fat content) in 0.05% TWEEN 20 in TBS (Tris buffer solution). This reduces non-specific binding of the antibody to binding sites on membrane proteins.

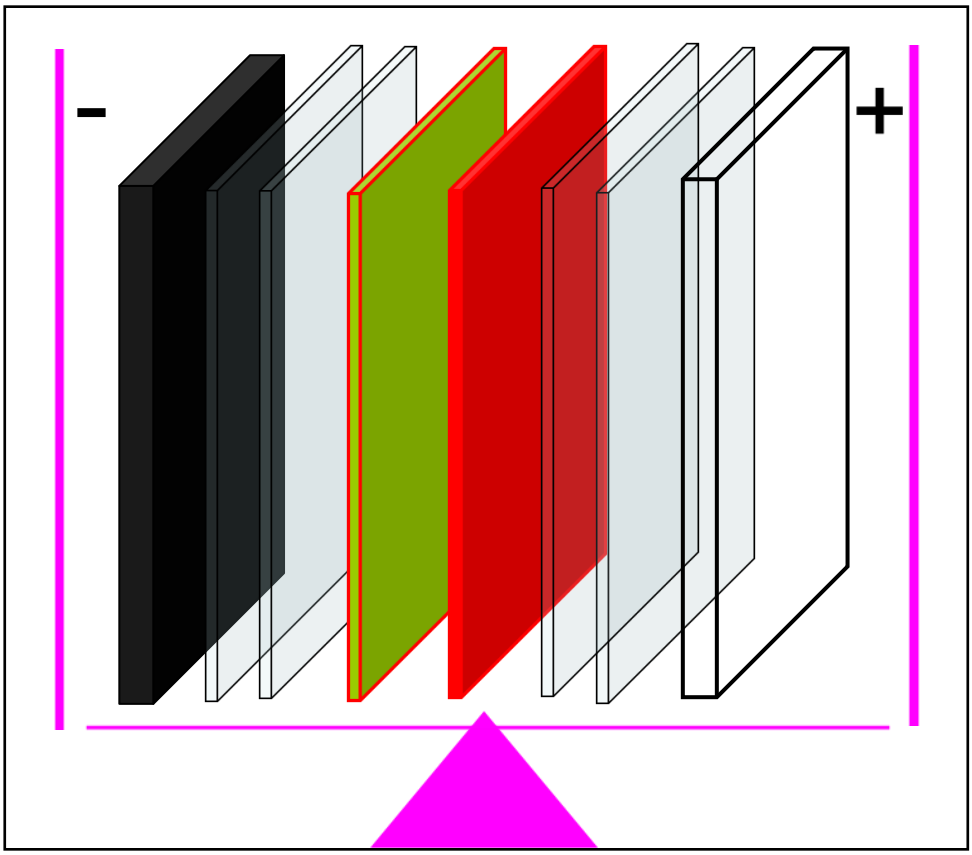

Figure 6.3 Western blot, gel to membrane transfer. The gel and membrane are clamped tightly together, and submerged in the transfer buffer, to which an electrical current is applied. The negatively charged proteins travel towards the positively charged electrode, but are stopped by and bind to the membrane. From left to right: gel holder (black), fibre pad, blotting paper, gel, nitrocellulose membrane, blotting paper, fibre pad, gel holder.

The membrane was incubated in the milk for 2 hours at room temperature (or overnight at 4°C for some experiments). All incubation and washing steps were performed on an orbital shaker. Nitrocellulose membranes may have a high capacity for binding proteins and antibodies. In addition, milk proteins have a low immunogeneity to antibodies.

The different primary antibodies were added to the milk solution according to individual recommended concentrations and following optimisation. Membranes were probed for the primary antibodies for 2 hours at room temperature. Table 6.2 shows the different antibodies used in western blotting.

Table 6.2 Antibodies used in western blotting. The table also shows antibodies which will require further optimisation. Different exposure times were attempted for these antibodies.

Antibody	Dilution	Incubation time
Primary antibodies		
Zyxin #Z0377 (Sigma Aldrich)	1:1000	2 hours
PIASx #Ab50226 (Abcam)	1:1000	2 hours
DR4 #Ab8415 (Abcam)	1:1000	2 hours
Loading controls		
A-tubulin #Ab7291 (Abcam)	1:2500	2 hours
GAPDH #Ab9485 (Abcam)	1:2500	2 hours
B-actin #Ab8227 (Abcam)	1:1000	2 hours
Secondary antibodies		
Anti-rabbit #A0545 (Sigma Aldrich)	1:1000	1 hour
Anti-mouse # A0944 (Sigma Aldrich)	1:1000	1 hour
Antibodies that require further optimisation		
Zyxin #Ab28720 (Abcam)	1:500 – 1:1500	2 hours - overnight
Zyxin #Ab58210 (Abcam)	1:1000 – 1:30, 000	2 hours - overnight
DR4 #Ab13890 (Abcam)	1:50 – 1:1000	2 hours - overnight
KPNA2 #Ab54489 (Abcam)	1:50 – 1:1000	2 hours - overnight
GFI-1 #Ab21061 (Abcam)	1:100 – 1:200	2 hours - overnight
Spread2 #Ab58127 (Abcam)	1:1000 – 1:10,000	2 hours - overnight

The membrane was washed 3 times, each for 5 minutes, in TBS-TWEEN, to remove any unbound primary antibody. It was then incubated in 5% milk-TWEEN and secondary antibody for 1 hour at room temperature. Precision Protein StrepTactin-HRP conjugate (#161-0380, Bio-Rad, UK) was also added to this mixture. This recognizes the Strep-tag sequence in the Precision Plus Protein Western C, to enable detection of ladder markers of molecular weight. The membrane was washed again in TBS-TWEEN for 3 times, 5 minute each, before image processing.

6.3.6 Image Development

The SuperSignal®West Pico Chemiluminescent Substrate (#34078, Thermo- Scientific, UK) was used. This is a substrate for detecting HRP, and has two components: Luminol/Enhancer solution, and Stable Peroxide solution. The two components were mixed together in a 1:1 ratio to create a working solution. We used 8 ml of each to produce enough solution that completely covers the nitrocellulose membrane. The membrane was incubated in the working solution for 5 minutes in the dark with slight agitation. The membrane was removed and placed in a plastic protector which covers both sides of the membrane. The plastic protector was placed in such a manner as to ensure absence of any bubbles which would interfere with the image development. It was then placed in a light-proof film cassette.

The membrane was exposed to X-ray films CL-XPosure (#34090, Thermo-Scientific) for 5 minutes originally, and then different intervals according to preliminary images. The films were developed by gentle agitation in a developer (#P7042-1GA, Sigma Aldrich), until images were detected. This was followed by immersion in 5% acetic acid, and finally a fixer (#P7167-1GA, Sigma Aldrich), each for 30 seconds. The films were then

washed under running tap water, and left standing to dry. The membranes were stored in cold PBS at 4°C, before being re-probed for loading controls.

6.3.7 Loading Controls

Membranes were re-blocked in 5% milk in TBS-TWEEN as previously described. Each membrane was incubated in 10 ml of 5% milk in TBS-TWEEN containing the loading control protein. Incubation was for 2 hours at room temperature. Membranes were washed in TBS-TWEEN 3 times, for 5 minutes each. They were then incubated with secondary antibodies in TBS-TWEEN for 1 hour at room temperature. A further 3 washes in TBS-TWEEN were performed before image development as described above (section 6.3.6).

6.3.8 Image Analysis

The dry films were scanned using a GS-800 laser densitometer (Bio-Rad). The Quantity-One software (version 4.6.1, Bio-Rad) was used for image analysis and protein quantification. Each band representing a protein sample was normalized to the corresponding band representing the loading control of the same sample. For each protein, the normalized values of the PN and RR samples were used to calculate a ratio. This ratio would reflect the fold change, and therefore the level of differential expression of individual proteins. A standard 2 fold cut-off (≥2 fold) was used to determine significant differential expression. A worked example of how the fold change is calculated is shown in Figure 6.4.

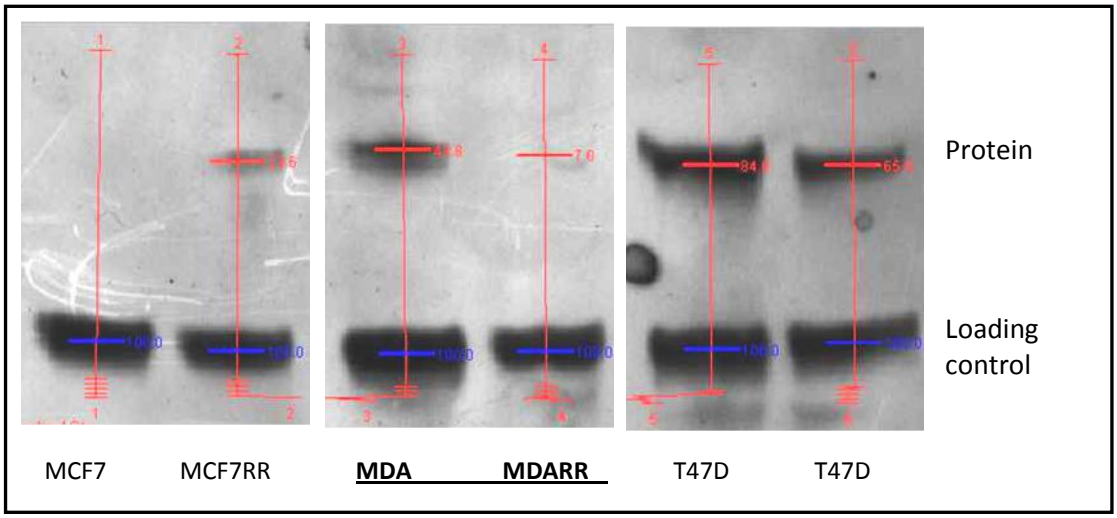

Figure 6.4 The fold change in differential expression between a radiosensitive and a radioresistant sample as measured using densitometry.

The exact normalised values of each cell line and the corresponding subline are supplied by the software. Calculation will then reveal the fold change, e.g. MDA/MDARR= 43.84/7.95 = 6.2, down-regulated (Figure 6.4).

6.4 Western blot Results

6.4.1 Protein Quantification

Protein extracted from the parent cell lines and their derivative radioresistant sublines (MCF7, MCF7RR, MDA-MB-231, MDA-MB-231RR, T47D, T47DRR, PE/CA-PJ41, PE/CA-PJ41RR ,PE/CA-PJ49, PE/CA-PJ49RR) was placed in a 96 well plate as described previously, and the orientation of the different replicates is shown in table 6.3 below.

Table 6.3 Western blot; the orientation of the protein lysates on the 96 well plate. The figure shows the orientation of the 10 samples; lysates from five cell lines and their derivative RR sublines (MCF7, MCF7RR, MDA-MB-231, MDA-MB-231RR, T47D, T47DRR, PE/CA-PJ41, PE/CA-PJ41RR ,PE/CA-PJ49, PE/CA-PJ49RR). Two replicates of the BSA standards (A and B) were used. Each sample lysate was represented by three replicates of 1:5 dilution and three replicates of 1:10 dilution. DH: distilled water used as a blank, S: BSA standards at different dilutions (0.1 – 2mg/ml).

A	DH	**S 2**	**S1.5**	**S0.75**	**S0.25**	**S 0.2**	**S 0.1**			
B	DH	**S 2**	**S1.5**	**S0.75**	**S0.25**	**S 0.2**	**S 0.1**			
C	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
D	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
E	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5	1/5
F	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10
G	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10
H	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10
	MCF7	**MCF7 RR**	**MDA**	**MDA RR**	**T47D**	**T47D RR**	**PJ41**	**PJ41RR**	**PJ49**	**PJ49 RR**

A spectrophotometer was used to determine the absorbancies of the different samples. The protein concentrations were thereafter determined from a standard curve of the absorbance vs. the standards' concentration (Figure 6.5).

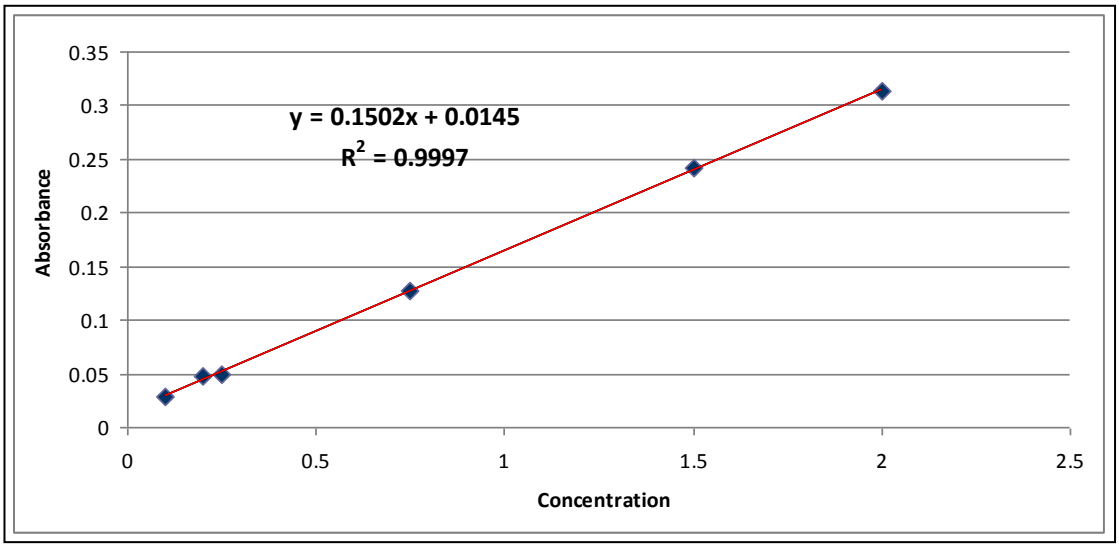

Figure 6.5 Standard Curve The standard curve is used to obtain the protein concentration (x) from the equation y=mx+c. In the above example, m (0.1502) and c (0.0145) are produced by the curve, while y is the protein absorbance as read by the spectrophotometer. R2 is the Pearson coefficient, and a value of >0.980 indicates the efficiency of the assay and data.

Below is an example of calculating the final protein concentration of the MCF-7RR protein lysates. The BSA standard curve is used to obtain the equation y=mx+c, which is then used to calculate the final protein concentration. In this example, the final concentration of the MCF7RR lysate was found to be 11.715mg/ml.

Sample and dilution	Absorbance of Unknown **(y)**	Concentration of unknown **(x)** mg/ml	Correct for Dilution (**x** X dilution)	Average Concentration (mg/ml)
MCF7RR **1/5**	0.375	2.4001	12.0007	12.0340
MCF7RR **1/5**	0.377	2.4134	12.0672	
MCF7RR **1/5**	Excluded	-	-	
MCF7RR **1/10**	0.188	1.1551	11.5513	11.3959
MCF7RR **1/10**	0.183	1.1218	11.2184	
MCF7RR **1/10**	0.186	1.1418	11.4181	

The gel loading volumes of the protein sample and diluting buffer were calculated to obtain a protein load of 20 µg in a total of 25 µl.

6.4.2 Loading controls

Alpha-tubulin was optimised as a loading control. A fresh membrane was probed for α-tubulin to ensure presence of protein within the samples, and satisfactory protein quantification before proceeding with testing for the target antibodies (Figure 6.6).

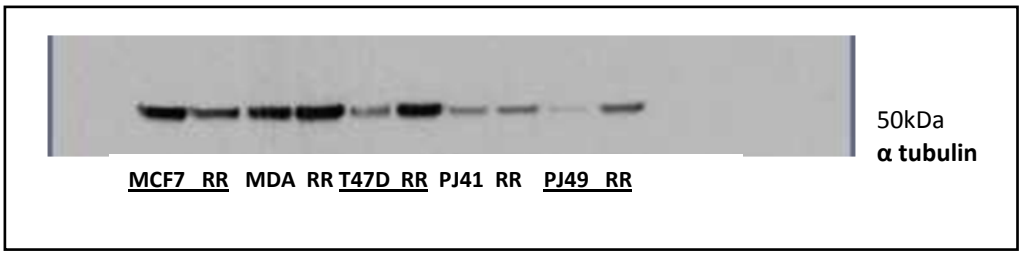

Figure 6.6 The expression α-tubulin in MCF7, MDA, T47D, PJ41, PJ49 cancer cell lines, and their radioresistant sublines. α-tubulin (#Ab7291), MW 50kD, used at 1:2500 dilution. Bands were detected at 50kDa, exposure for 3 seconds.

The appearance of bands at 50kD confirmed presence of protein within all ten sample lysates. However, some lysates showed unequal bands. The differences were more markedly observed between the T47D and T47DRR lysates, and the PJ49 and PJ49RR lysates (Figure 6.6). This pattern persisted on repeating the experiment. As the loading volumes were quite small, raising the possibility of human pipetting error, it was decided to repeat the experiment using higher volumes for making the sample/buffer solution. An example, MCF-7 cell line, is shown below. The ratio between the sample and buffer was kept constant, and the final loading volume was the same at 20µl.

	Sample	Conc mg/ml	Conc µg/ml (x)	Sample µl (20/x *1000)	Buffer µl	Final volume µl
Original vol.	MCF-7	11.8934	11893.4	1.7	23.3	25
Revised vol.	MCF-7	11.8934	11893.4	(1.7x2)= 3.4	(23.3x2)= 46.6	50

A fresh membrane was probed with α-tubulin (Figure 6.7). The three breast cancer cell lines demonstrated equal bands, however, the differences in, and between the oral cell lines were still maintained.

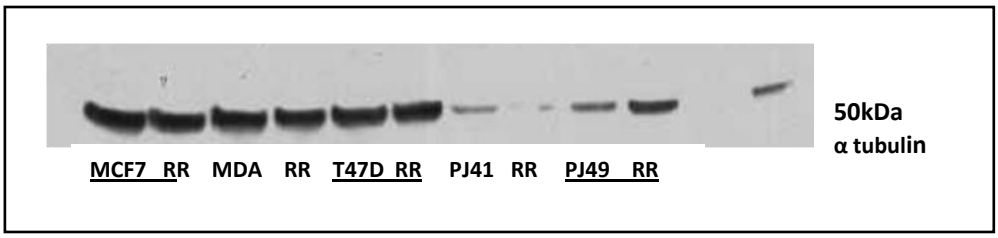

Figure 6.7 The expression of α-tubulin in MCF7, MDA, T47D, PJ41, PJ49 cell lines and their derivative radioresistant sublines – using higher loading volumes. α-tubulin (#Ab7291) at 50kDa. Larger volumes of sample and buffer were used. The figure shows equal bands amongst the three breast cancer cell lines and their radioresistant cell lines. However, variations in the oral cancer cell lines are demonstrated.

