THE UNIVERSITY OF HULL

Identification of Predictive Biomarkers of Resistance to Radiotherapy in Rectal Cancer

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by

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I dedicate this thesis to the loving memories of my late mother, who passed away on 10th March, 2010.

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Published abstracts

1. **S Mehmood,** VC Hodgkinson, L Scaife, AW Beavis, IA Hunter, L Cawkwell. **Predictive biomarkers of radioresistance in rectal cancer identified by antibody microarray: a possible role for DR4 protein.** European Journal of Surgical Oncology (EJSO), 37 (11): 1011 (2011).

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4. L Scaife, VC Hodgkinson, D ELFadl, **S Mehmood,** IA Hunter, GP Liney, AW Beavis, PJ Drew, MJ Lind and L Cawkwell. **Proteomic identification of putative biomarkers of radiotherapy resistance.** Radiotherapy and Oncology 103 (suppl 1): S216-S217 (2012).

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Abstract

Introduction and Aims:

Neoadjuvant radiotherapy (RT) provides local control of disease in rectal cancer, however, the ability to predict response to RT is limited. The aim of this study was to establish a novel radioresistant (RR) rectal cancer cell line model and identify putative biomarkers of radioresistance using a microarray-based comparative proteomic platform.

Methods:

The inherent radiosensitivity of SW-837 and HRA-19 rectal adenocarcinoma parental cells was initially assessed. Following irradiation at 0, 2, 4, 6, 8 and 10 Grey (Gy) single doses using a linear accelerator, modified colony counting assays were performed. Dose response curves (DRCs) were plotted from calculated survival fraction (SF) values. To induce radioresistance, parental SW-837 and HRA-19 cells were irradiated in 8 and 4 Gy fractions, respectively, to a total dose of 48 Gy to generate novel sub-lines (termed SW-837 RR and HRA-19 RR). Following comparison of the DRCs from untreated parental cells and novel RR cells, total protein was extracted from radiosensitive parental (PN) cells and novel RR cells. Protein lysates from respective PN and RR cells were co-incubated onto Panorama antibody microarray (AbMa) slides containing 725 antibodies. Proteins which demonstrated at least 1.8-fold difference in expression between PN and RR cells were considered significant. The proteins with consistent differential expression and a putative role in radioresistance were selected for validation by immunohistochemistry (IHC), as a pilot study, in an archival series of 33 rectal cancer tissues categorised into

‘good response’ and ‘poor response’ groups based on tumour regression grading following long course chemoradiotherapy.

Results:

The comparison of DRCs revealed SW-837 RR cells to be significantly more radioresistant at 4, 6, 8 and 10 Gy and HRA-19 RR cells at 4, 6 and 8 Gy single doses than their respective parental cells ($p<0.05$). Comparative AbMa analyses of respective PN and RR cells demonstrated 62 differentially expressed proteins (DEPs) common to both RR cell lines. Of these, two apoptosis related proteins - DR4 and Bcl-2 were chosen for preliminary immunohistochemical validation for their putative role in radioresistance. The DR4 protein was found to be equally expressed whereas Bcl-2 demonstrated a trend towards reduced expression in 'poor-responder' rectal cancers compared with 'good-responders' that was not statistically significant.

Conclusions:

A radioresistant rectal cancer model consisting of novel SW-837 RR and HRA-19 RR cell sublines was successfully established and comparative proteomic analysis revealed a number of DEPs. In a small pilot IHC study, initial validation experiments showed no significant expression difference for DR4 and Bcl-2 proteins between ‘poor-responder’ and ‘good-responder’ rectal cancer patients. A panel of novel biomarkers from antibody array data would require further validation to determine their association with radioresistance before any firm conclusions can be drawn.

Table of Contents

Table of Figures and Tables

LIST OF TABLES

Abbreviations

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
AIF	Apoptosis Inducing Factor
AKR1B10	Aldo-Keto Reductase Family 1 Member B10
AML	Acute Myelogenous Leukemia
ATM	Ataxia-Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3-related kinase
BAX	Bcl Associated X protein
Bcl-2	B-cell Lymphoma/Leukemia
B-CLL	B-cell Chronic Lymphocytic Leukemia
BSA	Bovine Serum Albumin
CD	Crohn's Disease
CDC	Cell Division Cycle
CDK	Cyclin Dependent Kinase
CDKI	Cyclin Dependent Kinase Inhibitor
CIMP	CpG Island Methylator Phenotype
CRM	Circumferential Resection Margin
CRT	Chemoradiotherapy
CS	Catalytic Subunit
DCC	Deleted in Colon Cancer
DDR	DNA Damage Response
DEP	Differentially Expressed Protein
DFS	Disease Free Survival
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-dependent Protein Kinase
DPD	Dihydropyrimidine dehydrogenase
DSB	Double Strand Break
Dub	De-Ubiquitinising
E1	Ubiquitin Activating Enzyme
E2	Ubiquitin Conjugating Enzyme
E3	Ubiquitin Protein Ligase
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked-Immunosorbent Assay
FFPE	Formalin-Fixed Paraffin Embedded
Gy	Gray
HER2	Human Epidermal Growth Factor Receptor 2
HNC	Head and Neck Cancer
HRR	Homologous Recombination
HSP	Heat Shock Protein
IAP	Inhibitor of Apoptosis Protein
IBD	Inflammatory Bowel Disease
ICAM-1	Intercellular Adhesion Molecule 1

IHC	Immunohistochemistry
IkB	Inhibitor of NF-Kb
IKK	IKB Kinase
IκB	Inhibitor of NFκB
KIP1	Cyclin Dependent Kinase Inhibitor 1B
LC	Liquid Chromatography
LOH	Loss of Heterozygosity
MALDI	Matrix-Assisted Laser Desorption/Ionisation
MDM2	Ubiquitin Protein Ligase E3
MLH	MutL Homologue
MMP	Mitochondrial outer Membrane Permeabilisation
MMR	Mismatch repair
MnSOD	Manganese Superoxide Dismutase
MS	Mass Spectrometry
MSH	MutS Homologue
Mu	Monitor units
NFκB	Nuclear Factor Kappa B
NHEJ	Non Homologous End Joining
NPC	Nasopharyngeal Cancer
NSCLC	Non–Small Cell Lung Carcinoma
OS	Overall Survival
PARA	Pro-Apoptotic Receptor Agonist
PBS	Phosphate-Buffered Saline
pCR	Pathological Complete Response
PI-3K	Phosphatidylinositol 3-kinase
RIDEP	Repeatedly Identified Differentially Expressed Protein
RT	Radiotherapy
SDS	Sodium Dodecyl Sulphate
SMAC/DIABLO	Second Mitochondria-derived Activator of Caspases / Direct Inhibitor of Apoptosis Binding protein with Low pI
SNP	Single nucleotide polymorphism
SSB	Single Strand Break
TBS	Tris-Buffered Saline
TGF	Transfroming Growth Factor
TGFα	Transforming Growth Factor α
TM	Trans-membrane
TME	Total Mesorectal Excision
TNM	Tumour Node Metastasis
TOF	Time-Of-Flight
TP	Thymidine phosphorylase
TS	Thymidylate Synthase
Ub	Ubiquitin
UC	Ulcerative Colitis
UPS	Ubiquitin-Proteasome System
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor

CHAPTER-1: Introduction

1.1 Epidemiology

Colorectal cancer (CRC) is the third most common cancer worldwide and in the United Kingdom (UK) (Haggar and Boushey 2009; CancerResearchUK 2012). Most CRCs are distributed in the colon (64%) with a third diagnosed in the rectum and the rectosigmoid region (34%) (Figure 1.1) (CancerResearchUK 2012).