This was re-enforced when probing a new membrane with a different loading control; GAPDH. GAPDH revealed equal bands between breast cancer cell lines and their corresponding RR sublines (Figure 6.8), however the oral cancer cell lines showed persistent differences.

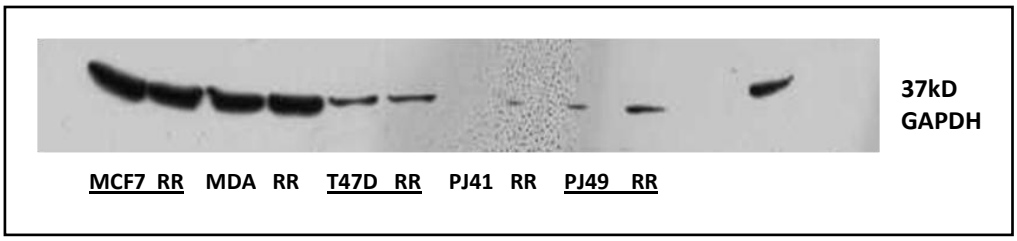

Figure 6.8 The expression of GAPDH in MCF7, MDA, T47D, PJ41, PJ49 cell lines and their derivative radioresistant sublines. GAPDH (Ab#9485) MW 37kD, dilution 1:2500, exposure for 5 minutes. The figure shows equal expression of GAPDH between each breast cancer cell line and its corresponding radioresistant subline. However, visible differences exist in the oral cell lines.

The results were pointing towards a possible quantification problem in the oral cancer cell lines. It was decided to repeat the quantification, and proceed at this stage with analysing the breast cancer cell lines only. This was secondary to time restrictions and diminishing volumes of breast cancer lysates available for the experiment.

The breast cancer cell lines' western blot results will be presented in the following sections.

The lysates were tested using loading controls α-tubulin and GAPDH. Equal bands were demonstrated when probing fresh membranes with α-tubulin and GAPDH (Figure 6.9).

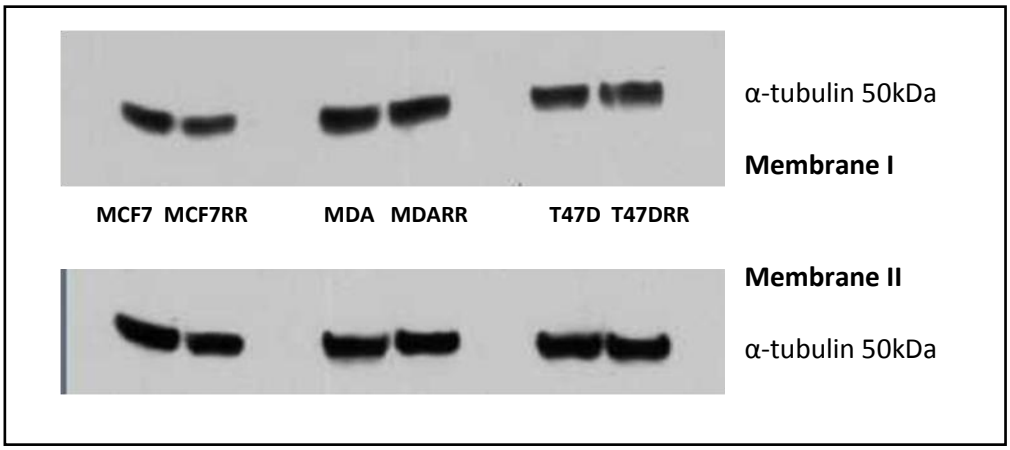

Figure 6.9 α-tubulin expression in MCF7, MDA, T47D breast cancer cell lines and their derivative radioresistant sublines. α-tubulin (#Ab7291) at 1:2500 dilution. The figure shows satisfactorily equal band sizes in the above mentioned cell lines.

6.4.3 Antibody Optimization – Breast cancer cell lines

Optimization was first performed either on the three breast cancer cell lines described above (MCF7, MDA, T47D), or on three experimental cell lines used solely for the purpose of optimisation. This was done to maximize the amount of protein sample available for the study purposes. Optimization was performed using protein lysates from a Daudi cell line - human derived Burkitt's lymphoma cell line (Klein et al. 1968), MCF7 cell line and T47D cell line.

Protein lysates had been previously extracted and quantified using the RC-DC method. Gels were loaded using equal amounts of protein lysates of 15 µg per well. The three samples were first probed with α-tubulin to ensure sufficient existing protein in the three experimental cell lysates.

Target antibodies were chosen for confirmatory testing. The choice of proteins for western blot confirmation was based on antibody microarray results. Proteins which showed differential expression of ≥2 fold across one or more cell lines were chosen.

Known functions of said proteins were also considered and their associations with other proteins.

6.4.4 Optimisation and individual antibodies

Some antibodies were tried, but optimisation was not achieved within the time available for this work. Spred2 optimisation will be demonstrated as such an example. Other antibodies with similar results will only be listed. Antibodies which were successfully optimised and utilised will be presented afterwards.

Spred2

AbMA has demonstrated a 2.1 fold differential expression of Spred2 in both MCF7RR and MDARR sublines relative to their parent cell lines. Western blot confirmation was sought; however, serial attempts at optimising Spred2 antibody in both the experimental cell lines (MCF7, Daudi, T47D), and the test samples (MCF7, MCF7RR, MDA, MDARR, T47D and T47DRR), have failed to produce satisfactory bands as demonstrated in Figures 6.10 and 6.11.

Antibodies which were not successfully optimised:

1. KPNA #Ab54489
2. GFI-1 #Ab21061
3. Zyxin #Ab28720
4. Zyxin #Ab58210
5. DR4 #Ab13890

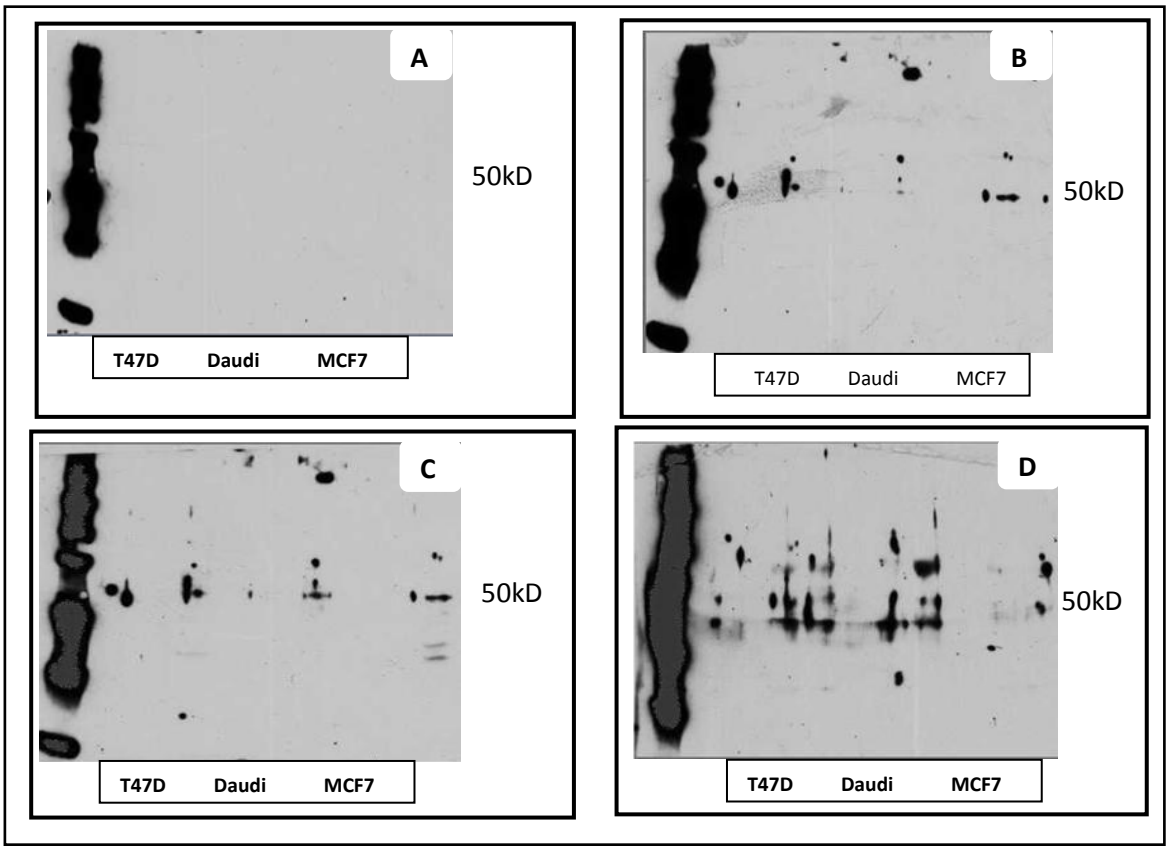

Figure 6.10 Optimisation of Spred2 using MCF7, Daudi and T47D cell lines. Spred2 (#Ab58127), MW 47kD. A: dilution 1:10,000, two hour and exposure for 30 minutes. No bands were detected. B: 1:10,000 dilution, overnight membrane incubation, exposure for 30 minutes. Background noise and unevenly shaped bands are demonstrated ~50kD. C: dilution 1:10,000, overnight membrane incubation, longer exposure. Further bands of smaller MW have appeared, with no improved characterisation of the bands at 50kD. D: 1:2000 dilution. Multiple indistinct bands are demonstrated.

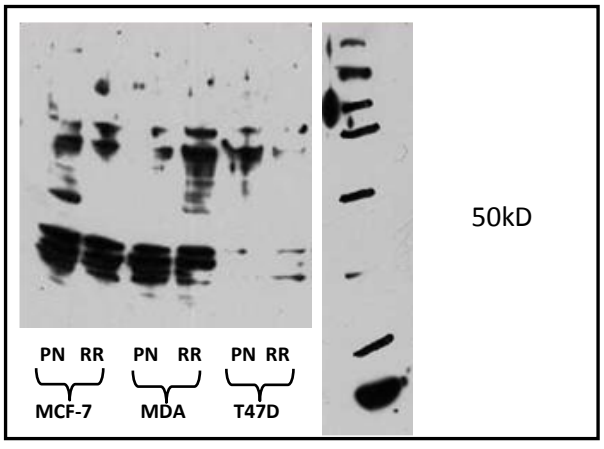

Figure 6.11 Spred2 expression in 3 breast cancer cell lines and the derivative radioresistant sublines (MCF7, MCF7RR, MDA, MDARR, T47D, T47DRR). Spred2 (#Ab58127) 1:1000 dilution. No predominant bands were demonstrated.

Zyxin

Zyxin showed significant differential expression in all three breast cancer radioresistant sublines (MCF7RR, MDARR, T47DRR) using AbMA, by 3.3 fold, 3 fold and 3.5 fold respectively. Zyxin #Ab28720 and zyxin #Ab58210 were tested, but did not yield satisfactory results to be utilised. Zyxin #Z0337 was then successfully utilised.

This antibody was chosen as it is the same antibody that was used in the Panorama Antibody microarray XPRESS Profiler725 utilised in the screening experiment described in chapter V. The antibody was first optimized using protein lysates from four human breast cancers (tissue extract TE1-TE4) – work performed by Dr V Hodgkinson (Figure 6.12). The antibody was then used to probe protein lysates from the three breast cancer cell lines (Figure 6.20).

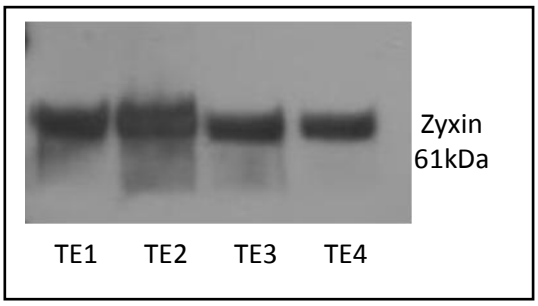

Figure 6.12 Zyxin expression in protein extracts from human breast cancer tissue. Zyxin (#Z0377), at dilution of 1:2500. Bands are demonstrated at ~61kDa in all four tissue extracts (TE).

A fresh membrane containing the three breast cancer cell lines and the radioresistant sublines was probed with Zyxin #Z0377 at 1:1000, Figure 6.19. Zyxin was differentially expressed in MCF7 and MDA. The experiment was repeated using, and B-actin was used as loading control (Figure 6.13).

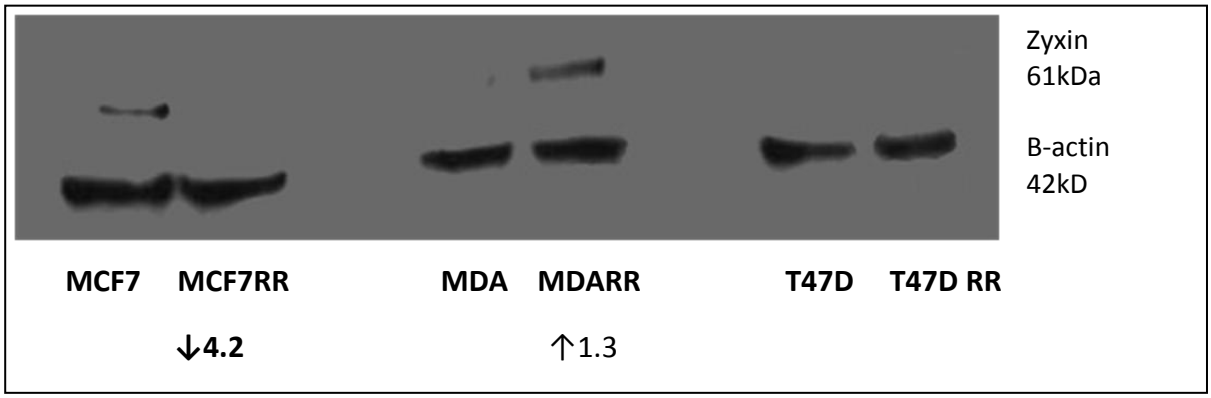

Figure 6.13 Zyxin expression in MCF7, MDA, T47D breast cancer cell lines and their derivative radioresistant sublines. Zyxin (#Z0377) at a dilution of 1:1000, β-actin (#) 1:1000. Bands are detected at 61kD MCF7 and MDARR, with MCF7RR showing evidence of significant down-regulation; fold change of >4.

The results were consistent in two membranes. Zyxin showed significant down-regulation in the MCF-7RR subline (4.2 fold), which is consistent with the result of the AbMA (Table 6.1). Zyxin was not detected by western blotting in the T47D cell line, neither was it detected in its derivative RR subline. It may be possible that the amount of Zyxin protein in the T47D cell lines was not sufficient enough for detection by western blotting. The result in the MDA and MDARR cell lines was however contradictory to the AbMA results where Zyxin expression was significantly down-regulated in the radioresistant subline. Using WB, it was found to have a trend towards upregulation (1.3 fold) in the RR subline, but this did not reach significance.

PIASx

PIAS is a nuclear protein. There are at least 5 members of the PIAS family (Jackson 2001). PIAS-x (alternative name PIAS2) has at least 3 isoforms produced by alternative splicing; alpha, beta and isoform 3. PIASxβ – the predominant isoform – has a molecular weight of ~ 68kDa, PIASxα shows at ~ 63kD. The third isoform can be detected at ~ 30kDa. PIASx is known to undergo post-translational modification

(sumoylation /modification by SUMO), and this can contribute to multiple bands on western blot.

Using AbMA (Table 6.1), PIASx was found to be significantly differentially expressed in T47DRR (2.7 fold) and MDARR (2.3 fold) sublines. There was no significant differential expression in the MCF7RR subline. For western blot, PIASx was first optimised using lysates from three cell lines as previously described; Daudi, MCF-7 and T47D. Three bands were detected, and these corresponded to the previously documented isoforms of PIASx (Figure 6.14).

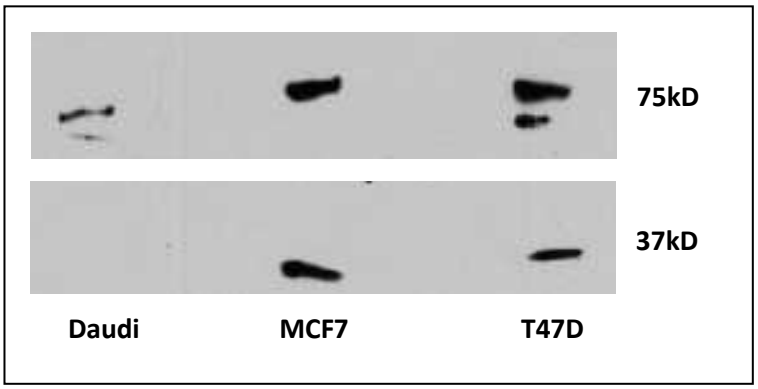

Figure 6.14 PIASx expression in Daudi, MCF7 and T47D cell lines. PIASx (#Ab50226) used at a dilution of 1:1000. Three bands were detected, corresponding to the three isoforms PIASxα, PIASxβ and isoform 3.

PIASx (#Ab50226) was incubated with the three study cell line lysates and the corresponding sublines (Figure 6.15).

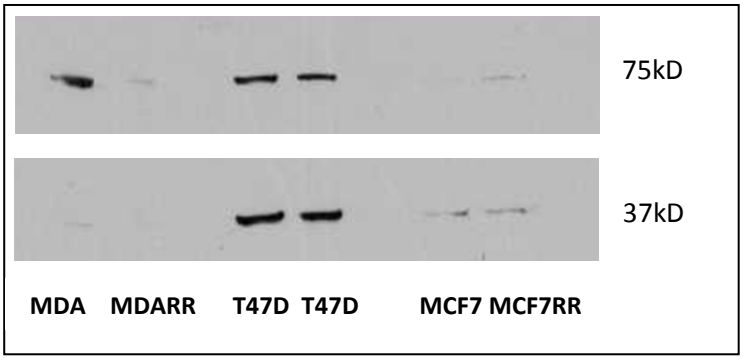

Figure 6.15 PIASx expression in MCF7, MDA, T47D breast cancer cell lines and their derivative sublines (1). PIASx (#Ab50226) used at a dilution of 1:1000 was detected , showing two isoforms at 75kD (predominant isoform) and 37kD.

PIASx was significantly down-regulated in MDARR and T47DRR sublines when using the AbMA technology (by 2.3 and 2.7 fold respectively). This difference in expression was confirmed in the MDARR subline, using western blotting, with a significant fold decrease (Figure 6.16 and 6.17). The difference in the T47DRR subline did not reach the two fold level of significance. Both AbMA and western blotting did not show a differential expression of PIASx in the MCF7RR subline when compared to the MCF7 breast cancer cell line.

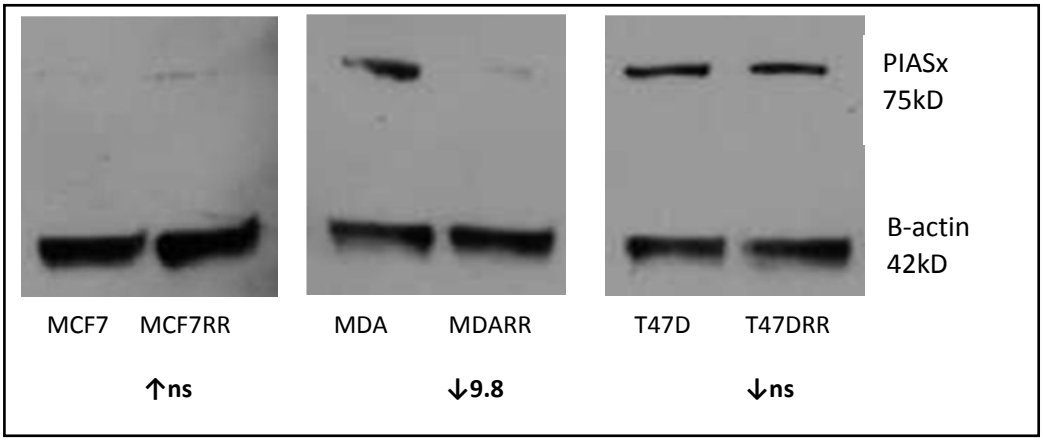

Figure 6.16 PIASx expression in MCF7, MDA, T47D breast cancer cell lines and their derivative sublines (2). PIASx (#Ab50226) 1:1000, β-actin 1:1000. PIASx shows significant down-regulation in the MDARR subline with a fold change of 9.8.

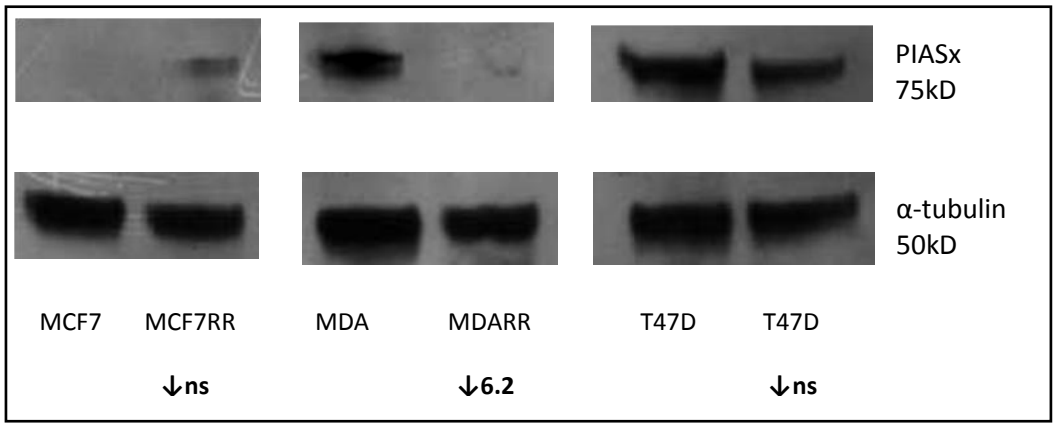

Figure 6.17 PIASx expression in MCF7, MDA, T47D breast cancer cell lines and their derivative sublines (3). PIASx (#Ab50226) at 1:1000, α-tubulin at 1:2500 dilution. PIASx showed significant down-regulation in MDARR subline with a fold change of 6.2.

DR4

Using AbMA, DR4 was differentially expressed in MCF7RR subline (4.1 fold) and T47DRR subline (5.1 fold). Its differential expression in MDARR subline did not reach significance.

DR4 (#Ab8415) was used at a dilution of 1:1000 on the three breast cancer cell lines. The AbMA results were confirmed using western blot when DR4 showed significant down-regulation in the MCF7RR subline (Figure 6.18). However, the differential expression in the T47D was not significant.

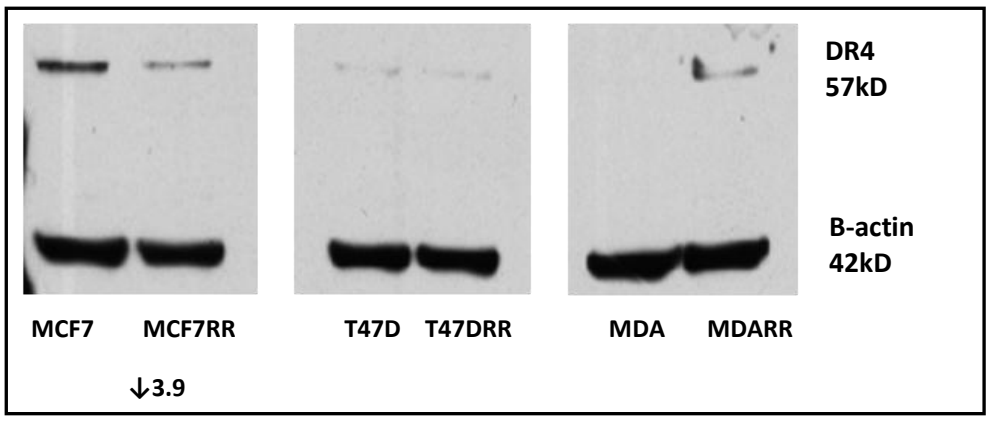

Figure 6.18 DR4 expression in MCF7, MDA, T47D breast cancer cell lines and their derivative sublines. DR4 (#Ab8415) 1:1000, β-actin (Ab8227)1:1000. DR4 shows significant down-regulation in the MCF7RR subline with a fold change of 3.9. It shows no significant differential expression in the other two radioresistant sublines.

6.5 Discussion

WB was successfully utilised to confirm the significant association between the proteins zyxin, PIASx and DR4, and radioresistant breast cancer sublines. GFI-1, Spred2 and Importin-α (KPNA2) will require further optimisation of the relevant antibodies before further tests of confirmation are attempted.

Spred2 has been reported as an inhibitor of the EGFR downstream effector molecule MAPK. The AbMA technology has shown a significant association between Spred2 and RR breast cancer sublines. Spred2 qualifies as a potential marker of RR, and further confirmatory tests are recommended.

Importin-α was differentially expressed in two breast cancer, and two oral cancer cell lines using AbMA. It was the only protein out of 725 proteins on the AbMA slide that showed differential expression across the two different cancer types. Importin-α has a known function in the transport, and therefore haemostasis of proteins involved in the cell cycle and apoptosis. It has been previously associated with poor prognosis in breast cancer, and pre-clinical studies have suggested a role in cell cycle arrest.

GFI-1 has well documented pro-survival properties. It may contribute to tumour progression through inhibiting apoptosis, increasing proliferation and causing uncontrolled proliferation secondary to interference with cell cycle checkpoints, especially G1 arrest. It would therefore be a logical candidate as a RR biomarker. We were not able to continue the process of optimising the relevant antibodies with regards to Importin-α and GFI-1 because of time constraints, however these two proteins are suitable candidates for further assessment and confirmation, and we therefore decided to continue the two processes in parallel.

Zyxin showed differential expression in all three breast cancer cell lines. It showed significant down-regulation in MCF7RR subline using western blot (>4 fold). This is in agreement with AbMA results. However, no such concordance was observed in MDARR or T47DRR sublines. As discussed previously, though zyxin has been suggested to be a stress protein, as evidenced by its repeatedly differentially expressed status, its suggested functions and associations with apoptosis make it a reasonable putative marker for further assessment. The RR sublines utilised in both AbMA and WB had been exposed to ionizing radiation. RT in itself causes cellular damage, injury and may stimulate stress proteins. This however does not preclude the possibility that zyxin may be associated with the non-irradiated but inherently RR cell. We therefore chose to clinically validate the association between zyxin and RR using IHC.

PIASx was confirmed to be down-regulated in the MDARR subline using WB. This is in accordance with the AbMA results which showed differential expression in MADRR and T47DRR sublines. We have not however demonstrated a significant differential expression in the T47D subline. Though the latter showed a trend towards downregulation, this did not reach the 2 fold cut-off for significance. DR4 differential down-regulation in the RR MCF7 subline was confirmed using WB. This was not confirmed in the T47DRR subline. DR4 and PIASx were both suitable candidates for clinical validation.

CHAPTER VII

Clinical Validation;

Methods & Results

Chapter Aim:

1. To identify a clinical sample comprising two groups, representing a radiosensitive and a radioresistant phenotype, and to obtain archival breast cancer tissue from these two groups

2. To utilise the immunohistochemistry technique and archival breast cancer tissue in the clinical validation of the association between radioresistance and previously identified potential biomarkers.

Clinical Validation; Methods & Results

7.1 Introduction

Clinical validation of the differential protein expression between radiosensitive and radioresistant phenotypes was sought using IHC as the third stage in the biomarker discovery pipeline (chapter IV). A list of potential protein biomarkers associated with RR in breast cancer cell lines was produced after utilising AbMA (chapter V). WB was utilised to confirm this differential expression in some proteins, and determine the direction of change (up-regulation vs. down-regulation). In this chapter we present the IHC analysis and clinical validation of these results. Two retrospective case series representing a radiosensitive group and a radioresistant group were selected (section 7.3). Archival breast cancer tissue of these cases was then obtained, and IHC performed to ascertain the differential expression of previously selected protein markers. The protein markers which will be assessed using IHC, and their selection process, are described in section 7.1.

7.2 Proteins selected for IHC

Eight proteins were selected for IHC; Zyxin, PIASx, DR4, GFI-1, KPNA2, Chk1, 26S proteasome and UBAE1.

Importin-α (KPNA2),GFI-1, zyxin, PIASx, DR4 and Chk1 were all identified as potential markers of RR in breast cancer cell lines using AbMA as described previously (chapter V). In addition zyxin, PIASx and DR4 were confirmed as putative biomarkers using WB. They were also suitable candidates considering their proposed functions and /or roles in molecular pathways.

Chk1 is a nuclear protein that is activated through phosphorylation by ATM and ATR as part of DDR pathway (Figure 2.2), and in turn inactivates Cdc25 phosphatase (cell division cycle). Cdc25 is responsible for the activation of cdk within the cell cycle. Chk1 can therefore result in the inhibition of cell cycle progression, mainly through S phase delay and G2/M arrest. IHC on normal fibroblasts have shown that its nuclear expression varies with the cell cycle, being positive during the S to M phases, but not in the G0 to early G1 phases (Kaneko et al. 1999). Cytoplasmic staining of Chk1 has also been observed in PTEN depleted cells, and UbcH7 depleted cells (Whitcomb et al. 2009). Previous IHC studies of Chk1 have demonstrated both nuclear and cytoplasmic staining in breast cancer tissue (Verlinden et al. 2007). Chk1 showed an association with triple negative breast cancers (Verlinden et al. 2007). Similarly, another study of pre-menopausal women with breast cancer has suggested Chk1 as a marker of aggressive tumours because of a positive correlation with larger, higher grade tumours, however no association was found with recurrence rates (Lundgren et al. 2008). Over expression of Chk1, was reported to be associated with RR in rat embryos (Hu et al. 2001).

In this project, Chk1 showed significant differential expression in only one cancer cell line using AbMA; MDARR with a fold change of 2. In the other two sublines, its fold change was ≤1.5. It was selected for further analysis due to its known role in cell cycle regulation which makes it a suitable and logical candidate protein for further confirmatory testing. Chk-1 would constitute a logical biomarker of the response of cancer cells to radiation. It was thus chosen as a potential candidate for IHC confirmation.

KPNA2 was detected by AbMA in both breast and oral cancer cell lines. WB confirmation was not accomplished because of technical issues regarding the commercial antibodies. As it was the only protein which showed differential expression across more than one cancer type, we chose to further analyse KPNA2 using IHC.

The 26S proteasome was not included in the antibody array (Panorama XPRESS Profiler 725, Sigma Aldrich) used for screening for potential markers of RR in this project. It was chosen for IHC analysis based on previous work undertaken in our laboratories that reported promising results. The 26S proteasome is a key component of the UPP. This is a major proteolytic mechanism that consists of ubiquitin and 26S proteasome, and plays an important role in the degradation of many proteins of apoptosis and cell cycle control including Bax, BcL-2, p53, p21, p27 and cyclins (section 2.3.1.4). The 26S proteasome had been previously identified as a strong potential marker of RR at our institution (Smith et al. 2009b). Previous work at our institution has created three radioresistant breast cancer cell lines (MCF-7RR, MDA-MB-231RR and T47DRR). Components of 26 S proteasome were shown to be down-regulated in all three RR cell lines using 2DE-MS, LC-MS/MS and expression microarray, namely PSMA1, PSMA2, PSMA7 and PSMB1 (Smith et al. 2009b). Further IHC was undertaken on pre-treatment biopsies from laryngeal cancer tissue. The sample comprised 22 patients who were free of recurrence three years after RT treatment, and 22 patients who suffered a recurrence within a year. The 26S proteasome was significantly down-regulated in the recurrence – radioresistant - group. The 26S proteasome was not included in the antibody array utilised in the screening phase of this project. However, considering the

above literature, it was considered as a potential marker, and the decision taken to further assess its expression in breast cancer tissue samples using IHC.

UBAE1 (ubiquitin activating enzyme 1) was selected for IHC because of its close association with UPP. It was not a target protein in the antibody array utilised in this project. UBAE1 is an integral regulatory enzyme responsible for the initial reaction that activates ubiquitin protein. This is a rate limiting step, and is responsible for subsequent protein ubiquitination and degradation. Previous studies have identified the localization of UBAE1 as both cytoplasmic and nuclear (Grenfell et al. 1994;Trausch et al. 1993). Furthermore, it has been suggested that this localization can alter with cell cycle progression (Grenfell et al. 1994).

7.3 Breast cancer clinical case series selected for IHC

A retrospective series of clinical samples of breast cancer tissue was sought through examining patient data bases and following strict inclusion criteria (section 7.4.1). These selected samples were analysed for the differential expression of protein biomarkers using IHC. Two patient populations representing a radiosensitive and a RR phenotype were selected as detailed in the methods section. Archival tissue was then obtained and IHC performed. Proteins were selected for testing based on AbMA and WB results, in addition to selecting other significant markers detected from previous work at our laboratory (section 7.1).

This work was approved by Hull and East Riding local research ethics committee (Ref LREC/10/03/216).

7.4 Methods

7.4.1 Patient Selection

All patients undergoing surgery for breast cancer at Hull & East Yorkshire NHS Trust were included in the original search. Prospectively maintained databases were utilised. These included the national BASO database and local hospital databases. Where available, paper records were used to obtain additional information in the final study sample. The search included all patients undergoing surgery for breast cancer at Castle Hill Hospital between 1988 and 2007. The final study sample included patients undergoing BCT and satisfying the following criteria. Archival tissue was then obtained from two identified patient groups as follows:

A. Test group representing a radioresistant sample.

Inclusion criteria:

1. Female patients
2. BCS for early stage invasive breast cancer (I and II).
3. Adjuvant fractionated external beam WBRT at a dose of 40-50 Gy (+/- boost to tumour bed).
4. Microscopically clear margins of excision; minimum margin 1 mm.
5. Ipsilateral LR of invasive disease (same quadrant or scar recurrence), or an ipsilateral axillary recurrence (preceded by axillary RT).
6. Recurrence of the same pathological description as the primary cancer (same ER status, different grades accepted)
7. Recurrence occurring within 4 years of completion of the RT treatment.

Exclusion criteria:

Patients with clinical or pathological factors known to be associated with LR were excluded.

1. Tumour size >50mm
2. Extensive ductal carcinoma insitu
3. Lymph node status; N2 or more

B. A control group representing a radiosensitive sample.

This group included patients who underwent BCT and who were disease free at 10 years following completion of RT treatment. These patients were matched to the test group as follows:

1. Age; <40 vs. age ≥40
2. Tumour size; ≤20 vs. 20-50 mm
3. Tumour type; ductal vs. other
4. Tumour grade; grade 3 vs. grade 1 & 2
5. DCIS; present vs. absent
6. Lymph node status; pN0 vs. pN1
7. LVI; present vs. absent
8. ER status; negative vs. positive

Further examination of paper records and pathology reports was undertaken. The final test and control groups were determined based on the above criteria. Many of the patients had breast surgery in the early 1990s, and the quality of the pathology reports was inferior to current standards and not compliant with current reporting

recommendations. A dedicated breast pathologist (E Long) provided the missing information after re-examining pathology slides. ER IHC examination was carried out by the histopathology laboratory technicians if not previously reported.

An Excel database was constructed incorporating all clinical and pathology details, and SPSS v. 17 was utilised for statistical analysis. The two groups were matched as described above. Tissue blocks of FFPE tissue were provided by the breast pathologist after examining relevant slides.

7.4.2 Immunohistochemistry technique

7.4.2.1 Antibody optimisation

Selected antibodies were first optimised to determine the most suitable concentration and duration of incubation with DAB. Optimisation was carried out on 5-10 slides. These consisted of breast cancer, oral cancer and colorectal cancer tissue slides. One negative control slide was included in each experimental run. These did not comprise part of the experimental clinical series.

7.4.2.2 Slide preparation

FFPE tissue blocks were obtained from the histopathology department at Hull Royal Infirmary. Suitable blocks containing representative cancer tissue were identified by a breast pathologist. Glass slides were pre-labelled using three identifiers to avoid errors; specimen number, tissue block number and year of surgery. The FFPE blocks were placed on ice, after which 4µm sections were cut using a microtome. These were cut onto the surface of a water bath and thereafter picked onto the glass slides. The slides were left to dry overnight in a 37°C incubator. Six slides were obtained from each tissue block.