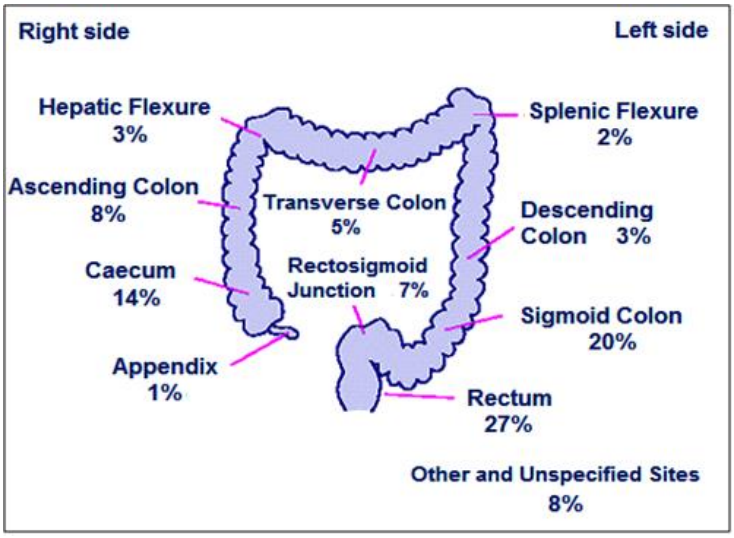

Figure 1.1: Distribution of CRC. A third of colorectal cancers are located in the rectum and the rectosigmoid region. (Source: Cancer research, UK)

In 2009, 41,142 new cases of CRC were registered in the UK. Rectal cancer represents approximately a third of the CRC. In 2009, 14,999 new cases of rectal cancer were registered in the UK. Of those, 8,975 were males and 6,024 were females (CancerResearchUK 2012).

In the UK, colorectal cancer is the second most common cause of cancer related deaths. In 2010, there were 16,013 deaths from CRC in the UK, 9,806 from the colon and 6,207 from the rectal cancer (CancerResearchUK 2012). The crude incidence and mortality rates of CRC, Colon, and Rectal cancer are shown in Table 1.1.

Table 1.1: Crude incidence and mortality rates of CRC in the UK.

Site of CRC	**Colon and Rectum**	**Colon cancer**	**Rectal cancer**
Crude Incidence Rates (per 100,000 population)			
Male	74.8	45.2	29.5
Female	58.7	39.5	19.2
Both	66.6	42.3	24.3
Mortality rates (per 100,000 population)			
Male	28.4	16.2	12.2
Female	23.1	15.3	7.8
Both	25.7	15.7	10

The incidence rates of CRC in the UK are estimated to be the 14th highest for men (56 per 100,00) and 12th highest for women (38 per 100,000) in Europe according to 2008 statistics (CancerResearchUK 2012). The highest European age standardised incidence rates for 2008 are estimated to be in Slovakia for men (91 per 100,000) and Denmark for women (50 per 100,000), while Greece has the lowest rates of CRC (24 per 100,000 for men and 17 per 100,000 for females) (Figure 1.2) (CancerResearchUK 2012). Such differences might be due to Mediterranean food consumption in Greece, and more meat and less fiber consumption in western European countries (La Vecchia 2004).

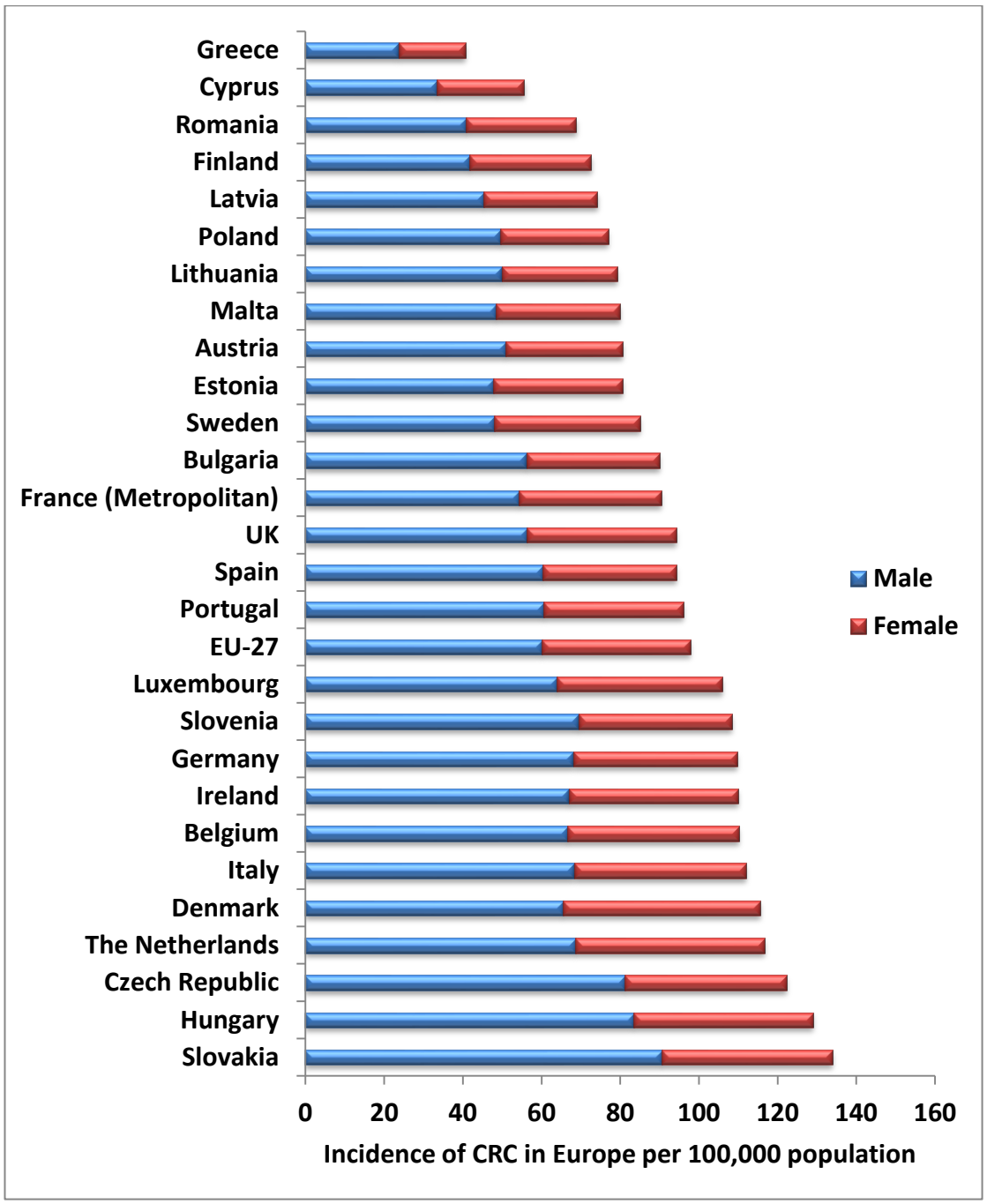

Figure 1.2: The European age standardised incidence of CRC. According to 2008 statistics, UK ranked 12th (women) and 14th (men) for age standardised incidence rates within 27 countries of the European Union.

1.2 Anatomy of the Rectum

The rectum begins where taenia coli of the sigmoid colon join to from a continuous outer longitudinal muscle layer (Williams, Bulstrode et al. 2008) (Figure 1.3). Its upper limit is indicated by the end of the sigmoid mesocolon

which is at the level of sacral promontory (ACPGBI_guidelines 2007). It has been agreed by the association of coloproctologists of GB & Ireland that any tumour whose distal margin is seen at 15cm or less from the anal verge using a rigid sigmoidoscope should be classified as rectal (ACPGBI_guidelines 2007) .

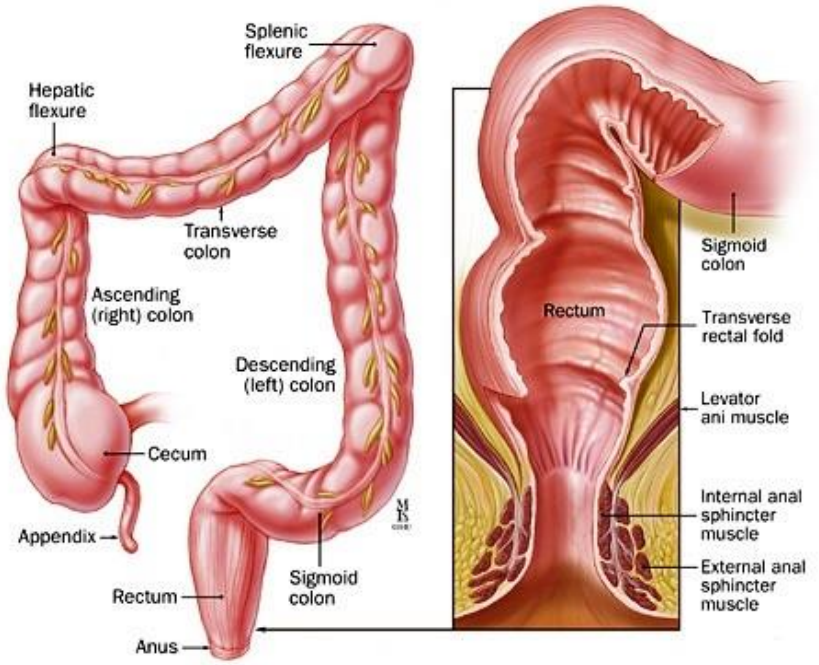

Figure 1.3: Normal anatomy of the colon and rectum. The rectum begins at the distal end of sigmoid colon, at the level of sacral promontory. (source: http://www.hopkins-gi.org/GDL_Disease)