Experiments on clinical samples were performed for one batch per day. A batch consisted of 15 test (radioresistant) slides, 16 control (radiosensitive) slides and 1 negative control slide. Test and control slides were mixed and placed onto two metal racks. Slides were labelled with the antibody name and specific concentration. The negative slide was labelled as such. Slides were first de-waxed and deparaffinised by immersing the metal rack in warm Histoclear II solution for 10 minutes (pre-warmed to 37°C by heating in a microwave for 1 minute). The metal rack was then immersed in two pots of Histoclear II at room temperature for 10 seconds each. Tissue rehydration was performed by consecutively immersing the slides into 3 pots of 100% ethanol for 10 seconds each. The above steps were carried out in a fume hood. The rack was then placed in a pot of tap water and transferred to the sink, where it was rinsed under running water for 1 minute. At this point, it was ensured that the slides were fully de-waxed.

The slides were incubated in a freshly prepared mixture of 8 ml of 3% v/v hydrogen peroxide in 400 ml of methanol. This ensures that endogenous peroxidase activity is blocked - peroxidase within tissue can react with DAB, thus resulting in non-specific staining and therefore false positives.

7.4.2.3 Antigen retrieval and labelling

The slides were rinsed in running tap water before antigen retrieval. This was performed by placing the slide rack in a pressurised cooker containing a boiling solution of 1500ml of distilled water, and 15ml of Vector Antigen unmasking solution low pH (#H-3300, Vector Laboratories). The slides were left in the pressurised cooker for 3 more minutes after it had reached full pressure.

The slides were allowed to cool under running water and then placed in a pot of TBS (Tris buffered saline). They were removed from the metal racks, assembled into plastic coverplates and placed in Sequenza® racks (Thermo-Scientific) - Figure 7.1. They were subsequently rinsed with TBS for 5 minutes.

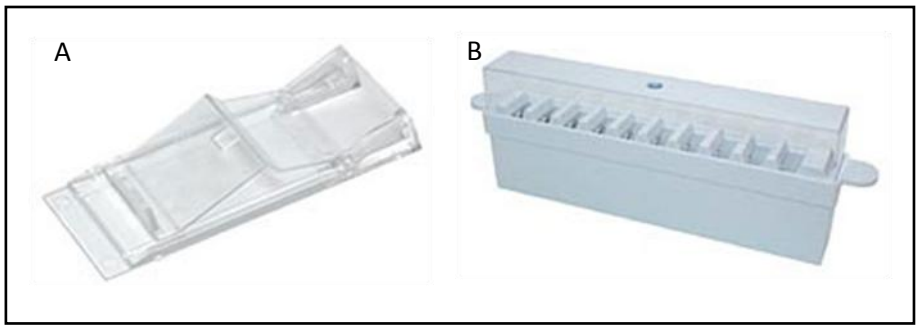

Figure 7.1 Plastic coverplate and Sequenza rack used for slide assembly in immunohistochemistry. A: plastic coverplate into which a single slide is placed, before multiple coverplates can be assembeled onto a Sequenza rack, B. In the Sequenza rack, slides can be incubated with antibodies or buffers, and washed before further incubations.

100 µl of casein 1x (10x casein #SP-5020 Vector – 10% in TBS) blocking solution was added to each slide reservoir and left to stand for 10 minutes. The slides were washed before the primary antibody was added. The primary antibody was diluted in 0.2x casein in TBS to achieve the required concentration based on optimisation results. Dilutions of the primary antibodies used are shown in Table 7.1. A volume of 100 µl of diluted antibody was added to each slide reservoir and incubated for 2 hours at room temperature. The negative control slide was incubated with 100 µl of 0.2xcasein only without the antibody. The slides were then rinsed in TBS twice, for 5 minutes each time.

Table 7.1 Antibodies used in IHC. The primary antibodies were diluted in casein with TBS solution to achieve the final concentrations. The durations at which they were incubated with DAB to allow visualisation are also shown. *Chk1 and GFI-1 will require further optimisation. All antibodies were supplied by Abcam.

Antibody	Dilution	Incubation in DAB
DR4 #Ab13890	1:50	10 min
26S P #Ab21165	1:50	30 min
KPNA2 #Ab70609	1:50	15 min
PIASx #Ab50226	1:50	20 min
UBAE1 #Ab24623	1:50	6 min
Zyxin #Ab58210	1:50	10 min
Chk1 #Ab2845 *	1:50	10 min
GFI-1 #Ab21061 *	1:50, 1:25	30 min

The DAKO kit was used for secondary antibody application; (StreptABC Complex/HRP Duet, Mouse/Rabbit – #K0 492, DAKO UK). 100 µl of 1% biotinylated secondary antibody in TBS was applied to each slide reservoir, and incubated for 30 minutes at room temperature. The slides were washed with TBS for 5 minutes. Each slide was then incubated for 30 minutes with Horseradish peroxidase (HRP) -Streptavidin conjugate (100 µl of 1% conjugate solution in TBS per slide reservoir). The slides were washed for a final time with TBS for 5 minutes before the visualisation step.

7.4.2.4 Slide staining

The slides were dismantled into a metal rack in a pot of TBS, and incubated in a freshly prepared mixture containing 3ml of DAB, 15 drops of 30% v/v hydrogen peroxide and 400 ml of distilled water. This step allows for the antigen-antibody reaction to be visualised; DAB is a chromogen which reacts with HRP to produce brown insoluble

precipitates. Hydrogen peroxide is used to eliminate the endogenous peroxidase activity that may exist in some tissues, and which can react with DAB thus giving rise to false positive results. Slides were momentarily removed at intervals and examined under the microscope to determine sufficient staining. The slides were allowed to incubate for a maximum of 30 minutes if required. Once sufficient staining was observed (brown colouration of the tissue), the slides were removed and rinsed in running water for two minutes.

The slides were immersed in copper sulphate (0.5% w/v copper sulphate in 0.9% w/v saline) for 5 minutes to enhance the staining. The slides were rinsed in running water, and counterstained by immersion in Harris haematoxylin solution (#HHS32, Sigma-Aldrich UK) for 20 seconds. The slides were again rinsed in water before being dipped 10 times into a pot of acid alcohol (1% HCl in 70% v/v ethanol). A final wash in water was performed before the final steps were carried out in the fume hood. The slides were dehydrated by three; 10 second immersions in ethanol, and cleared by two; 10 second immersions in Histoclear II. They were finally immersed in a third pot of Histoclear II ready for mounting. This was achieved using Histomount preparation, before the slides were left to dry overnight.

7.4.2.5 Slide visualization and scoring

The PALM®Microbeam Zeiss Axiovert 200 inverted microscope was used to view the slides. A scoring sheet was devised for each protein. Individual scoring methodologies were devised for each protein to be examined based on:
a. Literature search, which identified previous IHC reports and scoring methods.
b. Preliminary slide examination. All slides were examined, and the staining quality and feasibility of possible scoring methods determined.

Scoring was undertaken by two independent investigators (ELFadl and Hodgkinson), who were trained by a breast pathologist at the start of the project. They were blinded to clinical details at the time of examination. The results were checked and any discrepancies resolved by a third investigator (Cawkwell), also blinded to clinical details at the time of examination.

7.4.2.6 Statistical analysis

The final data from each protein, as agreed by all investigators, was summarised and entered into a 2x2 contingency table. Statistical significance was assessed using two tailed Fisher's exact test. *p* values were produced, and a value ≤0.05 was regarded as significant; i.e. an association between the particular protein and the radioresistant phenotype was proven.

7.5 Results

7.5.1 Breast cancer clinical series

A total 28 samples (14 test samples - representing a radioresistant group, and 14 control samples - representing a radiosensitive group) were included in the final analysis. After satisfying the inclusion and exclusion criteria, groups of patients were excluded from the final analysis because of the following reasons:
- exhibiting histological characteristics not in accordance with the original pathology reports. All the original slides were first assessed by an experienced breast pathologist before the tissue blocks were obtained and sections produced. This was to ensure that the tissues demonstrated all the characteristics described in the original pathology reports. If this was not the case, then these cases were excluded.
- corresponding tissue blocks were missing and could not be located for IHC

examination

- tissue obtained from FFPE blocks could not be used to obtain high quality slides, e.g. very small amount of invasive cancer demonstrated.

There were 14 test (radioresistant) and 14 control (radiosensitive) samples available for analysis. There were no significant differences between the two groups (Table 7.2). All patients underwent BCS for early stage breast cancer at Castle Hill Hospital between 1990 and 2006. Excision margins were clear. A margin was deemed clear if it was ≥ one millimetre, as per unit policy (previous and existing). This was followed by WBRT at a dose of 40 Gy in 15 sessions over a 3 week period. Patients who received a boost, received a dose of 10 Gy in 5 sessions over a one week period. All those who suffered an axillary recurrence had previously received RT to the axilla.

The majority of the invasive cancers were ductal carcinomas, except for two in the test group (metaplastic, mucinous), and 2 in the control group (2 lobular).

7.5.2 Characteristics of the recurrences

The characteristics of the recurrent cancers are shown in Table 7.3.

14 patients suffered LRR. The time to recurrence was calculated in months from the completion of RT. LR were defined as recurrence of the invasive cancer, constituting the same pathological characteristics (invasive type, ER status). Recurrences were located within the same quadrant as the original cancer or within/close to the surgical scar. Recurrences were incidentally identified during routine follow up mammography in 5 patients. They were detected by patients themselves in 3 occasions, and picked up by medical staff in 5 occasions. One recurrence was detected through MRI.

Table 7.2 Breast cancer clinical series; patients' characteristics. There were no significant differences between the test and control groups with regards to age and the main histological features of the tumours. LVI: lymphovascular invasion, LN: lymph node. *Fisher's exact test, ‡Chi square test.

		Cases (RR) n=14	Controls (RS) n=14	*p* value
Age	Mean <40 ≥40	54 2 12	55 1 13	1.000 *
Invasive type	Ductal Others	12 2	12 2	1.000 *
Invasive grade	1&2 3	6 8	9 5	0.449 *
Invasive size	≤20 21-30	8 6	12 2	0.209 *
Insitu component	Present Absent	8 6	10 4	0.695 *
LVI	Present Absent	5 9	7 7	0.704 *
Axillary surgery	Yes No	13 1	11 3	0.596 *
LN status	N1 N0 NA	4 9 1	3 8 3	0.548 ‡
Involved LN	<4 ≥4 NA	4 1 10	3 0 13	0.472 ‡
ER status	Negative Positive	8 6	8 6	1.000 *

Four patients suffered more than one LR, and three of them progressed to distant metastases, and eventually death. However, all had no evidence of distant metastases at the time of diagnosis of LRR.

Table 7.3 Breast cancer clinical series, characteristics of locoregional recurrences. Fourteen patients suffered locoregional recurrences. The sites, duration to recurrence and mortality are shown in this table.

Loco-regional recurrences	**N=14**
Local	8 (57.1%)
Axillary	4 (28.6%)
Local & axillary	2 (14.3%)
Mean duration to LRR (range in months)	21.1 (6-43)
Further: local recurrences Distant metastases	4 (28.6%) 3/4 - all after > 1 year
Alive	10 (71.4%)
Dead	4 (28.6%) 3 cancer deaths (metastases) 1 non-cancer death

7.5.3 Immunohistochemistry results; individual proteins

IHC slide images and individual protein differential expressions will be presented in this section. The slides interpreted were of high quality. This however might not have been reflected in the picture format presented in this thesis due to the limitations of the microscope camera.

KPNA2

Twenty seven slides were suitable for scoring. There was intense nuclear staining and a more widespread but less intense cytoplasmic staining. Nuclei were graded as positive if intense staining was reported in ≥10%, and as negative if reported in <10%. The

cytoplasm was scored as negative (if no or weak staining) or positive (if moderate or strong staining) – Figure 7.2.

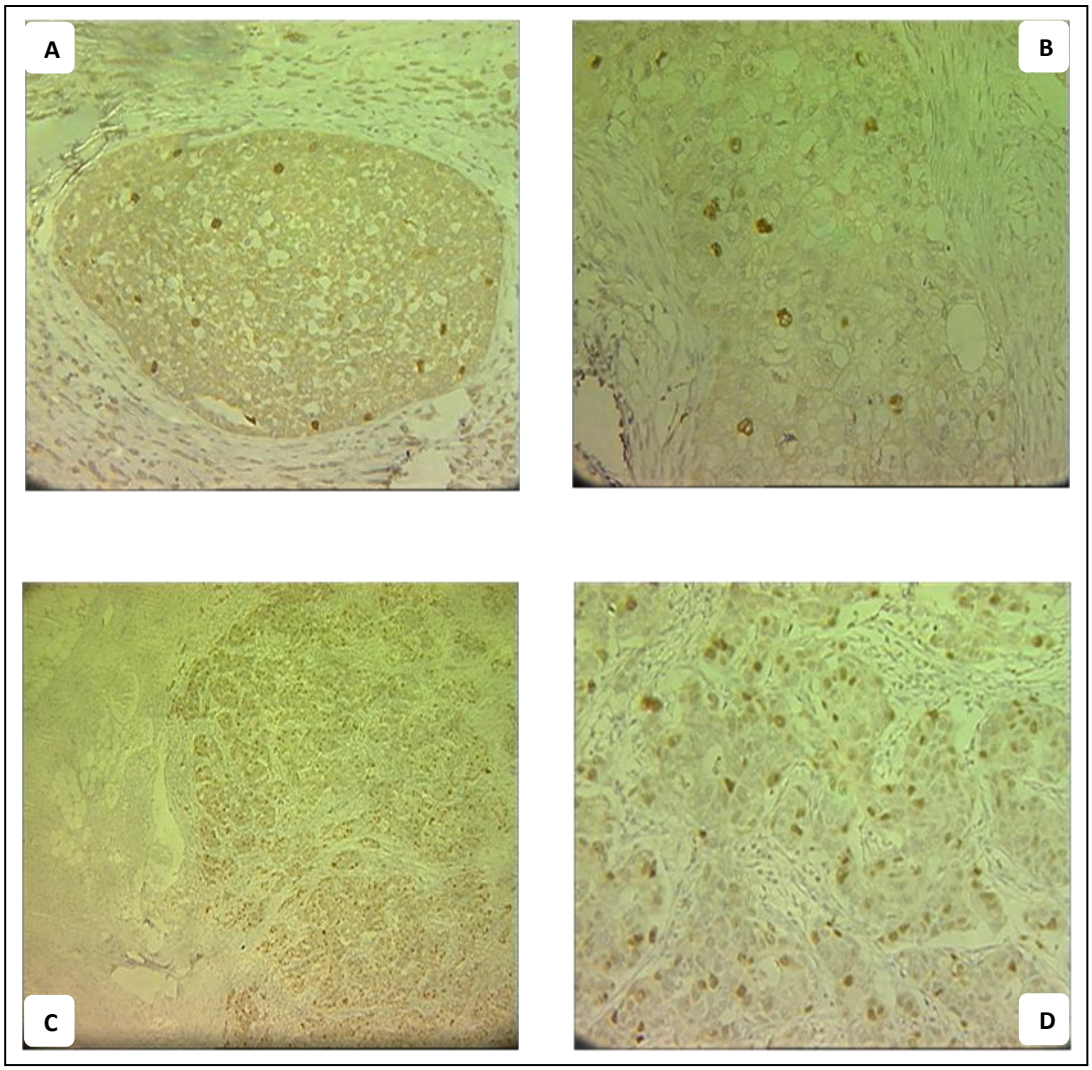

Figure 7.2 IHC of KPNA2 protein showing nuclear staining in breast cancer tissue. A: Negative nuclear staining <10% in DCIS x200. B: Negative nuclear staining in invasive breast cancer x200. C: Positive nuclear staining in invasive breast cancer tissue x50. D: Same slide as C, x200.

As demonstrated by the 2x2 contingency tables below, there was no significant correlation between the nuclear or the cytoplasmic expression of KPNA2 and the RR group (Table 7.4 and Table 7.5), *p* value of 1.00 and 0.44 respectively.

Table 7.4 Nuclear expression of KPNA2 in breast cancer clinical series using IHC. No significant correlation was found between nuclear expression of KNPA2 and radioresistant group; *p*=1.00 (2-sided Fisher's exact test)

	Negative <10% (low expression)	Positive ≥10% (high expression)	Total	p value
Radioresistant	9	4	**13**	**1.00**
Radiosensitive	9	5	**14**	
Total	**18**	**9**	**27**	

Table 7.5 Cytoplasmic expression of KPNA2 in breast cancer clinical series using IHC. No significant correlation was found between cytoplasmic expression of KNPA2 and radioresistant group; *p*=0.44 (2-sided Fisher's exact test)

	Negative (low expression)	Positive (high expression)	Total	p value
Radioresistant	6	7	**13**	**0.44**
Radiosensitive	4	10	**14**	
Total	**10**	**17**	**27**	

PIASx

Twenty six slides were suitable for scoring. Intense nuclear staining was detected, with less marked cytoplasmic staining. Nuclear lymphocytic staining was prominent (Figure 7.3D). Nuclei were graded as positive if intense staining was reported in ≥10%, and as negative if reported in <10%. The cytoplasm was scored as negative (if no or weak staining) or positive (if moderate or strong staining).

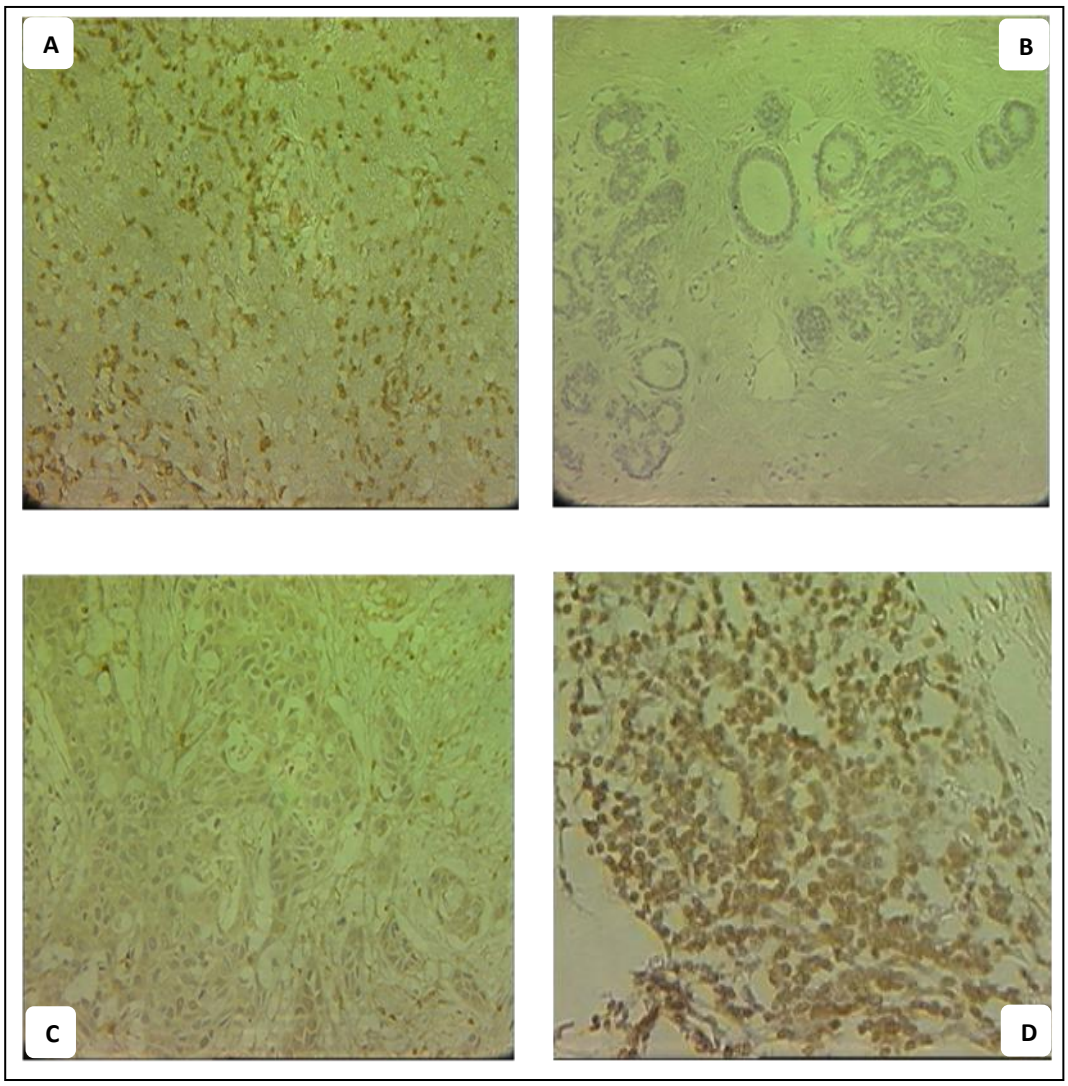

Figure 7.3 IHC of PIASx protein showing nuclear staining in breast cancer tissue. A: Positive nuclear staining in invasive cancer x200. B: Negative nuclear staining in normal breast tissue x200. C: Negative nuclear staining in invasive breast cancer tissue x200. D: Strong nuclear staining in lymphocytes x200.

There was no significant correlation between the nuclear or the cytoplasmic expression of PIASx and the RR group, as demonstrated by the contingency tables and Fisher's exact test (Table 7.6 and Table 7.7); *p* value of 0.41 and 1 respectively.

Table 7.6 Nuclear expression of PIASx in breast cancer clinical series using IHC. No significant correlation was found between nuclear expression of PIASx and radioresistant group; *p*=0.41 (2-sided Fisher's exact test)

	Negative <10% (low expression)	Positive ≥10% (high expression)	Total	p value
Radioresistant	10	3	**13**	
Radiosensitive	7	6	**13**	**0.41**
Total	**17**	**9**	**26**	

Table 7.7 Cytoplasmic expression of PIASx in breast cancer clinical series using IHC. No significant correlation was found between cytoplasmic expression of KNPA2 and radioresistant group; *p*=0.44 (2-sided Fisher's exact test)

	Negative (low expression)	Positive (high expression)	Total	p value
Radioresistant	7	6	**13**	
Radiosensitive	7	6	**13**	**1.00**
Total	**14**	**12**	**26**	

Zyxin

Twenty eight slides were suitable for scoring. The staining was mainly cytoplasmic (Figure 7.4), occasional nuclear and membranous staining was notes, but was not consistent enough to be scored. Cytoplasmic expression was scored as negative (if no or weak staining) or positive (if moderate or strong staining).

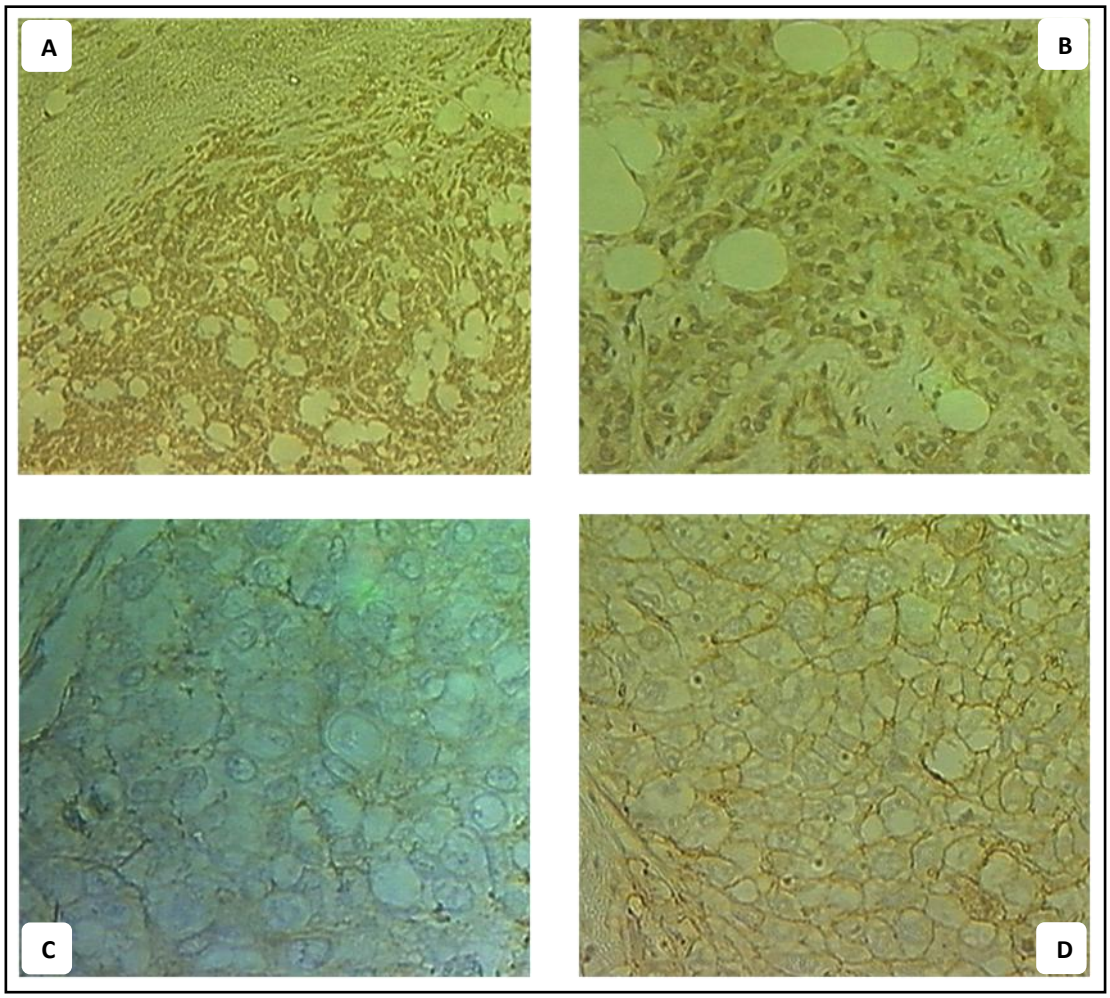

Figure 7.4 IHC of Zyxin protein showing cytoplasmic staining in breast cancer tissue. A: Positive cytoplasmic staining in invasive cancer x50. B: Same section as A, positive cytoplasmic staining x200. C: Negative cytoplasmic staining in invasive breast cancer tissue x400. D: Section showing positive membranous staining in invasive breast cancer tissue x200.

There was no significant correlation between the cytoplasmic expression of zyxin and the radioresistant group (Table 7.8), with a *p* value of 0.165.

Table 7.8 Cytoplasmic expression of zyxin in breast cancer clinical series using IHC. No significant correlation was found between cytoplasmic expression of zyxin and radioresistant group; *p*=0.165 (2-sided Fisher's exact test)

	Negative (low expression)	Positive (high expression)	Total	p value
Radioresistant	13	1	**14**	
Radiosensitive	9	5	**14**	**0.165**
Total	**22**	**6**	**28**	

UBAE1

There was widespread intense nuclear staining in invasive cancer, in situ cancer, normal breast tissue, in addition to lymphocytes and stroma. There were no marked differences between slides, and scoring was not possible. There was less intense cytoplasmic staining that was more specific to the cancer tissue; however no differences were detected between the slides (Figure 7.5).

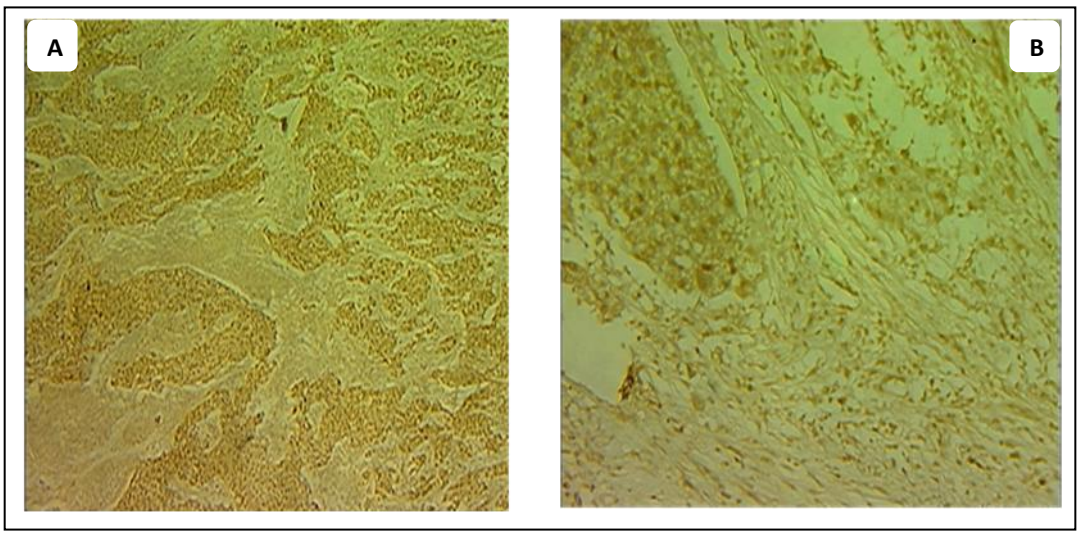

Figure 1 Immunohistochemistry of UBAE1 protein. A and B: widespread intense nuclear staining, and less marked cytoplasmic staining, in normal breast tissue and invasive cancer, x50 and x200 respectively.

Chk1

Optimization of primary antibody ChK-1 was carried out on breast cancer cell lines. Chk-1 was used at a dilution of 1:50 and incubated in DAB for 30 minutes. Staining was not sufficient (Figure 7.6). Further optimization of this protein would be required.

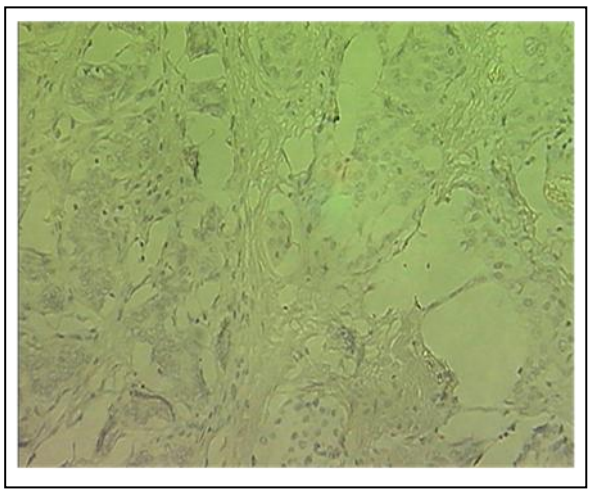

Figure 7.6 IHC of Chk-1 protein in invasive breast cancer tissue. No staining was demonstrated at 1:50 dilution with maximum incubation.

GFI-1

Optimization of primary antibody GFI-1 (ab21061, Abcam UK) was carried out on 5 breast cancer slides. GFI-1 was used at a 1:50 and 1:25 dilution, and incubated in DAB for 30 minutes. Staining was not sufficient (Figure 7.7). Further optimization of this protein would be required.

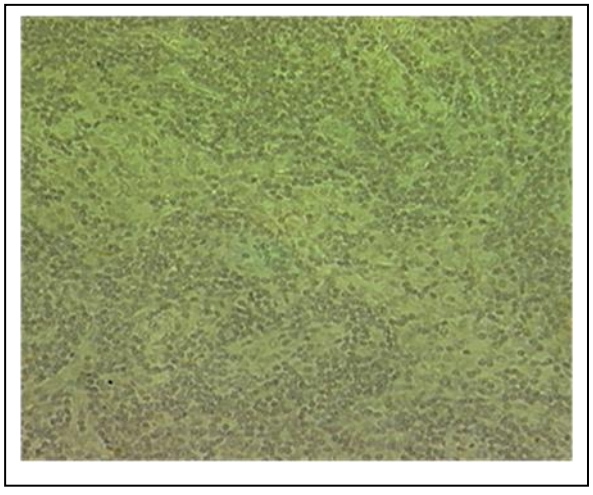

Figure 7.7 IHC of GFI-1 protein in invasive breast cancer tissue. Insufficient staining was demonstrated.

DR4

Twenty seven slides were suitable for scoring. The staining was mainly cytoplasmic, with occasional nuclear and rarely nuclear membrane staining (Figure 7.8B). It was strongest in normal breast tissue and DCIS, and weaker in cancer cells. Cytoplasmic scoring was undertaken of the invasive tissue only as negative (if no or weak staining), or positive (if moderate or strong staining), Figure 7.8.

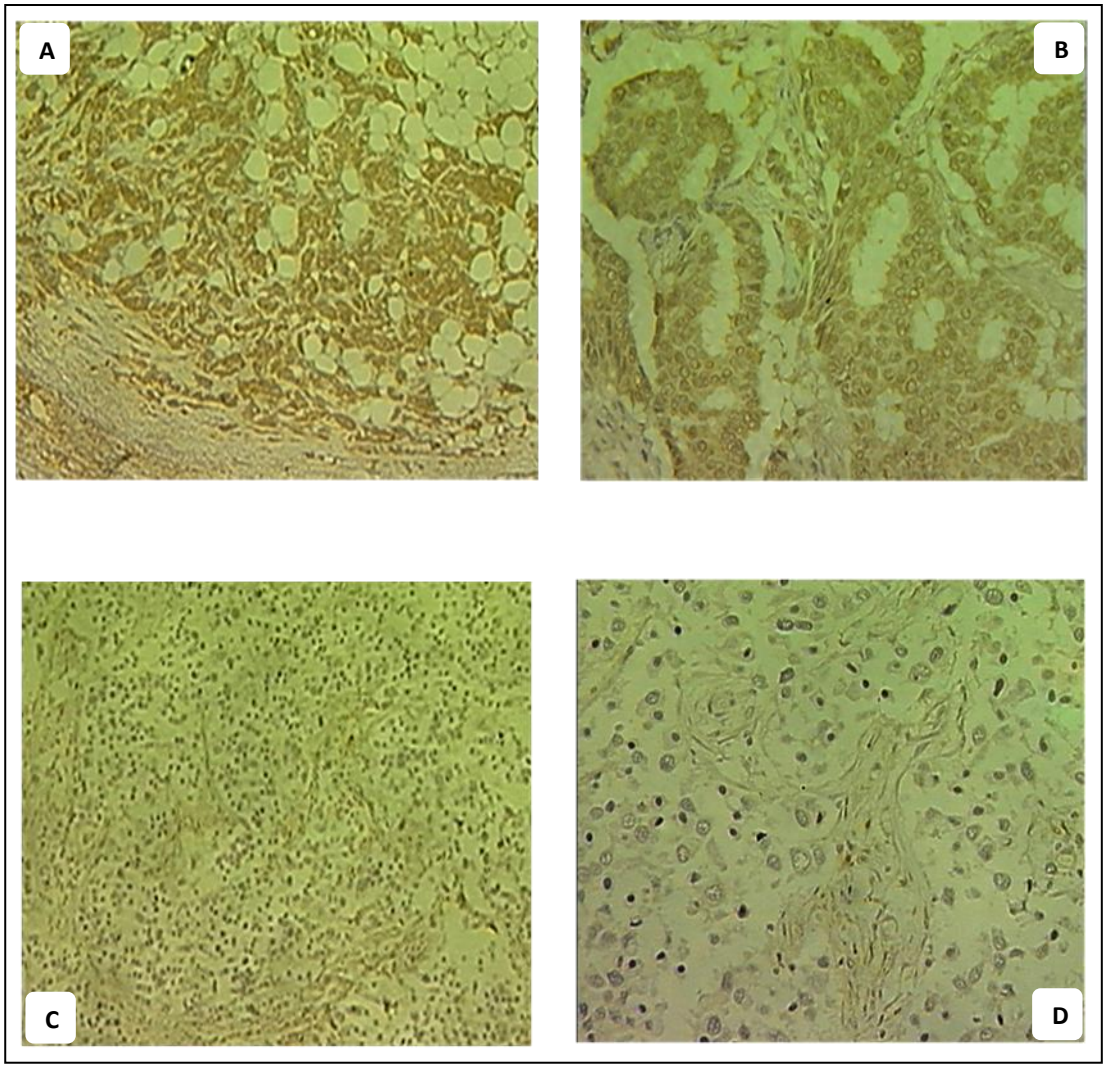

Figure 7.8 IHC of DR4 protein showing cytoplasmic staining in breast cancer tissue. A: Positive cytoplasmic staining x50. B: Positive cytoplasmic and nuclear membrane staining x200. C: Negative cytoplasmic staining x50. D: Negative cytoplasmic staining x200.