The adult rectum may vary in length from 12 to 18 cm and is divided into three equal parts (Figure 1.4). The upper third is intraperitoneal, middle is covered by peritoneum only anteriorly and the lower third is extraperitoneal. The middle and lower third of the rectum are covered by the mesorectum laterally and posteriorly. The lower third lies in mesorectal fascia, separated in front from prostate or vagina by Denonvillier's fascia, and behind from sacrum and coccyx by Waldeyer's fascia. These fascial layers provide barriers to invasive cancer. The rectum lies in the curvature of the sacrum and has three side to side curves

that are marked as semi-circular folds on the luminal aspect, called the Houston's valves. Upper and lower curves are convex to the right, and the middle is convex to the left (Williams, Bulstrode et al. 2008). The rectum ends at the anorectal junction where puborectalis muscle encircles the posterior and lateral aspects to create an anorectal angle of 120^0 (Williams, Bulstrode et al. 2008).

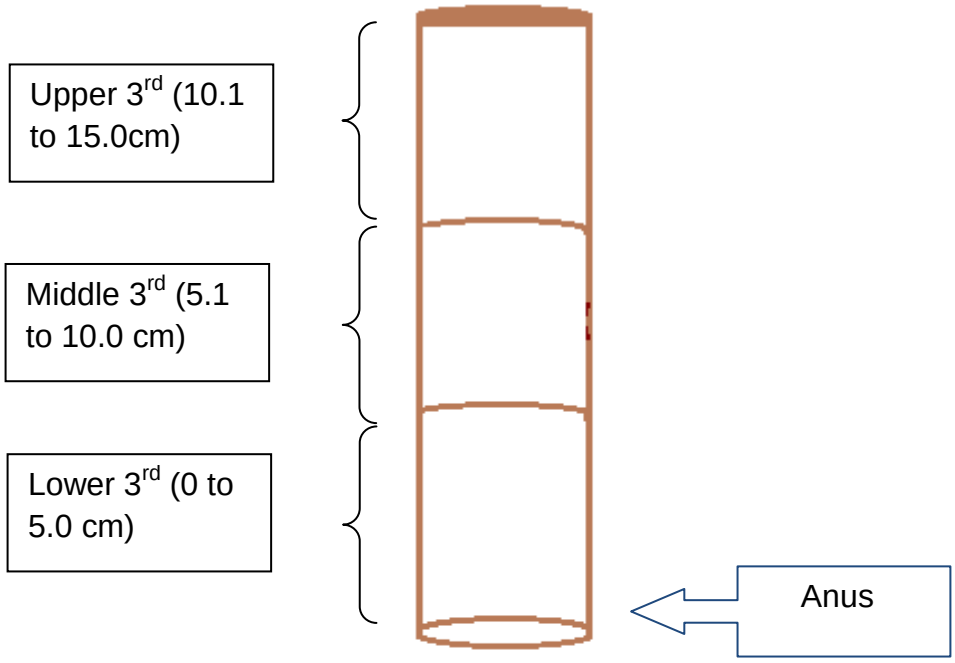

Figure. 1.4: Anatomic divisions of the rectum. The rectum is divided into three equal parts from anal verge and a determination of the site of lesion is facilitated by digital rectal examination and proctoscopy. Site of the lesion determines the suitability for a sphincter preserving procedure which is currently achievable even for low rectal cancer with the use of circular stapling gun.

1.3 Blood supply

1.3.1 Arterial supply and venous drainage

The rectum is supplied by superior, middle and inferior rectal arteries. Superior rectal artery is the direct continuation of inferior mesenteric artery and is the

main source of blood supply to rectum (Williams, Bulstrode et al. 2008). The middle rectal artery arises from the internal iliac artery whereas the lower rectal artery is a branch of internal pudendal artery.

The venous drainage of the upper half of rectum is to the inferior mesenteric vein, via superior rectal vein, into the portal system. Veins from the lower half of the rectum are drained by the middle and inferior rectal veins into the systemic system via the internal iliac veins (Williams, Bulstrode et al. 2008). The venous rectal drainage may explain why tumours of the lower rectum and anal canal can directly establish pulmonary metastases without hepatic metastases (Schafer 2010). The relationship of the ureters to the inferior mesenteric artery and superior rectal artery is of particular importance to the colorectal surgeon. Because the trunk deviates to the left, it passes close to the left ureter and left spermatic vessels, which are in danger during ligation of the inferior mesenteric artery (Schafer 2010).

1.3.2 Lymphatic drainage

Numerous small nodes are located adjacent to and on the rectal wall. The lymphatic trunks of the rectum accompany the inferior, middle and superior rectal arteries and are referred to as the inferior, middle and superior trunks. The former two collectively drain into perineo-pelvic-parietal nodes. However, the superior trunk follow the superior rectal／inferior mesenteric arterial axis to drain into abdominal nodes (Bell, Sasaki et al. 2009).

1.4 Aetiology of rectal cancer

Various genetic, endogenous and exogenous predisposing factors have been implicated in the development of CRC. Adenocarcinomas comprise a vast majority (98%) of the CRC. Other rare cancers occurring in the rectum include carcinoids, lymphomas, and sarcomas. In this thesis, the term 'rectal cancer' refers to rectal adenocarcinoma unless specified otherwise. The origin of CRC is either sporadic (~ 85%) or hereditary (~ 10 - 15%) (Ponz de Leon, Benatti et al. 2004; Weitz, Koch et al. 2005; Soreide, Nedrebo et al. 2009).

1.4.1 Sporadic rectal cancer

The vast majority of rectal cancers arise sporadically caused by any of a wide range of endogenous and exogenous risk factors. Whatever the aetiological association, the initial lesion is an adenoma which transforms into cancer many years down the line (Section 1.5). Various factors predisposing to development of rectal cancer are enlisted in table 1.2.

Rectal cancer has clear gender predisposition as it is up to twice as common in men as in women (Potter 1999). Such gender predisposition is considered to be due to the protective effect of the oestrogen in females and weight related increased incidence in men (Nelson, Dollear et al. 1997). The incidence of rectal cancer increases with age, some 15 times higher in adults 50 years and older than in those 20 to 49 years (AmericanCancerSociety 2011).

Table 1.2: Factors predisposing to development of CRC. A range of familial, exogenous and endogenous causative factors of CRC are outlined. For details see text (adapted from Weitz et al. 2005)

Older age	Inflammatory bowel disease
Male sex	Occupational hazards (eg, asbestos exposure)
Diet rich in meat and fat, and poor in fibre, folate, and calcium	High alcohol intake
Sedentary lifestyle	*Personal history of sporadic tumours*
Obesity	History of colorectal polyps
Diabetes mellitus	History of colorectal, small bowel, endometrial, breast, or ovarian cancer
Smoking	*Familial colorectal cancer (20%)*
Cholecystectomy	First or second degree relatives with CRC
Ureterocolic anastomosis	
Previous irradiation	

A wide range of dietary factors, including fibre, fruit and vegetables, fat, meat, folate and calcium have all been associated with the CRC development, albeit without definitive scientific evidence in pathogenesis of CRC (Ponz de Leon, Benatti et al. 2004). There is evidence that higher red and processed meat consumption increases the risk of both the Colon and Rectal cancer development (AmericanCancerSociety 2011; Chan, Lau et al. 2011) whilst increased fibre and fish intake may reduce such risk (Bingham, Day et al. 2003). A recent systematic review supported limiting red and processed meat consumption as one of the dietary recommendations for the prevention of CRC (Chan, Lau et al. 2011). The dietary fibre is protective of rectal cancer (Schatzkin, Mouw et al. 2007) and the resistant starch has been linked to the chemoprevention of CRC (Young and Le Leu 2004). Resistant starch has been shown to affect the colonic luminal environment and facilitate apoptotic deletion of genetically damaged cells in the bowel mucosa thereby promoting colorectal mucosal health (Young and Le Leu 2004).

Similarly, increased body mass index is a recognised predisposing factor of cancers (Lukanova, Bjor et al. 2006). It has been reported that such risk increases by 15% and 33% in overweight and obese subjects, respectively (Bergstrom, Pisani et al. 2001). Conversely, serum polyunsaturated fatty acids are associated with a reduced risk of the CRC (Kojima, Wakai et al. 2005). Another major risk factor for rectal cancer is smoking which represents one of the most significant public health threats. It remains a major preventable risk factor for a range of conditions. In rectal cancer, it is associated with incidence and mortality that is higher in men compared with women. There is consistent evidence, as revealed in recent meta-analyses, that for both incidence and mortality the association is stronger for rectal cancer compared with colon cancer (Botteri, Iodice et al. 2008; Tsoi, Pau et al. 2009). Smoking is associated with an absolute risk increase of 10.8 cases per 100,000 person/years in addition to having a statistically significant dose-relationship with an increasing number of pack-years and cigarettes per day. However, such association is significant only after 30 years of smoking (Botteri, Iodice et al. 2008).

Inflammatory bowel disease (IBD) is a recognised premalignant condition in colorectal carcinogenesis accounting for up to 2% of cases (Weitz, Koch et al. 2005). Herrinton et al. reported that over a 12-year follow-up, the incidence of CRC among individuals with either Crohn's disease or Ulcerative Colitis was 60% higher compared with the general population and was stable over time (Herrinton, Liu et al. 2012). In a population based study over 16 years, Bernstein et al. found that UC was associated with an increased risk of developing rectal carcinoma whilst CD was related to increased risk of developing small bowel cancer and lymphomas (Bernstein, Blanchard et al.

2001). A large, national population based Danish study concluded that over a 30-year follow-up, the diagnosis of UC or CD was not related to increased risk of CRC except for select subgroups of UC (Jess, Simonsen et al. 2012). Those subgroups remaining at increased risk include patients with paediatric or adolescent onset UC, those with long duration of disease, and those with concomitant primary sclerosing cholangitis. The declined risk for CRC from 1979 to 2008 is most likely to have resulted from improved therapies for patients with IBD (Jess, Simonsen et al. 2012).

Approximately 20% of all patients who develop sporadic CRC are estimated to have some degree of familial predisposition without fulfilling the strict criteria for hereditary CRC. Family history of first or second degree relatives is therefore relevant in such cases.

1.4.2 Hereditary rectal cancer

Hereditary rectal cancer occurs as part of the hereditary CRC syndromes. Hereditary syndromes have germline mutations in specific genes that substantially increase the life time risk of development of CRC. Two most common hereditary CRC syndromes are Familial Adenomatous Polyposis (FAP) syndrome and Hereditary Non Polyposis Colorectal Cancer (HNPCC) syndrome (Soreide, Nedrebo et al. 2009). The relatively uncommon types of hereditary CRC syndromes include Peutz-Jeghers syndrome, juvenile polyposis syndrome and Cowden syndrome (Weitz, Koch et al. 2005).

FAP syndrome is an autosomal dominant disorder and carries a very high penetrance. It is caused by mutations in the APC gene. It accounts for less than 1% of all CRC and patients have widespread colonic and rectal polyps that inevitably progress into malignant disease (Vasen, Moslein et al. 2008). Most of the genetics studies for CRC are based on the studies on FAP patients and their family members. It is characterised by appearance of numerous small polyps throughout colon and rectum. Conventionally, presence of 100 or more polyps is diagnostic of FAP. The treatment is proctocolectomy with ileal pouch–anal anastomosis (Vasen, Moslein et al. 2008). First degree relatives of FAP patients should undergo genetic testing for APC mutation. The family members that are found to carry the mutation would require periodic endoscopic surveillance for polyposis of colon and rectum from early teenage. Prophylactic surgery is recommended and has been shown to reduce FAP related mortality (Vasen, Moslein et al. 2008; Half, Bercovich et al. 2009).

The HNPCC syndrome, also known as Lynch syndrome, contains mutations in mismatch repair (MMR) genes. Two most common mutations involve MSH-2 and MLH-1 genes. Mutations of a few other genes have also been described. It is an autosomal dominant disorder with an incidence of 1:2000. Most of the colorectal cancers in HNPCC are on the right side and rectal cancer is rare in these subjects (Umar, Boland et al. 2004).

1.5 Pathogenesis and molecular biology of rectal cancer

1.5.1 The adenoma-carcinoma sequence

It is believed that colorectal cancers arise as an adenoma many years before transformation into a carcinoma. This development of CRC from a benign lesion, termed the adenoma-carcinoma sequence, may take several decades. The risk of CRC begins to increase after the age of 40 years and rises sharply at the ages 50 to 55 years. After that age, the risk of CRC occurrence doubles with each succeeding decade, the highest incidence noted at age 75 years (Soreide, Nedrebo et al. 2009). There is evidence that only 10% of the adenomas transform into malignancy after 10 years (Scholefield 2000). Adenomas can be detected on colonoscopy in up to 20% of patients and 90% of those are amenable to endoscopic removal (Scholefield 2000).

The model of genetic alterations responsible for the adenoma-carcinoma sequence presented some fundamental features (Vogelstein, Fearon et al. 1988). The Vogelgram suggested that colorectal carcinogenesis results from mutational inactivation of tumour suppressor genes coupled with the activation of oncogenes. It has also been proposed that mutations in at least four or five genes are required for malignant transformation and it is the total accumulation of changes that is responsible for the tumour's biological behaviour (Vogelstein, Fearon et al. 1988). The adenoma-carcinoma sequence can be seen as a spectrum that starts from normal epithelium through hyperproliferation to dysplasia and finally invasive cancer (Figure 1.5).

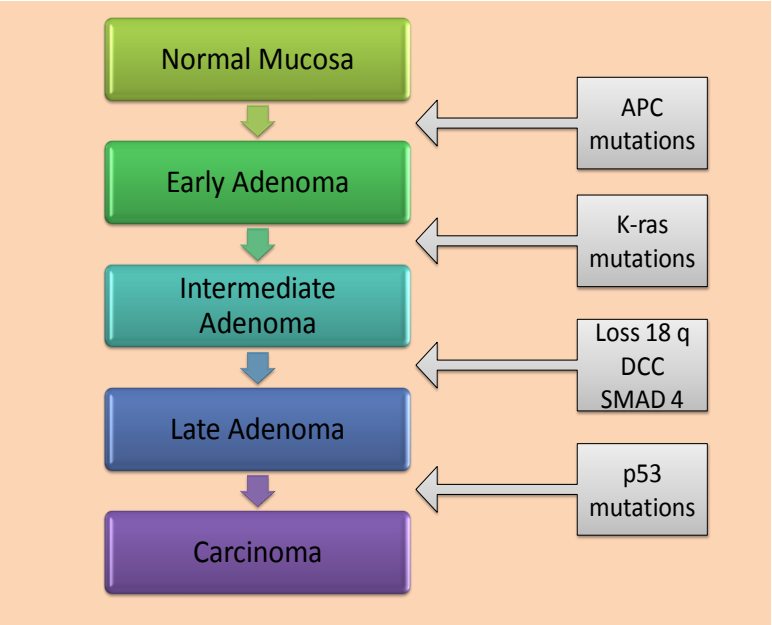

Figure 1.5: The Vogelgram - A genetic model for colorectal carcinogenesis. The model of adenoma-carcinoma sequence as proposed by Bert Vogelstein with a snapshot of molecular alterations occurring in colorectal carcinogenesis. Mutation of the familial polyposis gene APC constitutes the initiating event for both the hereditary CRC and most cases of the sporadic colorectal cancer. Classical Vogelgram depicted the role of mutations in APC gene, genes on 18q, and the K-ras and p53 genes in contributing to the evolution of colorectal cancer. The adenomas are stratified according to the architectural changes and presence of dysplasia (low versus high grade dysplasia). Additional genetic lesions are associated with advancing adenomas. Loss of p53 is usually considered the point that marks the transition to invasive cancer. Alternative pathways to above chromosomal instability pathway are microsatellite instability pathway with defects in MMR genes (MSH2, MLH1 and others), and CIMP pathway with methylator phenotype (See text).

The colorectal carcinogenesis is triggered by a combination of genetic alterations, which form the basis of the adenoma-carcinoma sequence hypothesis (Ogino and Goel 2008). The relative timing of the genetic events in the adenoma-carcinoma sequence is indicated by corresponding stages of the colorectal tumour development. Loss of heterozygoisty (LOH) on chromosome 5q and/or somatic mutation of the APC gene may represent the earliest events (Fearon and Vogelstein 1990; Fearon and Jones 1992).