There was a significant difference between the two groups with only 14% of the control group showing low expression of DR4 as compared with 54% of the RR group, *p*=0.04. Down-regulation of the protein expression was significantly associated with occurrence of LRR and therefore with RR (Table 7.9).

Table 7.9 Cytoplasmic expression of DR4 in breast cancer clinical series using IHC. Significant Down-regulation of the DR4 protein is associated with the radioresistant group; *p*=0.04 (2-sided Fisher's exact test).

	Negative (low expression)	Positive (high expression)	Total	p value
Radioresistant	7	6	**13**	
Radiosensitive	2	12	**14**	**0.04**
Total	**9**	**18**	**27**	

26S Proteasome

Twenty eight slides were suitable for scoring. The staining was mainly cytoplasmic, with occasional nuclear staining; this latter was not scored. The staining was generally strongest within normal breast tissue, less within DCIS, and least within cancer cells (Figure 7.9). This suggests decreased expression within cancer tissue when compared with normal tissue. The staining was scored as negative (if no or weak staining) or positive (if moderate or strong staining).

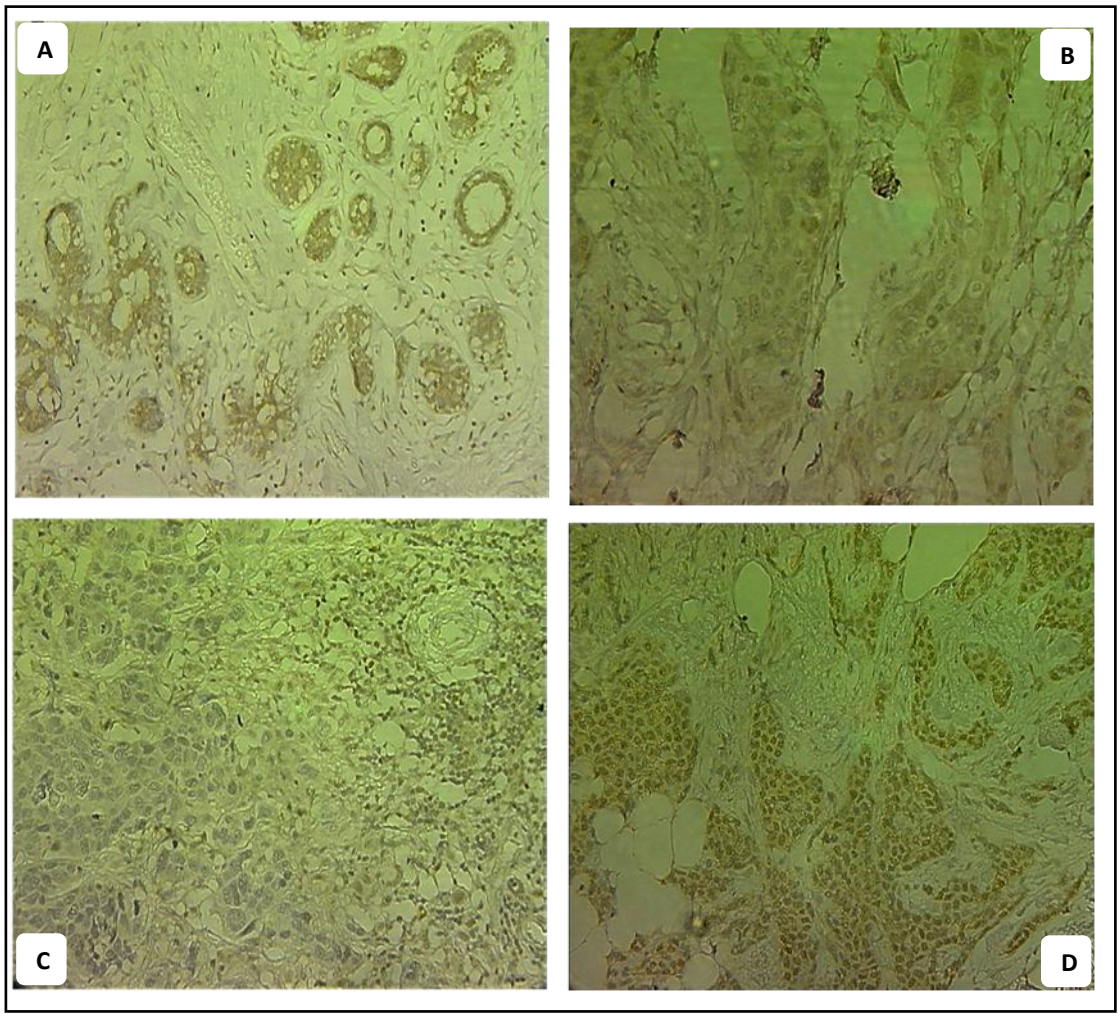

Figure 7.9 IHC of 26S proteasome protein. A: Positive cytoplasmic staining in normal breast lobules x200. B: Weak cytoplasmic staining in invasive cancer, classified as negative x200. C: Negative cytoplasmic staining in invasive breast cancer tissue (left), and positive cytoplasmic staining in lymphocytes (right) x200. D: Positive staining in invasive cancer tissue x200.

A total of 12/14 (85%) RR samples demonstrated decreased expression of the 26S proteasome in the invasive carcinoma compared with 5/14 (35%) samples from the radio-sensitive group.

Down-regulation of the protein expression was significantly associated with occurrence of LRR and therefore with resistance to RT (Table 7.10), *p* value of 0.018.

Table 7.10 Cytoplasmic expression of 26S proteasome in breast cancer clinical series using IHC. Significant Down-regulation of the 26S Proteasome associated with the radioresistant group; *p*=0.018 (2-sided Fisher's exact test).

	Negative (low expression)	Positive (high expression)	Total	p value
Radioresistant	12	2	**14**	**0.018**
Radiosensitive	5	9	**14**	
Total	**17**	**11**	**28**	

7.5.4 Summary of IHC results

IHC was performed on eight proteins to identify the association between them and the radioresistant samples. We have successfully identified the association between RR phenotypes and five potential protein markers; DR4, 26S proteasome, KPNA2, zyxin and PIASx (Table 7.11). We were not able to successfully utilise GFI-1, UBAE1 and Chk1, and the relevant antibodies would need to be further optimised.

A significant association between RR and the under-expression of DR4 and 26S proteasome proteins has been identified. No association was found between the RR samples and zyxin, PIASx or KPNA2.

7.6 Discussion

Clinical validation of eight proteins that were identified as potential markers of radioresistance was attempted. The IHC technique was successfully utilised as outlined in the methods section.

Table 7.11 Summary of AbMA, WB and IHC results. IHC was attempted in eight proteins, and the association with radioresistance was defined in five proteins; DR4, 26S proteasome (26S P), KPNA2, Zyxin and PIASx. *Recurrence vs. recurrence free, representing radioresistant and radiosensitive populations respectively. The arrows in the breast cancer tissue indicate significant down-regulation in the radioresistant group.

	AbMA					**WB**			**IHC**
	MCF7 **RR**	MDA **RR**	T47D **RR**	PJ41 **RR**	PJ49 **RR**	MCF7 **RR**	MDA **RR**	T47D **RR**	**Breast cancer tissue***
Zyxin	↓3.3	↓3.0	↓3.5	ns	ns	↓4.2	ns	ns	ns
PIASx	Ns	↓2.3	↓2.7	ns	ns	ns	↓9.8	ns	ns
DR4	↓4.1	ns	↓5.1	ns	ns	↓3.9	ns	ns	↓
GFI-1	↓2.5	↓2.4	↓3.6	ns	ns	Not optimised			Not optimised
KPNA2	↓3.5	ns	↓2.1	↓2.7	↓2.2	Not optimised			ns
Chk1	ns	↑2	ns	ns	ns	Not tested			Not optimised
26S P	-	-	-	-	-	Not tested			↓
UBAE1	-	-	-	-	-	Not tested			Not optimised

The eight proteins were chosen after careful consideration of their known associations and roles in cellular proliferation and /or apoptosis. The frequency of differential expression in the AbMA experiments was also considered. In addition, two proteins, 26S proteasome and UBAE1 were selected without being part of the first two phases of the project.

Trials of antibody optimisation were not successful with regards to GFI-1, UBAE and Chk1. Optimisation of the GFI-1 antibodies was not successful in both WB and IHC. GFI-1 has a well documented role in promoting cell survival. As GFI-1 was differentially expressed in three breast cancer lines using AbMA, it certainly constitutes a suitable

candidate for further analysis. Similarly Chk-1 antibody optimisation in IHC was not successful, but considering its well documented roles in cell cycle arrest, its further optimisation is recommended. UBAE1 is the triggering enzyme for the ubiquitin proteasome proteolytic pathway. Especially considering the confirmatory results of 26S proteasome, it would make a logical argument to further attempt UBAE1 analysis.

Zyxin has been identified as a potential biomarker of RR in AbMA (three breast cancer sublines), and in WB (one breast cancer subline). It failed to show any significant association with RR using IHC. Zyxin has been suggested to play important roles in promoting cellular apoptosis, in addition to its role in cellular adhesion, migration and mitosis. It down-regulation in the RR sublines would be in keeping with its pro-apoptotic role. Conversely, zyxin was shown to be a RIDEP, therefore suggesting that its differential expression might be secondary to a stress response. Our findings support this; zyxin was differentially expressed in a RT treated breast cancer subline, but not in the non treated breast cancer tissue. This would suggest that zyxin should be regarded as a differentially expressed stress protein. It would be interesting to confirm this by comparing pre-radiation breast tissue with tissue obtained from recurrence samples belonging to the same patient.

Importin-α (KPNA2) was not confirmed using WB due to technical issues, but was deemed an interesting candidate as it was the only protein differentially expressed across breast and oral cancer cell lines using the AbMA. It however, failed to show any significant association using IHC. Similarly, PIASx was confirmed as a putative biomarker using WB, but failed to show an association when using IHC.

The 26S proteasome had been identified as a potential biomarker of resistance using proteomic discovery techniques other than AbMA; 2D mass spectrometry. It had also

been identified as a potential marker of RR in laryngeal cancer tissue using IHC. It was therefore considered a suitable candidate for this project. Indeed, the 26S proteasome was found to be significantly associated with the RR breast cancer tissue samples. This is therefore a strong putative marker of resistance to RT, and further research on larger samples should be considered.

DR4 is a pro-apoptotic protein which is activated as part of the extrinsic pathway, and which results in the activation of the caspase pathway which leads to cell fragmentation and death. DR4 has passed through all stages of the biomarker discovery pipeline, being proven to be differentially expressed and associated with the radioresistant phenotype using AbMA, WB and IHC. It is therefore a strong putative biomarker of RR, and wider scale studies are recommended.

CHAPTER VIII

Discussion

Discussion

The aim of this project was to identify molecular protein markers associated with tissue resistance to radiation, and which can be used to guide treatment options in breast cancer. The study was designed based on the biomarker discovery pipeline, which involves screening for putative markers, followed by confirmation and finally clinical validation.

For the first two phases, five cancer cell lines and their novel RR sublines were analysed for the differential protein expression. These constituted three breast cancer lines, and two oral cancer lines. The oral cancer cell lines were chosen for multiple reasons. RR sublines were already available. In addition, the study sought to identify biomarkers that would show differential expression across more than one cancer type. For future research, the oral cancer would pose an attractive model for IHC confirmation, as many patients with oral cancer are offered RT as the primary and only treatment modality.

A global technique is required to identify an array of markers that could potentially be associated with a certain disease process such as radioresistance. This could be a genomic, a transcriptomic or a proteomic technique. The latter has been identified as superior to gene and mRNA techniques (section 4.2). Different proteomic techniques have been identified and extensively utilised. The most well established and widely used high throughput proteomic technique is 2-D polyarylamide gel electrophoresis (2-DE) coupled with mass spectrometry. Proteins are separated based on their electrical charge, and secondly based on their molecular weight; thus defining and comparing their expression patterns in different protein samples. Mass spectrometry is an analytical technique that characterises these proteins based on their amino acid

sequence. This information can be used to identify the specific protein by testing against a protein database. The technique has many limitations. It is expensive, and requires a high level of expertise and training. Compared to AbMA, it takes a considerably longer time to complete, and consumes larger volumes of protein lysates. In addition, reproducibility can be poor, partly due to inherent differences in gels (Debernardi S et al. 2005). External proteins such as keratins can contaminate the gels, while low abundance and hydrophobic proteins may escape detection altogether (Debernardi et al. 2005, Rabilloud and Lelong 2011). AbMA is a relatively new technique, with promising results, and its high throughput yield makes it ideal for the screening phase. It is based on the same principles as DNA microarrays; the latter having being extensively, and successfully utilised (Sotiriou and Pusztai 2009), and recently introduced into clinical trials and the bedside (Mook et al. 2007, Rutgers et al. 2011, Kunz 2011).

AbMa has proved to be user friendly and time efficient. Satisfactory progress in the experimental procedure is ensured at every step through established minimal requirements that need to be satisfied before further progress can be made. Detailed data analysis is vital, but is well supported by an efficient software programme. This guides all the human applied steps, and ensures error is kept to a minimum. The most operator dependant, and time consuming step, is the manual feature manipulation to adjust the different spots to the gridded areas which will later be analysed. This has to be performed with utmost accuracy to exclude background noise and ensure correct analysis. Robotic deposition of nanolitre quantities of antibodies to the slide surface is supposed to produce uniform application. However, unexplained variations in spot quality and shape can be attributed to this process, and constitute a step which cannot

be altered through experimental design. The nitrocellulose coated slide has a 3-D surface. This increases the binding capacity, but can lead to inconsistency in sample deposition and spot morphology.

The antibodies on the array correspond to proteins belonging to a wide array of cellular processes, including apoptosis, cell signalling, cell adhesion and proliferation. The comparatively small number (725), does however exclude many known proteins (estimated at approximately 100,000), and even much more unknown ones. This therefore, limits the remainder of the discovery pipeline to those specific proteins. However, self assembly of chosen proteins, as is practised by certain institutions, would limit the “discovery” nature of this process. Fluorescent dyes may interfere with the antigen antibody interactions. They do however constitute the most superior agents in labelling technology because of the high resolution staining that they provide.

Global proteomic techniques tend to produce false positive results, and confirmation using methods such as western blotting is vital. In addition to excluding false positive results, it is possible that some markers fail the confirmation stage secondary to experimental design differences. The exact discordance rate between AbMA and WB is not known, as studies tend to report on the confirmed proteins only and a list of the non confirmed ones is rarely produced. In this project, cell culture was carried out simultaneously for AbMA and WB. Protein extraction was carried out using two different extraction buffers. These could have different strengths, and may therefore yield different protein pools to start with. The freeze-thaw cycles for proteins and antibodies were kept to a minimum. Similarly, antibodies were divided into aliquots to avoid repeated thawing. WB uses a heat denatured protein sample. This process can

expose different epitopes through unfolding of the protein structure thus leading to different antigen antibody interactions. It is possible that protein expression profiles change depending on phase of the cell cycle. Extracting protein at different times within a given 24 hour period may thus increase the AbMA protein yield. In addition, a direct correlation between protein quantity and protein activity may not always exist.

All proteomic techniques have been used in the *in vitro* setting, and mainly using cell lines. Transfer to clinical practice might prove challenging as the biological microenvironment is certain to produce differences in protein behaviour. A bridging technique is IHC, which demonstrates the importance of clinical validation using human tissue. Reproducing the disease microenvironment remains a challenge in routine research work. Another approach to confirming protein expression is the use of functional assays which demonstrate protein functions and interactions, thus leading to linking different proteins and identifying pathways of disease.

AbMA has yielded a total of 63 potential markers of RR. The most frequently differentially expressed proteins were further assessed through examining the literature and some were chosen for verification using WB. This step was limited by the availability and the quality of commercially available antibodies. Three proteins were confirmed using WB; zyxin, PIASx and DR4.

Immunohistochemistry and archival breast cancer tissue was utilised for clinical validation. The samples were subject to strict clinical criteria to represent a radiosensitive (control - disease free at 10 years) and a radioresistant (test - recurrence within 4 years) phenotype. The main difference between this and the model used in the first two phases was that the archival tissue in the test sample had not been exposed to RT. Any protein expression would therefore reflect an inherent RR state.

Should a molecular marker be recognised for clinical applications, it would be examined in pre-treatment biopsies obtained during the original diagnostic work up. This protein expression in the archival tissue would therefore represent such a state. On the other hand, the protein expression in the RR sublines possibly represents the state of the breast cancer cells after completion of the radiation treatment, and therefore, the state at which recurrences are expected to develop. The differential expression of zyxin might represent the above situation. This protein was differentially expressed in the RR sublines, but not in the archival tissue. It could perhaps contribute to RR after RT is completed, and therefore recurrences. Zyxin, however, has been identified as a potential marker of all states of cellular stress, and might not be associated with RT only.

The 26S proteasome is an important proteolytic protein that is capable of degrading, and therefore, controlling a large number of cellular proteins, of which a few are identified. These include pro-apoptotic proteins, and regulators of the cell cycle. The 26S proteasome can thus affect the response to radiation, depending on which effecter proteins are affected by increased or decreased proteolysis. For example, an increase in the level of caspase enzymes and p53 may encourage cancer cell apoptosis, and encourage the effects of radiation. However, an increase in the cyclin/cdk complexes may encourage cellular progression through the cell cycle and increased proliferation. Under-regulation of components of the 26S proteasome had previously been associated with RR using 2D-MS and expression microarrays. Under-regulation of the whole 26S proteasome had also been associated with RR. This association was confirmed using IHC and archival breast cancer tissue. The 26S proteasome is a promising biomarker of RR and further research is warranted. As a first step, IHC

studies on a larger cohort of tissue would affirm the above results. Further research into the specific functions and interactions of the 2S proteasome would identify closely related proteins that could also be implicated in RR. In addition, it would enable research into possible therapeutic agents.