The first recognisable manifestation of the adenoma-carcinoma sequence is the development of aberrant crypt foci that are small hyperplastic or dysplastic lesions precursors of the adenomas. Three stages of adenomas represent the progress in tumour size, dysplasia, and villous content. Different sizes at each stage of adenomas have been suggested; early adenomas < 1 cm, intermediate adenomas ≥ 1 cm, and late adenomas are greater than 1 cm in size containing foci of carcinoma (Fearon and Jones 1992). LOH involving chromosomes 18q and l7p and the mutations of the DCC and p53 genes occur more frequently at the later stages of carcinogenesis and trigger transformation into cancer (Fearon and Vogelstein 1990). Two major molecular pathways have been described: the chromosomal instability pathway and the microsatellite instability pathway (Leslie, Carey et al. 2002; Soreide, Nedrebo et al. 2009).

1.5.2 Chromosomal Instability Pathway

The Chromosomal Instability (CIN) pathway follows progression of genetic alterations in the form of chromosomal losses and gains. Such alterations culminate usually in corresponding histological changes. The genomic changes include activation of proto-oncogenes K-ras and inactivation of tumour suppressor genes APC, p53, DCC and loss of heterozygosity for the long arm of chromosome 18 (18q LOH).

APC is the most common initial gene mutated in hereditary and sporadic colon cancer. The APC gene is a tumour suppressor gene located on chromosome 5q21 (Rowan, Lamlum et al. 2000). The APC mutations in both hereditary and sporadic cancer have been consistently reported, suggesting that the APC gene

was an early target or gatekeeper gene in the development of colorectal cancer (Fearon and Vogelstein 1990). The subjects with APC mutations are also at the risk of developing extracolonic tumours. Chromosomal instability or microsatellite instability (MSI) can be observed in adenomas. Thus, genetic instability appears to be present during the initiation of an adenoma, before APC gene mutation and progression to frank malignancy. Several genes have been found to be commonly mutated in colorectal cancers (Table 1.3) (Soreide, Janssen et al. 2006; Soreide, Nedrebo et al. 2009; Armaghany, Wilson et al. 2012).

Table 1.3: Common genetic alterations in colorectal carcinogenesis. (Adapted from Armaghany, Wilson et al. 2012, Soreide, Nedrebo et al. 2009, Soreide, Janssen et al. 2006)

Genes	Chromosome	Role / Function
Genes involved in CIN pathway		
APC	5q21-22	Tumour suppressor gene: Mutated in FAP
K-ras	12p12.1	Oncogene: Early event CRC
p53	17p13	Tumour suppressor: Late event CRC
DCC	18q21.3	Tumour suppressor gene
SMAD4	18q21.1	Tumour suppressor gene
Genes involved in MSI pathway		
MSH2	2p16	DNA mismatch repair: HNPCC
MLH1	3p21	DNA mismatch repair: HNPCC; epigenetically silenced in sporadic CRC
MSH6	2p16	DNA mismatch repair: HNPCC
PMS2	7p22	DNA mismatch repair HNPCC; rare
PMS1	2q31-33	DNA mismatch repair
MLH3	14q24.3	DNA mismatch repair

Mutation of the K-ras gene confers neoplastic properties in transfected cell lines, and is found in half of the colorectal carcinomas and adenomas greater

than 1cm in size (Vogelstein, Fearon et al. 1988). Smaller adenomas are less likely to have a K-ras mutation and therefore this oncogene may be significant at the later stages of tumour progression.

The deleted in colon cancer (DCC) gene is expressed in normal colon, but in 75% of large adenomas and carcinomas a somatic mutation leads to absence or reduced activity of its gene product suggesting the gene product acts as a tumour suppressor (Vogelstein, Fearon et al. 1988). The p53 tumour suppressor gene is a target in colorectal carcinogenesis (Mehigan, Ashman et al. 2006). In colorectal cancer it appears that a mutation of p53 that leads to its inactivation is a crucial step in the development from benign adenoma to invasive malignancy. It has been shown that high-level expression of the p53 protein correlates with the presence of point missense mutations. It was noted that when p53 is detected by immunohistochemistry in tumours, it is mutant, i.e. the over-expression of p53 is synonymous with mutation (Rodrigues, Rowan et al. 1990). In addition, deletion at 1p and 8p, as well as LOH of 17p and 18q, are frequent in CRC. Such alterations are the characteristics of the CIN pathway (Soreide, Janssen et al. 2006).

Contrary to the linear model of genetic alterations in colorectal carcinogenesis involving the APC, p53 and K-ras genes, as proposed by Fearon and Vogelstein, it is now accepted that up to 10% of cases would have mutations in all of above genes. Therefore there are alternative, multiple, mutational pathways for colorectal cancer development as above mutations are rarely found in combination (Smith, Carey et al. 2002; Soreide, Janssen et al. 2006).

1.5.3 Microsatellite Instability Pathway

The second pathway, accounting for 15% of sporadic colorectal cancers is referred to as the MSI pathway (Ogino and Goel 2008). Tumours belonging to this pathway display frame shift mutations and base-pair substitutions that are commonly found in short, tandemly repeated, nucleotide sequences known as microsatellites, scattered throughout the genome (Leslie, Carey et al. 2002; Ogino and Goel 2008). The nucleotides mismatches occur due to insertion of the wrong bases in newly synthesized DNA by the DNA polymerase. Such mismatches routinely occur at the time when two strands of DNA replicate, however, almost all such errors are quickly corrected by a molecular proofreading mechanism controlled by the MMR genes. These genes play a critical role in the identification and correction of such nucleotide errors (Leslie, Carey et al. 2002; Bedeir and Krasinskas 2011).

Essentially, the mismatch repair system works as a ‘spell checker’ that identifies and corrects the mismatched base pairs in the DNA. Failure of the mismatch repair apparatus, i.e. mutations in MMR genes, leads to persistence of errors and an alteration in the length of a microsatellite sequence, a process described as MSI (Soreide, Janssen et al. 2006; Bedeir and Krasinskas 2011). Further, there are two different mechanisms through which MSI occurs in hereditary and sporadic CRC. In hereditary tumours, the cause is a germline mutation in a mismatch repair enzyme. Several homologues of the bacterial MMR genes, hMSH2, hMSH3, hMSH5, hMSH6, hMLH1, hMLH3, hPMS1, and hPMS2, have been identified in humans (Table 1.3).

The alterations of the MutS homologue 2 (MSH2) and MutL homologue 1 (MLH1) mismatch repair genes account for more than 90% of HNPCC patients. On the other hand, the MSI in sporadic colorectal cancer occurs due to loss of expression of one of the MMR genes, most commonly MLH1, caused by epigenetic silencing (Mitchell, Farrington et al. 2002; Soreide, Janssen et al. 2006; Soreide 2007). MSI is most commonly seen in the right sided cancers (Mehigan, Ashman et al. 2006). It is also understood that CRCs of the left side (including rectal cancers) most likely originate from the CIN pathway (Soreide, Janssen et al. 2006; Soreide, Nedrebo et al. 2009). Therefore rectal cancers developing through the CIN pathway usually possess functional MMR genes.

Moreover, a subset of CRC does not exhibit genetic aberrations of CIN or MSI pathways. In such cancers epigenetic changes marked by DNA methylation are increasingly recognised. Transcriptional inactivation by cytosine methylation at promoter CpG islands of tumour suppressor genes is thought to be the mechanism of carcinogenesis in these cases. A number of tumour suppressor genes have been shown to be silenced by promoter methylation in CRC (Ogino and Goel 2008). This pathway of intense DNA hypermethylation is described as the CpG island methylator phenotype (CIMP) pathway (Imai and Yamamoto 2008; Issa 2008). Such epigenetic silencing provides a theoretical alternative to the genetic instability in driving the molecular evolution of cancer. In addition to the differences in genetic instability compared with the CIN cases, the CIMP cases tend to have BRAF and KRAS mutations and a fewer APC and p53 mutations (Issa 2008).

1.5.4 Are colon and rectal cancer the same entity?

Colorectal cancer has traditionally been regarded as one entity. However, interest has grown to classify the CRC into right and left sided cancers in relation to splenic flexure due to their embryological origin from mid-gut and hind-gut, respectively (Iacopetta 2002; Benedix, Kube et al. 2010). Rectal cancer is generally considered part of the left sided cancers (Iacopetta 2002). There are several different biological and histological characteristics that distinguish between the right sided and left sided cancers. Moreover, right sided colorectal cancers carry a worse prognosis than left sided and rectal cancers, possibly because of more advanced staging and fewer curative resections (Benedix, Kube et al. 2010; Suttie, Shaikh et al. 2011).

Accumulating evidence suggests that right sided cancers and left sided colorectal cancers differ in various molecular features. It is reported that the right sided cancers demonstrate higher MSI compared with left sided and rectal cancers. Likewise, more right sided cancers exhibit CIMP phenotype than the left sided cancers. KRAS and BRAF mutations are also reported to be higher in right sided cancers compared with left sided and rectal cancers (Yamauchi, Morikawa et al. 2012). It is also understood that the left sided cancers (including rectal cancers) usually originate from the CIN pathway and most will possess functional MMR genes (Soreide, Janssen et al. 2006; Soreide, Nedrebo et al. 2009). More recently, it has been proposed that the molecular differences are gradual changes from caecum to rectum in contrast to the abrupt change at splenic flexure suggested by most of the studies over the last two decades (Yamauchi, Morikawa et al. 2012). Different epidemiological, molecular, and

histological characteristics differentiating right and left sided cancers are summarised in table 1.4 (Iacopetta 2002; Benedix, Kube et al. 2010).

Table 1.4: Features of right and left sided (& rectal) colorectal cancers (Source of data: Iacopetta 2002; Benedix, Kube et al. 2010)

Feature	**Right sided cancers**	**Left sided cancers**
Age at diagnosis	Older	Younger
Gender	More females	More males
Mucinous tumours	Frequent	Infrequent
Familial cancer syndrome	HNPCC	FAP
Ploidy	Mostly diploid	Mostly aneuploidy
Loss of heterozygosity	Infrequent	Frequent
p53 mutation	20–30%	50–60%
MSI+	25%	2–3%
CIMP+	25–40%	3–10%

Similarly, clinico-pathological differences have been described. Most of the patients with HNPCC have right sided cancer and rectal cancer is rare in these subjects (Umar, Boland et al. 2004). Autosomal dominant FAP syndrome manifests as polyposis of rectum that spreads proximal along the left colon. Right sided neoplastic lesions may be depressed type whereas the left sided and rectal cancers are usually non-depressed type adenomatous lesions. Whereas adenoma-carcinoma sequence may underpin the progression of mucosal lesions to invasive cancers in the left colon and rectum, a de novo pathway from depressed type lesions may be implicated in some cancers of the right colon due to absence of adenomas (Konishi, Fujii et al. 1999).

1.6 Staging of rectal cancer

In 1932, the British pathologist Cuthbert Dukes described a staging system which, for over three quarters of a century, remains a valuable staging

classification for rectal cancer (Dukes 1932) (Table 1.5). The Dukes classification was based on the extent of tumour spread and originally described three stages (Figure 1.6).

Table 1.5: Dukes' classification of colorectal cancer. (* Stage D was not included in the original Dukes classification but was later included to account for distant metastases)

Stage	Extent of disease
Dukes A	Carcinoma limited to rectal wall
Dukes B	Direct spread of carcinoma to extra-rectal tissue, regional nodes not involved
Dukes C	Carcinoma has invaded the regional lymph nodes
D*	Distant metastases present

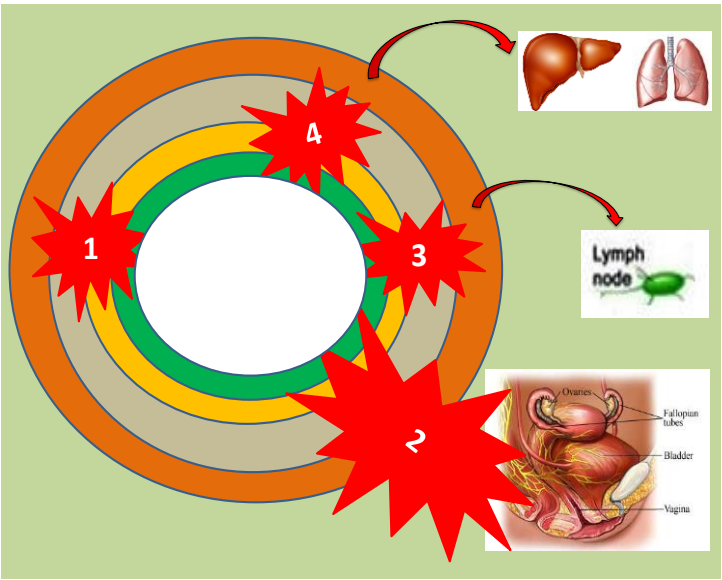

Figure 1.6: Dukes' stages of CRC. 1=Dukes A (cancer limited to bowel wall), 2=Dukes B (direct spread of cancer to extra-rectal tissues), 3 =Dukes C (cancer with lymph node metastasis, 4=Stage D (cancer with distant metastasis). Figure drawn by the author with images of organs sourced from http://www.cancer.gov accessed 10/05/2012.

The Dukes' staging, however, has limitations while planning preoperative treatment. The modern management of rectal cancer is based on Tumour,

Node and Metastasis (TNM) system. The TNM staging system provides the most accurate assessment of tumour size and behaviour and therefore is the most useful tool in planning treatment. Accurate pathologic staging in rectal cancer is fundamental to understanding the results of treatment interventions and outcomes (Compton and Greene 2004). The extent of surgical resection is commonly included in the final classification to facilitate further treatment. Residual tumour after primary surgical resection is categorised by a system known as the R classification. Various TNM and R levels and their definitions are given in Table 1.6 (Nelson, Petrelli et al. 2001; Compton and Greene 2004; Glimelius and Oliveira 2008).

Table 1.6: TNM and R classification system in rectal cancer (Nelson, Petrelli et al. 2001)

TNM	**Level**	**Definition**
Primary Tumour (T)	Tx	Primary tumour cannot be assessed
	T0	No evidence of primary tumour
	Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
	T1	Tumour invades submucosa
	T2	Tumour invades muscularis propria
	T3	Tumour invades through muscularis propria into the subserosa or into nonperitonealised pericolic or perirectal tissues
	T4	Tumour perforates visceral peritoneum or directly invades other organs or structures
Regional Lymph Nodes (N)	Nx	Regional lymph nodes could not be assessed
	N0	No regional lymph node metastases
	N1	Metastases in one to three regional lymph nodes
	N2	Metastases in four or more regional lymph nodes
Distant Metastases (M)	Mx	Distant metastases could not be assessed
	M0	No distant metastases
	M1	Distant metastases
Extent of resection (R)	Rx	Presence of residual tumour cannot be assessed
	R0	No residual tumour
	R1	Microscopic residual tumour
	R2	Macroscopic residual tumour

A prefix “p” represents the pathological determination of the T, N and M components which is based on gross and microscopic examination of the resection specimen of a previously untreated primary tumour, lymph nodes and distant metastases, respectively. Prefix “c” with a TNM component denotes clinical assessment of that component and is based on evidence acquired through physical examination, radiologic imaging, endoscopy, biopsy and surgical exploration. Prefix “y” refers to the same TNM staging following neoadjuvant treatment (Compton and Greene 2004).

The TNM system forms the basis of Union for International Cancer Control UICC) classification of the CRC which is internationally recognised and standardised classification system. Table 1.7 gives an overview of the UICC, TNM and Dukes staging classifications (Compton and Greene 2004; Glimelius and Oliveira 2008).

Table 1.7: Clinical stages in rectal cancer. (Adopted from Compton and Greene 2004, Glimelius and Oliveira 2008) and prognosis (Weitz et al. 2005)

UICC Stage	**TNM stage**	**Dukes stage**	**Tumour extension / spread**	**5 year survival**
Stage 0	Tis, N0,M0	N/A	Carcinoma in situ	-
Stage I	T1, N0, M0	A	Submucosa	80 – 95%
	T2, N0, M0		Muscularis propria	
Stage II-A	T3,N0, M0	B	Subserosa / perirectal tissue	72 – 75%
Stage II-B	T4, N0, M0		Perforation into perirectal tissue or invasion to other organs	65 – 66%
Stage III-A	T1,T2, N1, M0	C	1–3 regional nodes involved	55 – 60%
Stage III-B	T3,T4, N1, M0		1–3 regional nodes involved	35 – 42%
Stage III-C	Any T, N2, M0		4 or more regional nodes involved	25 – 27%
Stage IV	Any T, Any N, M1	D	Distant metastases	0 – 7%

Recent developments in non-invasive staging techniques have raised the possibility of a change of the nomenclature to represent the accurate imaging modality (Skandarajah and Tjandra 2006). Moran et al. recommended further prefixes to be added to reflect modern imaging developments (Moran, Brown et al. 2008). They suggested that the following TNM prefix terminology should be applied in all reporting of rectal cancer staging to clarify current ambiguity: ‘p’ – pathologist, ‘u’ – ultrasound, ‘ct’ – CT scan, ‘mr’ – MRI scan, ‘y’ – any staging following neo-adjuvant therapy which can be applied to radiology in a similar way to the yp classification.