DR4 has a well documented role in promoting apoptosis, and under-regulation may thus decrease apoptosis and encourage cell survival and proliferation. DR4 is known to function in the extrinsic pathway, but this does not preclude the possibility that its differential expression may disturb the balance of the whole process, including the intrinsic pathway. DR4 has emerged as a strong putative biomarker of RR, as it was the only protein that satisfied all three phases of the biomarker discovery pipeline.

Most patients with breast cancer present in the early stages, and the standard treatment constitutes BCT. Although RT has improved the outcomes of BCT, postoperative recurrence still constitutes a significant problem, and is associated with considerable morbidity, mortality and high cost implications. RT also forms an integral part of the treatment strategies for many cancers; whether in the adjuvant, neo-adjuvant, palliative setting, or even as the only treatment option. Failure of RT treatment could have multiple aetiological factors, one of which is radioresistance. Although numerous studies exist, especially examining the significance of different molecular pathways in the aetiology of RR, a clear understanding of the exact mechanism remains to be identified. Numerous members of these signalling pathways and potential biomarkers have previously been tested for their association with RR. However, none exists in routine clinical practice, as yet, and further research is required. Isolating such markers would potentially allow the preoperative identification of patients who are unlikely to respond to radiation treatment. This

would then enable the treating physicians to address this issue, e.g. administering an alternative form of therapy.

Identifying molecular biomarkers of the response to RT can be a huge step towards individualising the treatment of breast cancer, and potentially other cancers. If such markers are identified in the pre-treatment stage; by testing core biopsies obtained in the original diagnostic work-up, patients can be informed that their disease might not respond to radiation, and therefore they could be offered other treatment modalities that otherwise would not have been indicated. It would also enable the patients to avoid an un-necessary form of treatment which has many side effects. A marker of RR need not be restricted to breast cancer, but can potentially be responsible for radiation failure in treating other types of cancer. The majority of patients with early stage breast cancer are offered BCT only. If markers of RR are identified, patients can be offered more aggressive treatments in the first instance, such as mastectomy. This would improve local control, and decrease recurrence rates. Alternatively, radiosensitisers can be added to RT in these patients to try and produce a more effective therapeutic result. The field of biomarker research has a huge potential for improving oncological outcomes and enhancing patients' quality of care, in breast and other cancers.

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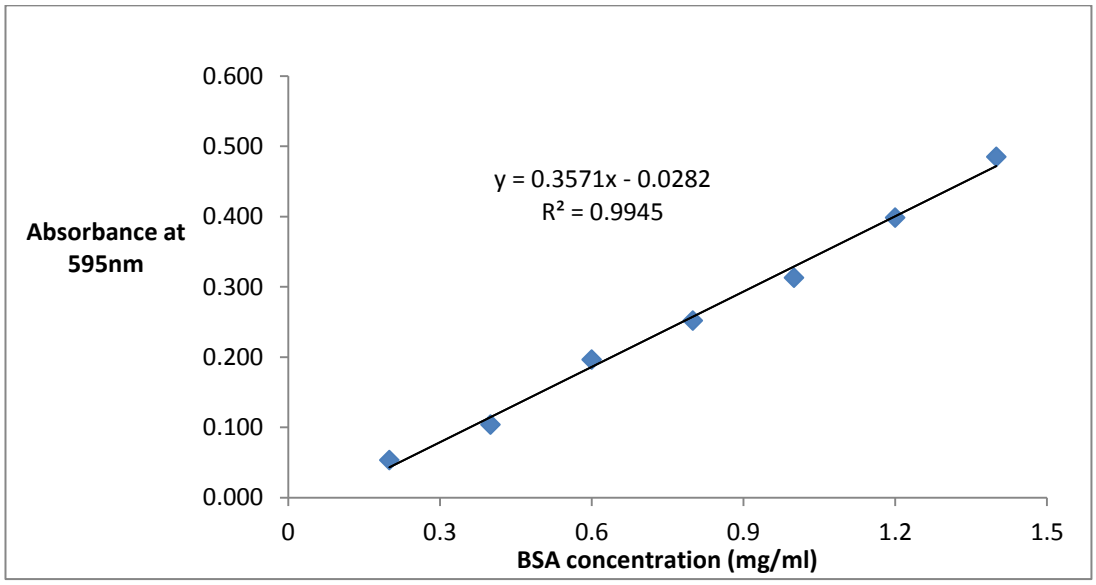
Appendix A: AbMA protein calculations

The following tables and diagram demonstrate an example of the calculation of the un-labelled and labelled protein concentration for the MCF-7 **RR** subline.

Bradford assay un-labelled protein quantification.

	Blank	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Blank	**0.000**	**0.029**	**0.054**	**0.102**	**0.192**	**0.244**	**0.323**	**0.410**	**0.480**
Blank	**0.004**	**0.030**	**0.053**	**0.106**	**0.201**	**0.260**	**0.303**	**0.387**	**0.490**
Neat	**0.477**	**0.460**	**0.441**						
1:5	**0.099**	**0.099**	**0.101**						

Standard curve of absorbance versus BSA concentration



Calculating the protein concentration using the equation y=mx+c

Sample	Absorbance of unknown (y)	Concentration of unknown (x) mg/ml	Final Conc (mg/ml)	Average (mg/ml)
T neat	0.477	1.4790	1.478966346	1.42588141
T neat	0.460	1.4279	1.427884615	
T neat	0.441	1.3708	1.370793269	
T 1.5	0.099	0.3431	1.715745192	1.725761218
T 1:5	0.099	0.3431	1.715745192	
T 1:5	0.101	0.3492	1.745793269	

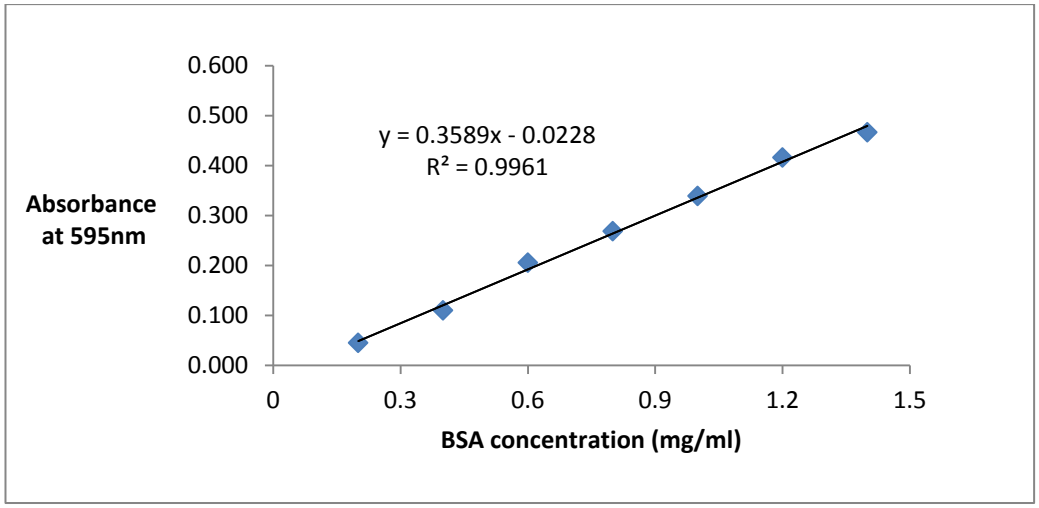
The final protein concentration of un-labelled MCF-7**RR** was found to be **1.575 mg/ml.**

The protein lysates were each diluted in Buffer A to achieve a concentration of 1 mg/ml, and the labelling step performed as described in the methods section. The labelled samples were then re-quantified using the Bradford Assay as described. Only neat samples were used, as prior knowledge of the concentrations makes the use of a 1:5 dilution unnecessary.

MCF-7 RR labelled with Cy5 Bradford assay protein quantification

	Blank	**0.1**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**1.2**	**1.4**
Blank	**0.000**	**0.023**	**0.046**	**0.113**	**0.199**	**0.262**	**0.351**	**0.417**	**0.469**
Blank	**0.004**	**0.018**	**0.044**	**0.107**	**0.212**	**0.275**	**0.327**	**0.415**	**0.464**
Neat	**0.409**	**0.392**	**0.378**						

Standard curve of absorbance versus BSA concentration



Calculating the protein concentration using the equation y=mc+x

Sample	Absorbance of unknown (y)	Concentration of unknown (x) mg/ml	Final Conc (mg/ml)	Average (mg/ml)
T neat	0.409	1.2031	1.203120646	1.158539983
T neat	0.392	1.1558	1.155753692	
T neat	0.378	1.1167	1.116745612	

The final concentration MCF-7**RR** Cy5 labelled= **1.16 mg/ml**.

ABMA; Five cancer lines and their radioresistant counterparts, with the corresponding D/P values and slide scanning details.

	MCF-7	MCF-7 RR	MDA	MDA RR	T47D	T47D RR	PJ-41	PJ-41 RR	PJ-49	PJ-49 RR
D/P ratio	2.53	3.08	2.96	3.80	2.55	2.95	4.81	4.87	5.9	4.3
PMT gains	270/220		290/230		280/220		280/210		280/210	
Count ratio	1		1		0.9		0.88		1.04	

Appendix B: AbMA protein list and AbMA slide details

Panorama AbMA XPRESS Profiler725 - protein list- lot 038k4787

ANTIBODY	*SIGMA No.*
_14-3-3 θ/τ	T5942
Acetylated Protein	A5463
Actin	A5060
Actin	A3853
Actin, α-Smooth Muscle	A5228
β-Actin	A1978
β-Actin	A2228
α-Actinin	A5044
Actopaxin	A1226
ADAM-17 (TACE), C-Terminal	T5442
β1 and β2-Adaptins	A4450
I-Afadin	A0349
AFX	A8975
AFX (FOXO4)	A5854
AKR1C3	A6229
Aly	A9979
β-Amyloid	A8354
Amyloid Precursor Protein, C-Terminal	A8717
Amyloid Precursor Protein, N-Terminal	A8967
Amyloid Precursor Protein, KPI Domain	A8842
Androgen Receptor	A9853
Annexin V	A8604
Annexin VII	A4475
Anti Cy3+Cy5	
AOP1	A7674
AP-1	A5968
AP-2α	A0844
AP Endonuclease	A2105
Apaf1, N-Terminal	A8469
Apoptosis Inducing Factor (AIF)	A7549
APRIL, Extracellular Domain	A1726
APRIL, Extracellular Domain 2	A1851
ARC, C-Terminal	A8344
ARNO (Cytohesin-2)	A4721
Arp1α/Centractin	A5601
ARP2	A6104
ARP3	A5979
ARTS	A3720
ARTS	A4471
ASAP1/Centaurin β4	A4227
ASC-2	A5355
ASPP1	A4355
ASPP2	A4480
ATF-1	A7833
ATF2	A4086
phospho-ATF-2 (pThr^{69,71})	A4095
ATM	A6093
Anti Cy3+Cy5	
ATM	A6218
Aurora-B	A5102
BACE-1	B0806
BACH1	B1310
BAD	B0559
BAF57	B0436

ANTIBODY	*SIGMA No.*
BAK	B5897
BAP1	B9303
Bax	B3428
Bax	B8429
Bax	B8554
Bax	B9054
Bcl-10	B7806
Negative Control	
Bcl-10	B0431
Negative Control	
Bcl-2	B9804
Bcl-2	B3170
Bcl-x	B9304
Bcl-x_L	B9429
BID	B4305
BID	B3183
Bim	B7929
Anti Cy3+Cy5	
BLK	B8928
Bmf, N-Terminal	B1684
Bmf, C-Terminal	B1559
BNIP3	B7931
BOB.1/OBF.1	B7810
Brg1/hSNF2β	B8184
BTK, C-Terminal	B0811
BTK, N-Terminal	B0686
BUB1	B0561
BUBR1	B9310
c-Abl	A5844
c-Cbl	C9603
c-erbB-2	E2777
c-erbB-3	E8767
c-erbB-4	E5900
phospho-c-Jun (pSer^{63})	J2128
phospho-c-Jun (pSer^{73})	J2253
c-Myc	M4439
c-Myc	C3956
Uvomorulin/E-Cadherin	U3254
N-Cadherin	C2542
N-Cadherin	C2667
Pan Cadherin	C1821
Anti Cy3+Cy5	
Calbindin-D-28K	C7354
Calcineurin (α-Subunit)	C1956
Caldesmon	C6542
Calmodulin	C7055
Calnexin	C4731
Calponin	C2687
Calreticulin	C4606
Calretinin	C7479
Claspin	C7867
CaM Kinase IV (CaMKIV)	C2851
CaM Kinase Kinase α (CaMKKα)	C7099
CaM Kinase IIα (CaMKIIα)	C6974
CaM Kinase IV (CaMKIV)	C9973
CASK/LIN2	C4856
Casein Kinase 2β	C3617

ANTIBODY	*SIGMA No.*
Caspase 2	C7349
Caspase 3	C9598
Caspase 3, Active	C8487
Caspase 4	C4481
Caspase 4	C3392
Caspase 5	C6979
Caspase 6	C7599
Caspase 7	C7724
Anti Cy3+Cy5	
Caspase 7	C1104
Caspase 8	C3101
Caspase 8	C2976
Caspase 8	C4106
Pro-Caspase 8	C7849
Caspase 9	C7729
Caspase 9	C4356
Caspase 10	C8351
Caspase 10	C1229
Caspase 11	C1354
Caspase 12	C7611
Caspase 13 (ERICE)	C8854
Catalase	C0979
α-E-Catenin	C8114
α-N-Catenin	C8239
α–Catenin	C2081
β-Catenin	C7207
β-Catenin	C7082
phospho-β-Catenin (pThr^{41})	C8616
phospho-β-Catenin (pSer^{33}/pSer^{37})	C4231
phospho-β-Catenin (pSer^{45})	C5615
phospho-β-Catenin (pSer^{33})	C2363
δ-Catenin/NPRAP	C4864
Anti Cy3+Cy5	
Cathepsin D	C0715
Cathepsin L	C2970
Caveolin-1	C3237
CD40	C5987
Cdc14A	C2238
Cdc25c	C0349
Cdc25A	C9479
Cdc27	C7104
Cdc6	C0224
Cdc7 Kinase	C6613
Cdh1	C7855
Cdk1^{p34cdc2}	C4973
Cdk3	C9987
Cdk4	C8218
Cdk6	C8343
Cdk-7/cak	C7089
Negative Control	
CENP-E	C7488
Centrin	C7736
Chk1	C9358
Chk2	C9108
Chk2	C9233
Chondroitin Sulfate	C8035
Anti Cy3+Cy5	
Ciliated Cell Marker	C5867
CIN85	C8116
Casein Kinase 2α	C5367
Clathrin Light Chain	C1985
Clathrin Heavy Chain	C1860
CNPase	C5922
Cofilin	C8736
Coilin	C1862

ANTIBODY	*SIGMA No.*
Collagen, Type IV	C1926
Connexin 32	C3470
Negative Control	
Connexin- 32	C6344
Connexin- 43	C8093
Connexin- 43	C6219
β-COP	G6160
Cortactin	C6987
Corticotropin Releasing Factor	C5348
COX II	C9354
Crk-L	C0978
Crk II	C0853
Csk	C7863
CtBP1, N-Terminal	C9491
CtBP1, C-Terminal	C8741
Anti Cy3+Cy5	
CUG-BP1	C5112
Cyclin A	C4710
Cyclin B_1	C8831
Cyclin D_1	C5588
Cyclin D_1	C7464
Cyclin D_2	C7339
Cyclin D_3	C7214
Cyclin H	C5351
Cystatin A	C3095
Cytohesin-1	C8979
Cytokeratin peptide 4	C5176
Cytokeratin CK5	C7785
Cytokeratin peptide 7	C6417
Cytokeratin 8.12	C7034
Cytokeratin 8.13	C6909
Cytokeratin peptide 13	C0791
Cytokeratin Peptide 17	C9179
Cytokeratin peptide 18	C1399
Cytokeratin peptide 19	C6930
Pan Cytokeratin	P2871
DAPK	D2178
phospho-DAPK (pSer^{308})	D4941
DAP Kinase 2	D3191
Anti Cy3+Cy5	
Daxx	D7810
DcR1	D3566
DcR2	D3188
DcR3	D1814
DEDAF	D3316
Desmin	D1033
Desmosomal Protein	D1286
Destrin/ADF	D8940
Dnase I	D0188
Dnase II	D1689
DNMT1	D4567
DNMT1	D4692
DOPA Decarboxylase	D0180
DP2	D7438
DR3	D3563
Negative Control	
DR4	D3813
DR5	D3938
DR6	D1564
DRAK1	D1314
Dystrophin	D8168
Dystrophin	D8043
E2F1	E9026
Anti Cy3+Cy5	
E2F1	E8901

ANTIBODY	*SIGMA No.*
E2F2	E8776
E2F3	E8651
E2F4	E8526
E6AP	E8655
EGF receptor	E3138
ERK5 (Big MAPK-BMK1)	E1523
Elastin	E4013
ELKS	E4531
Endothelial Cell Protein C Receptor	E6280
Endothelial Cells	E9653
Endothelin	E0771
Epidermal Growth Factor	E2520
Episialin (EMA)	E0143
ERP57	E5031
Estrogen Receptor	E0521
Estrogen Receptor	E1396
Exportin T	E1531
Ezrin	E8897
F1A	F3428
FADD	F8053
Focal Adhesion Kinase (pp125^{FAK})	F2918
FAK Phospho (pSer^{772})	F9051
Anti Cy3+Cy5	
phospho-FAK Phospho (pSer^{910})	F9301
phospho-FAK (pTyr^{397})	F7926
phospho-FAK (pTyr^{577})	F8926
Falkor/PHD1	F5303
Fas (CD95/Apo-1)	F4424
Fas Ligand	F2051
Fas Ligand	F1926
FBI-1/PAKEMON	F9429
Fibroblast Growth Factor-9	F1672
Fibronectin	F0791
Fibronectin	F3648
Fibronectin	F7387
Filamin	F1888
Filensin	F1043
FKHR (FOXO1a)	F6928
FKHRL1 (FOXO3a)	F2178
FKHRL1 (FOXO3a)	F1304
FLIPγ/δ, C-Terminal	F9925
FOXC2	F1054
FOXP2	F6304
FANCD2	F0305
FXR2	F1554
FRS2 (SNT-1)	F9052
Anti Cy3+Cy5	
G9a Methyltransferase	G6919
Glutamic Acid Decarboxylase 65 (GAD 65)	G4913
Glutamic Acid Decarboxylase 65 (GAD 65)	G5038
Glutamic Acid Decarboxylase (GAD65/67)	G5163
GADD 153 (CHOP-10)	G6916
GAP1^{IP4BP}	G6666
GAPDH	G8795
GATA-1	G0290
Gelsolin	G4896
Gemin 2	G6669
Gemin 3	G6544
GFAP (Glial Fibrillary Acidic Protein)	G9269
GFAP (Glial Fibrillary Acidic Protein)	G3893
Growth Factor Independence-1 (GFI)	G6670
Glutamate receptor NMDAR 2a	G9038
Glutamine Syntethase	G2781
Glycogen Synthase Kinase3β (GSK-3β)	G7914
Glycogen Synthase Kinase-3 (GSK-3)	G4414