1.7 Treatment of rectal Cancer

Surgery has traditionally remained the mainstay of treatment of rectal cancer. Over the last two decades, significant development has been seen in the management of rectal cancer. Introduction of the surgical technique of total mesorectal excision (TME) and improvement in the radiotherapy (RT) regimens have substantially improved the treatment standards. Treatment ranges from surgery alone in stage-I, surgery and RT / chemoradiotherapy (CRT) in stage II/III to either or any combination of (primarily palliative) surgery, RT, and chemotherapy +/- molecular treatment in stage IV disease (McCourt, Armitage et al. 2009).

1.7.1 Surgical management of rectal cancer

Traditional surgery in rectal cancer revolved around whether a sphincter preserving procedure could be undertaken. Advent of the circular stapling guns

has now made it possible to achieve that even for cancers previously considered too low for a primary anastomosis. There are two standard elective procedures for the oncological resection of rectal cancer, both based on TME principle. The sphincter preserving anterior resection of rectum (AR) is generally offered to patients with upper to middle rectal tumours, whereas most patients with low rectal cancer would undergo abdominoperineal excision of rectum (APER).

1.7.1.1 Total Mesorectal Excision (TME)

Over the past few decades, significant advancements have been made in the oncological, operative and peri-operative management of rectal cancer. It was in early 1990s that the mesorectal planes were defined aiming at achieving an optimum level of oncological resection for rectal cancer - a major breakthrough in the management of rectal cancer (Heald 1995; Heald 1995). The fundamental principle of this technique is to achieve a radical resection by sharp dissection within the true pelvis around the intact mesorectum under direct vision, thus enveloping the entire rectum, including the tumour.

The TME approach has been shown to significantly reduce the rates of local recurrence (LR) in rectal cancer (Garcia-Granero, Faiz et al. 2009; Quirke, Steele et al. 2009). Five-year LR rate following TME surgery is reduced to 10-15% (Bonadeo, Vaccaro et al. 2001; Wibe, Syse et al. 2004), and reported as low as 4% (Havenga, Enker et al. 1999). TME surgery also provides survival benefit in improving the overall survival (OS). Five years OS in stage I rectal cancer following surgery alone is more than 93% whereas it is as low as 0 – 7%

in stage IV (Sauer 2002). Dutch TME trial recently reported the long term data on survival and recurrence in patients treated with TME alone versus RT and TME, thereby providing the most reliable data about survival outcomes related to TME (Table 1.8) (van Gijn, Marijnen et al. 2011).

Table 1.8: Stage stratified survival from rectal cancer. Five-year local failure and overall survival rates after curative TME surgery (R0) alone according to tumour stage (Sauer 2002; van Gijn, Marijnen et al. 2011)

UICC Stage	**5-Year LR rates (%)**	**5-Year overall-survival (%)**
All patients	10.9	63.5
UICC stage I		
pT1 pN0 (n = 60)	1.7	94.9
pT2 pN0 (n = 145)	6.5	87.9
UICC stage II		
pT3a/b pN0 (n = 128)	4.4	87.8
pT3c pN0 (n = 60)	14.8	74.4
pT3d pN0 (n = 43)	18.0	67.2
pT4 pN0 (n = 20)	10.6	63.5
UICC stage III		
pT1-4 pN1 (n = 183)	18.3	66.8
pT1-4 pN2 (n = 137)	32.3	35
UICC stage IV		
Any T, Any N, M1		0-7%

The Dutch TME trial reported overall survival of 63.5% and 40% at 5 and 10 years, respectively, in the TME only group (Peeters, Marijnen et al. 2007; van Gijn, Marijnen et al. 2011). The 10-year risk of local recurrence in TME alone was found to be 11%, which dropped further to 5% when performed following neoadjuvant RT (van Gijn, Marijnen et al. 2011). Evidence is now emerging from randomised studies that the oncological benefits of TME appear to be comparable between the open and laparoscopic approaches (Kang, Park et al. 2010; Ng, Lee et al. 2014).

1.7.1.2 Mesorectal grading

TME is now established as a gold standard principle of the surgical resection of rectal cancer with proven survival benefits. TME is the required standard in rectal resections for cancer. Three grades of TME have been described that are reported to predict local and distant recurrence (Figure 1.7) (Nagtegaal, van de Velde et al. 2002; Maslekar, Sharma et al. 2007; Garcia-Granero, Faiz et al. 2009).

- **Complete or grade III TME:** This is the best grade mesorectal excision which is characterised by intact mesorectum containing only minor irregularities (not deeper than 5mm) of a smooth mesorectal surface.
- **Nearly complete or grade II TME:** Intermediate grade, nearly complete mesorectal excision is obtained with moderate bulk to the mesorectum, however, it contains irregularity of the mesorectal surface.
- **Incomplete or grade I TME:** This is the poorest grade mesorectal excision that provides little bulk to the mesorectum with defects down onto muscularis propria and/or very irregular circumferential resection margin.

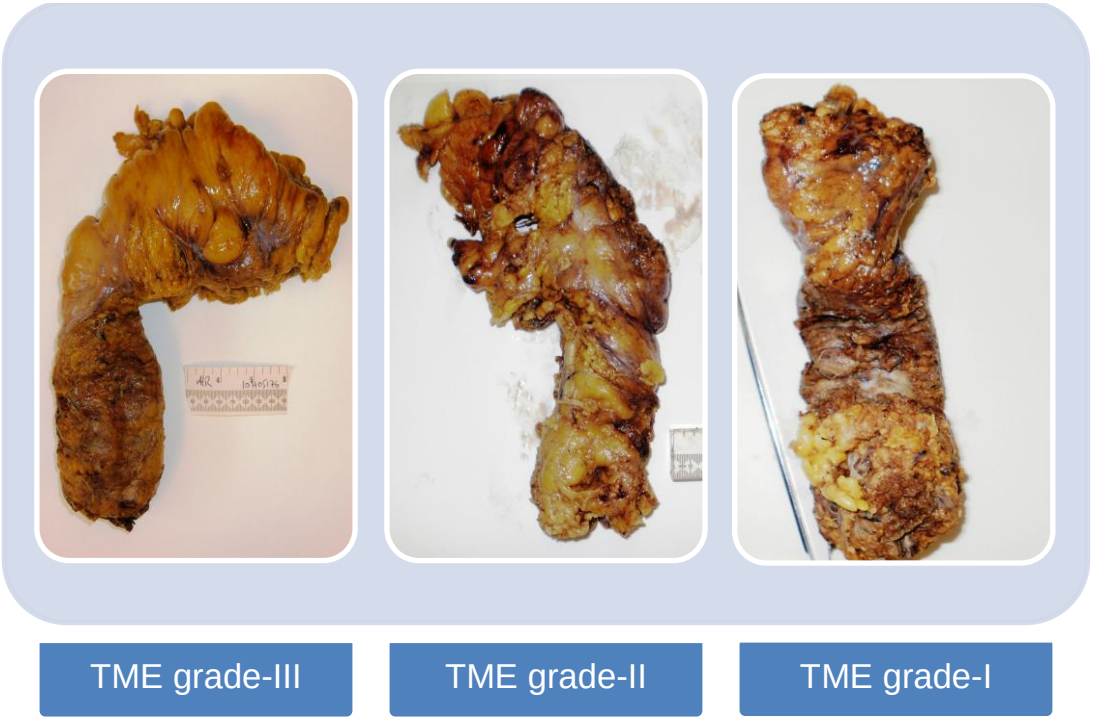

Figure 1.7: Mesorectal grading system in rectal cancer. Source: Archives of rectal cancer samples from the patients included in this project – courtesy Dr. MacDonald, colorectal pathologist, Castle Hill Hospital

1.7.1.3 Local resection

The local resection of rectal cancer has received some popularity in recent years in a highly select population. A number of studies, in the recent past, have attempted to investigate the role of local resection of rectal cancer. Four different methods of local resection of rectal cancer have been described (McCourt, Armitage et al. 2009). These include transanal resection, transanal endoscopic microsurgery (TEM), transsacral resection (Kraske procedure) and transsphincteric resection. In the past the management of rectal cancer with local resection approach was reserved mainly for highly select early rectal cancer, for patients unfit for radical surgery or for local palliation in metastatic disease (Bonnen, Crane et al. 2004). However, local resection with curative intent is increasingly being sought as an appropriate option in locally advanced rectal cancer following neoadjuvant CRT. Recent large studies have shown that

it could be a safe alternative with comparable oncological outcomes especially in selected patients with complete pathological response following neoadjuvant CRT (Callender, Das et al. 2010; Issa, Murninkas et al. 2012).