ANTIBODY	*SIGMA No.*
Glycogen Synthase Kinase-3 (GSK-3)	G6414
Granzyme B	G1044
Grb-2	G2791
GRK 2	G7670
GRP1	G6541
Anti Cy3+Cy5	
GRP 75	G4170
GRP78/BiP	G8918
GRP94	G4420
hABH1	A8103
hABH2	A8228
hABH3	A8353
hBRM/hSNF2α	H9787
HAT1 (Histone acetyltransferase 1)	H7161
HDAC-1	H3284
HDAC-1	H6287
HDAC-2	H3159
HDAC-2	H2663
HDAC-3	H6537
HDAC-3	H3034
HDAC-4	H9411
HDAC-4	H9536
Negative Control	
HDAC-5	H4538
HDAC-5	H8163
HDAC-6	H2287
HDAC-7	H2537
HDAC-7	H6663
HDAC-8	H6412
Anti Cy3+Cy5	
HDAC-10	H3413
HDAC-11	H2913
HDRP/MITR	H9163
Heat Shock Factor 1	H4163
Heat Shock Factor 2	H6788
Heat Shock Protein 25	H0148
Heat Shock Protein 27	P1498
Heat Shock Protein 27/25	H2289
Heat Shock Protein 70	H5147
Heat Shock Protein 90	H1775
Heat Shock Protein 110	H7412
Heat Shock Protein 110	H7287
Acetyl Histone H3 (Ac-Lys^{9})	H9286
Acetyl Histone H3 (Ac-Lys^{9})	H0913
Acetyl- & phospho-Histone H3 (Ac-Lys^{9}, Ser^{10})	H9161
Acetyl- & phospho-Histone H3 (Ac-Lys^{9}, Ser^{10})	H0788
Dimethyl Histone H3 (diMe-Lys^{4})	D5692
Dimethyl Histone H3 (diMe-Lys^{9})	D5567
methyl-Histone H3 (Me-Lys^{9})	H7162
phospho-Histone H2AX (pSer^{139})	H5912
phospho-Histone H3 (pSer^{10})	H6409
phospho-Histone H3 (pSer^{28})	H9908
phospho-Histone H3 (pSer^{10})	H0412
Anti Cy3+Cy5	
SUV39H1 Histone Methyl Transferase	S8316
HMG-1	H9537
hMps1	M5818
hnRNP-A1	R4528
hnRNP-A1	R9778
hnRNP-A2/B1	R4653
hnRNP-C1/C2	R5028
hnRNP-K/J	R8903
hnRNP-L	R4903
hnRNP-Q	R5653
hnRNP-U	R6278
hnRNP M3-M4	R3777

ANTIBODY	*SIGMA No.*
hPlk1	P5998
hPlk1	P6123
hSNF5/INI1	H9912
iASPP	A4605
IFI-16	I1659
IκBα	I0505
IKKα	I6139
ILK	I0783
ILK	I1907
ILP2	I4782
Negative Control	
Anti Cy3+Cy5	
Importin-α1	I9658
Importin-α3	I9783
Importin-α5/7	I9908
INCENP	I5283
ING1	I3659
α-Internexin	I0282
JAB 1	J3395
JAB 1	J3020
JAK 1	J3774
c-Jun N-Terminal Kinase	J4500
JNK, Activated (Diphosphorylated JNK)	J4750
KCNK9 (TASK-3)	K0514
Kaiso	K4263
KIF17	K3638
KIF3A	K3513
KSR	K4261
Ku Antigen	K2882
L1CAM	L4543
l/s-Afadin	A0224
Laminin	L9393
Laminin-2 (α-2 Chain)	L0663
LAP2 (TMPO)	L3414
Leptin	L3410
Anti Cy3+Cy5	
LIM Kinase 1	L2290
LIN-7	L1538
LIS1	L7391
LKB1	L7917
LDS1	L4793
Mad1	M8069
Mad2	M8694
MADD	M5683
MAFF	M8194
MAGI-1	M5691
MAGI-2	M2441
MAP Kinase, Activated/Monophosphorylated (Phosphothreonine ERK-1&2)	M7802
MAP Kinase, Monophosphorylated Tyrosine	M3682
MAP Kinase, Activated (Diphosphorylated ERK-1&2)	M9692
MAP Kinase, Monophosphorylated Threonine	M3557
MAP Kinase (ERK-1)	M7927
MAP Kinase (ERK1+ERK2)	M5670
MAP Kinase Activated Protein Kinase-2 (MAPKAPK-2)	M3550
MAP Kinase Phosphatase-1 (MKP-1)	M3787
MAPK non phosphorylated ERK	M3807
MAP Kinase 2 (ERK-2)	M7431
MAP Kinase Kinase (MEK, MAPKK)	M5795

ANTIBODY	*SIGMA No.*
MAP2 (2a+2b)	M2320
Anti Cy3+Cy5	
MAP1	M4278
MAP1 (Light Chain)	M6783
MAP1b	M4528
MAP2	M9942
MBD1	M6569
MBD2a	M7568
MBD2a,b	M7318
MBD4	M9817
MBDin/XAB1	M1944
MBNL 1	M3320
MCH	M8440
Mcl-1	M8434
MDC1	M2444
MDM2	M8558
MDM2	M4308
MDM2	M7815
MDMX	M0445
MeCP2	M9317
MeCP2	M7443
MeCP2	M6818
MEKK4	M7194
Melanocortin-3 Receptor	M4937
MGMT	M3068
Anti Cy3+Cy5	
Mint2	M3319
MRP1	M6565
MRP1	M9192
MRP2	M3692
α-MSH	M0939
MSH6	M2445
MSH6	M2820
MSK-1	M5437
MTA 2	M7569
MTA1	M1320
MTA1	M7693
MTA2/MTA1L	M7818
MTA3L	M0819
MTBP	M3566
mTOR	T2949
Munc-18-1	M2694
Munc-13/1	M6194
MyD88	M9934
Myosin	M1570
Myosin Iβ (Nuclear)	M3567
Myosin IIA	M8064
Myosin IX/Myr5	M5566
Negative Control	
Anti Cy3+Cy5	
Myosin Light Chain Kinase	M7905
Myosin Va	M4812
Myosin Va	M5062
Myosin VI	M0691
Myosin VI	M5187
NBS1 (Nibrin)	N9287
NBS1 (Nibrin)	N3037
NBS1 (Nibrin)	N3162
Nck-2	N2911
Nedd 8	N2786
Nerve Growth Factor-β	N3279
Nerve Growth Factor Receptor	N5408
Nerve growth factor receptor (NGFR p75)	N3908
Neurabin I	N4412

ANTIBODY	*SIGMA No.*
Neurabin II (C-terminal)	N5037
Neurabin-II	N5162
Neurofibromin	N3662
Neurofilament 160	N2787
Neurofilament 200	N4142
Neurofilament 200	N0142
Neurofilament 200	N5389
Neurofilament 68	N5139
Neurofilament 160/200	N2912
Anti Cy3+Cy5	
NF-κB	N8523
NAK (NFκB-Activating Kinase)	N2661
NG2	N8912
Nicastrin	N1660
Nitric Oxide Synthase, Brain (b-NOS)	N2280
Nitric Oxide Synthase, Brain (b-NOS)	N7155
Nitric Oxide Synthase, Endothelial (e-NOS)	N9532
Nitric Oxide Synthase, Endothelial (e-NOS)	N3893
Nitric Oxide Synthase, Endothelial (e-NOS)	N2643
Nitric Oxide Synthase, Inducible (i-NOS)	N7782
Nitric Oxide Synthase, Inducible (i-NOS)	N9657
Notch1	N6786
Nitrotyrosin	N0409
NTF2	N9527
Nuf2	N5287
O-GlcNAc Transferase	O6264
OP-18/Stathmin	O0138
Ornithine Decarboxylase (ODC)	O1136
p115/TAP	P3118
$p120^{ctn}$	P1870
$p130^{CAS}$	C0354
$p14^{arf}$	P2610
$p16^{INK4a/CDKN2}$	P0968
Anti Cy3+Cy5	
$p19^{INK4d}$	P4354
p21WAF1/Cip1	P1484
p300/CBP	P2859
$p34^{cdc2}$	C3085
p35 (Cdk5 Regulator)	P9489
p38 MAP Kinase, Non-Activated	M8432
p38 MAPK	M0800
p38 MAPK activated (diphosphorylated p38)	M8177
Negative Control	
p53	P5813
p53	P6874
phospho-p53 ($pSer^{392}$)	P8982
p53DINP1/SIP	P4868
p53R2l	P4993
p53 BP1	B4561
p53 BP1	B4436
$p57^{kip2}$	P2735
p63	P3362
p63	P3737
PABP	P6246
PAD14	P4749
phospho-PAK1 ($pThr^{212}$)	P3237
Par-4 (Prostate Apoptosis Response-4)	P5367

ANTIBODY	*SIGMA No.*
Anti Cy3+Cy5	
γ Parvin	P5746
Parkin	P6248
PARP	P7605
Paxillin	P1093
PCAF	P7493
Proliferating Cell Nuclear Antigen (PCNA)	P8825
PDK 1	P3110
Pen-2	P5622
Peripherin	P5117
Peroxiredoxin 3	P1247
PERP	P5243
Phospholipase A2 group V	P5242
Phosphoserine	P5747
Phosphothreonine	P6623
Phosphotyrosine	P1869
Phospholipase C γ1 (PLC γ1)	P8104
PhosphatidylSerine Receptor (PSR)	P1495
Negative Control	
PIAS-x	P9498
PID/MTA2	P5118
PINCH-1	P9371
Protein Kinase Bα /Akt1	P2482
Protein Kinase Bα /Akt1	P1601
Anti Cy3+Cy5	
phospho-PKB ($pSer^{473}$)	P4112
phospho-PKB ($pThr^{308}$)	P3862
Protein Kinase C (PKC)	P5704
Protein Kinase Cα	P4334
Protein Kinase $C\beta_1$	P3078
Protein Kinase $C\beta_1$	P6959
Protein Kinase $C\beta_2$	P3203
Protein Kinase $C\beta_2$	P2584
Protein Kinase Cγ	P8083
Protein Kinase Cδ	P8333
Protein Kinase Cε	P8458
Protein Kinase Cζ	P0713
Protein Kinase Cη	P8090
Protein Kinase D	P3987
PKR	P0244
Plakoglobin (Catenin γ)	P8087
Platelet-Derived Growth Factor Receptor β	P7679
Plectin	P9318
PML	P6746
Presenilin-1 (S182)	P7854
Prion Protein	P5999
PRMT1	P6871
PRMT1	P6996
Anti Cy3+Cy5	
PRMT2	P0748
PRMT3	P9370
PRMT4	P4995
PRMT5	P0493
PRMT6	P6495
PRMT6	P2996
Proliferating Cell Protein Ki-67	P6834
Protein Phosphatase 1α	P7979
Protein Phosphatase 1α	P7607
Protein Phosphatase 2Aα (PP2Aα)	P8998
Protein S	P4555

ANTIBODY	*SIGMA No.*
Protein Tyrosine Phosphatase PEST	P9109
PSF	P2860
PTEN	P7482
PTEN	P3487
PUMA/bbc3, C-Terminal	P4618
PUMA/bbc3, N-Terminal	P4743
Pyk2	P3902
phospho-Pyk2 (pTyr^{579})	P7114
phospho-Pyk2 (pTyr$^{579/580}$)	P6989
phospho-Pyk2 (pTyr^{580})	P6739
Negative Control	
Rab5	R7904
Anti Cy3+Cy5	
Rab 7	R8779
Rab9	R5404
RAD1	R5029
Rad17 (C-terminal)	R8029
Raf-1/c-Raf	R2404
Raf-1	R5773
phospho-c-Raf (pSer^{621})	R1151
RAIDD, Internal Domain	R9775
RAIDD	R5275
RALAR	R8529
Ran	R4777
Negative Control	
RAP1	R8154
RbAp48/RbAp46	R3779
Reelin	R4904
Retinoblastoma	R6775
phospho-Retinoblastoma (pSer^{795})	R6878
RhoE	R6153
RICK, C-Terminal	R9650
RIP (Receptor Interacting Protein)	R8274
RNase L	R3529
ROCK-1	R6028
ROCK-2	R8653
Anti Cy3+Cy5	
Rsk1	R5145
S-100	S2644
S-100 (α-Subunit)	S2407
S-100 (β-Subunit)	S2532
S-Nitrosocysteine	N5411
S6 Kinase	S4047
SAPK3	S0315
Spectrin (α and β)	S3396
Serine/Threonine Protein Phosphatase 2 A/A	P8109
Serine/Threonine Protein Phosphatase 1β	P7484
Serine/Threonine Protein Phosphatase 1γ1	P7609
Serine/Threonine Protein Phosphatase 2 A/Bγ	P5359
Serine/Threonine Protein Phosphatase 2 A/B′ pan2	P8359
Serine/Threonine Protein Phosphatase 2C α/β	P8609
Negative Control	
SGK	S5188
SH-PTP2 (SHP-2)	S3056
Siah2	S7945
Sin3A, N-terminal	S4445

ANTIBODY	*SIGMA No.*
Sin3A, C-Terminal	S6695
Sir2	S5313
SIRPα1 (SHPS-1)	S1311
Sirt1	S5196
Anti Cy3+Cy5	
SKM1 (Skeletal Muscle Type 1)	S9568
SKK2 (SAPKK2, MKK3, MEK3)	S5308
SLIPR/MAGI-3	S1190
SLIPR/MAGI-3	S4191
Smad4 (DPC4)	S3934
SMC1L1	S6446
SMN	S2944
α/β -SNAP, C-terminus	S9444
SNAP-23	S2194
SNAP-25	S9684
SNAP- 29	S2069
Sos1	S2937
Sp1	S9809
Spred-2	S7320
Striatin	S0696
Substance P Receptor	S8305
SMAC/Diablo	S0941
SUMO-1	S8070
SUMO-1 (C-terminal)	S5446
Survivin	S8191
Synaptotagmin	S2177
Synaptopodin	S9442
Synaptopodin	S9567
Anti Cy3+Cy5	
SynCAM	S4945
α1 Syntrophin	S4688
α1 Syntrophin	S4813
Syntaxin	S0664
Syntaxin 6	S9067
Syntaxin 8	S8945
α-Synuclein	S3062
Negative Control	
Tal	T1075
Tal	T1200
TAP	T1076
Tau	T9450
phospho-Tau (pSer$^{199/202}$)	T6819
Tau	T5530
Tenascin	T2551
Thimet Oligopeptidase 1	T7076
TIS7	T2576
Tumor Necrosis Factor Soluble Receptor II	T1815
Tob	T2948
TOM22	T6319
Topoisomerase-I	T8573
TRAIL	T3067
TRAIL	T9191
Anti Cy3+Cy5	
Transforming Growth Factor-β, pan	T9429
Transportin 1	T0825
TRF1	T1948
Tropomyosin	T2780
Tropomyosin (Sarcomeric)	T9283
Tryptophane Hydroxylase	T0678
TSG101	T5826

ANTIBODY	*SIGMA No.*
α–Tubulin	T6074
α–Tubulin	T6199
β-Tubulin	T5201
β-Tubulin I	T7816
β-Tubulin I+II	T8535
β -Tubulin III	T5076
β-Tubulin IV	T7941
γ–Tubulin	T5326
γ–Tubulin	T3559
γ -Tubulin	T3320
ε-Tubulin	T1323
Tubulin, Polyglutamylated	T9822
Tubulin, Tyrosine	T9028
Tumor Necrosis Factor-α	T8300
Tumor Necrosis Factor-α	T2824
Negative Control	
Anti Cy3+Cy5	
TWEAK Receptor/Fn-14	T9700
Tyrosin hydroxylase	T2928
$U2AF^{65}$	U4758
Ubiquitin	U0508
Ubiquitin C-terminal Hydrolase L1	U5133
Ubiquitin C-terminal Hydrolase L1	U5258
Negative Control	
Vanilloid Receptor-1	V2764
VDAC/Porin	V2139
Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1)	V4762
Vesicular GABA Transporter	V5764
VGLUT 1	V0389
VGLUT 2	V2639
Vimentin	V6389
Vinculin	V4505
Vitronectin	V7881
WAVE	W0392
WSTF	W3516
Y14	Y1253
ZAP-70	Z0627
Zip Kinase	Z0134
Zyxin	Z0377
GAPDH	G8795

AbMA: Summary of the slide analysis details for each of the five datasets (MCF7/MCF7RR, MDA/MDARR, T47D/T47DRR, PJ41/PJ41RR and PJ49/PJ48RR)

Substances matched denote those of superior quality that were included in the analysis. Substances matching the criteria denote proteins that were differentially expressed by more than 2 fold in the RR lysate.

	Substances matched	**Total substances**	**% Substances**	**Substances matching the criteria**
MCF7	724	766	95	21/724
MDA	716	766	93	9/716
T47D	695	766	91	24/695
PJ41	707	766	92	11/707
PJ49	707	766	92	13/707

Appendix C: Western blot buffers

Western blot extraction buffer:

4ml distilled water

1ml 0.5M Tris:HCl pH 6.8

0.8 ml glycerol

1.6 ml 10% SDS

200µl0.05%bromophenol blue

Western blot extraction buffer with enzyme inhibitors

10µl protease inhibitor (#806501-23, Amersham),

10µl phosphatase inhibitor I (#P2850, Sigma Aldrich)

10µl phosphatase inhibitor II (#P5726, Sigma Aldrich)

50µl β-mercaptoethanol (#M7522, Sigma Aldrich)

1ml of western extraction buffer