1.7.2 Radiotherapy in rectal cancer

1.7.2.1 Overview

Surgical resection has remained the mainstay of treatment in patients with rectal cancer for decades. Last two decades have seen significant development in the role of RT in rectal cancer (Glimelius 2002). Neoadjuvant RT with or without sensitising chemotherapy (i.e. CRT) has been the standard treatment approach for locally advanced rectal cancer in Europe for over a decade. Whereas the postoperative RT was a commonly practised regimen in the United States until recently, the neoadjuvant CRT has now become a standardised treatment regimen on both sides of the Atlantic (McCourt, Armitage et al. 2009; Wadlow and Ryan 2010).

1.7.2.2 Radiotherapy regimens

Radiotherapy is offered to patients with rectal cancer in the pre-operative (neoadjuvant) setting. A patient may be offered either of the following two RT regimens:

1. Long-course chemoradiotherapy (LCCRT): LCCRT is delivered via a conventionally fractionated dosage schedule spread over five weeks aiming to deliver a total RT dose of 45 to 50.4 Gray (Gy) (1.8 Gy per fraction, five fractions a week). This is combined with either intravenous 5-fluorouracil (5-FU),

or oral Capecitabine based sensitising chemotherapy. The primary aim of the LCCRT is to achieve down-sizing or down-staging of the tumour to improve resectability (ACPGBI_guidelines 2007). Surgery is delayed for 6-8 weeks following the completion of LCCRT to allow optimum tumour regression (Section 1.7.2.8).

2. Short-course radiotherapy (SCRT): SCRT is a hypofractionated RT regimen in which a total of 25 Gy of RT is delivered within a short duration of 5 daily fractions (5 Gy per fraction) over 1 week. Surgery is performed in the following week, before the onset of acute side-effects of radiotherapy (Bujko, Nowacki et al. 2006). The short interval between RT and surgery does not allow any significant tumour shrinkage prior to the surgical resection. SCRT is aimed at reduction of LR and is offered only if a cancer is clinically and radiologically assessed to be resectable (ACPGBI_guidelines 2007).

1.7.2.3 RT and Surgery versus Surgery alone

Radiotherapy is widely accepted to provide therapeutic benefit additional to surgical treatment. In pre-TME era, the Swedish rectal cancer trial demonstrated a reduction in LR (11% v 27%) and improvement in OS (58% v 48%) in patients treated with RT and surgery compared to those treated with surgery alone (SwedishRectalCancerTrial 1997). The Swedish Trial involved conventional surgical approach and thus needed the confirmation of the results in the setting of quality controlled TME surgery.

In a large prospective Dutch trial, SCRT followed by the TME surgery was compared with the TME surgery alone (van Gijn, Marijnen et al. 2011). The long-term (10 years) data from the trial found more than 50% reduction in the

rates of LR (5% v 11%) in the irradiated group. No differences in OS were observed, however, a survival benefit (50% v 40%) was demonstrated in stage III patients with negative circumferential margin treated with preoperative SCRT and the TME surgery compared with those treated with TME surgery alone (van Gijn, Marijnen et al. 2011). The rates of LR were similar to those reported at 5 years (Peeters, Marijnen et al. 2007). The toxicity data from the trial was previously published. Long term complications included significant bowel dysfunction in the irradiated patients compared with the non-irradiated patients that included; increased rates of faecal incontinence (62% v 38%), anal blood loss (11% v 3%), and mucus loss (27% v 15%) (Peeters, van de Velde et al. 2005). The total number of acute complications was slightly increased in the irradiated group (p =0.008) that included increased operative blood loss and increased risk of perineal wound complications. No difference was observed in the postoperative mortality or in the number of re-interventions (Marijnen, Kapiteijn et al. 2002).

1.7.2.4 RT versus CRT

Addition of sensitising chemotherapy to the preoperative RT provides better local control forming the present-day standard of neoadjuvant CRT regimen with worldwide acceptance. In a trial of 733 patients randomised to receive either preoperative RT (45 Gy in 25 fractions) or CRT (45 Gy RT + 5-FU) followed by postoperative chemotherapy, reduced rates of local recurrence were observed in the CRT arm (16.5% v 8.1%, p=0.004) without any survival difference. The pathological complete response (pCR) rate was significantly higher in the group treated with CRT compared with the RT group (11.4% v 3.6%, p<0.0001) (Gerard, Conroy et al. 2006).

A Cochrane review and meta-analysis of preoperative CRT versus RT alone for stage II and III resectable rectal cancer found that the addition of chemotherapy to preoperative RT provided better oncological outcomes (Ceelen, Fierens et al. 2009; Ceelen, Van Nieuwenhove et al. 2009). The incidence of LR at five years was significantly lower in the CRT group compared with RT group. No statistically significant differences were observed in the DFS or OS at five years. Compared to the preoperative RT alone, CRT significantly increased the rate of pCR although this did not translate into a higher sphincter preservation rate. However, CRT was associated with significantly increased grade III and IV acute toxicity while no differences were observed in postoperative morbidity or mortality.(Ceelen, Van Nieuwenhove et al. 2009).

A more recent meta-analysis (n=2312) summarising data from six randomised clinical trials compared preoperative RT with preoperative CRT for stage II and III resectable rectal cancer (Latkauskas, Paskauskas et al. 2010). The pCR rate was significantly higher in the CRT group compared with the RT group. However, the pooled data did not show any statistically significant difference between the groups in OS, cancer-related survival, LR, resectability, curative resectability, sphincter preservation, postoperative mortality and postoperative morbidity (Latkauskas, Paskauskas et al. 2010).

1.7.2.5 Pre-operative versus Post-operative RT/CRT

Pre-operative (neoadjuvant) RT / CRT regimens assume the advantages of improved patient compliance, potential down-staging of tumour, and the possibility of increased rates of curative resections and sphincter preserving

resections. RT is understood to be more effective in the preoperative setting owing to preserved tissue oxygen status. There is now a body of evidence to suggest that the neoadjuvant RT / CRT is superior to postoperative RT / CRT in providing better oncological outcome with comparable toxicity (Sauer, Becker et al. 2004; Wong, Tandan et al. 2007).

A randomised trial by the German Rectal Cancer study Group (CAO/ARO/AIO-94 trial) investigated the oncological outcomes and the toxicity profile between preoperative and postoperative CRT regimens (Sauer, Becker et al. 2004). The trial found reduced five-year cumulative incidence of LR (6% v 13%, p=0.006), grade 3 or 4 acute toxic effects (27% v 40%, p=0.001), and long term toxic effects (14% v 24%, p=0.01) in the preoperative CRT group compared with the postoperative CRT group. There was, however, no difference in the rates of OS (Sauer, Becker et al. 2004).

The MRC CR07 trial randomised 1350 patients with operable rectal cancer to preoperative SCRT (25 Gy in five fractions) or initial surgery with selective postoperative CRT (45 Gy in 25 fractions with concurrent 5-FU) restricted to the patients with involvement of the circumferential resection margin (Sebag-Montefiore, Stephens et al. 2009). The trial found a reduction in the rates of LR (4% v 11%, p<0.0001) and improved DFS (73% v 66%, p<0.013) in patients who received preoperative RT compared with those treated with postoperative CRT. However, trial did not demonstrate difference in OS between the groups (Sebag-Montefiore, Stephens et al. 2009).

1.7.2.6 Pre-operative SCRT versus Pre-operative LCCRT

In recent years, focus has shifted to the studies aimed at determining the best preoperative RT regimen. Hypofractionated SCRT has been studied to examine its safety and efficacy against the standard LCCRT. The main difference in two regimens is that the SCRT is primarily aimed at reduction in LR whilst LCCRT is used to shrink the tumour and possibly down-stage it prior to surgical resection (ACPGBI_guidelines 2007). Bujko et al. found the comparable long term results of preoperative conventionally fractionated LCCRT and preoperative SCRT in the Polish trial, the only such trial in the TME era (Bujko, Nowacki et al. 2006). The study randomised 312 patients to receive either SCRT (25 Gy in five fractions of 5 Gy) followed by the TME surgery within 7 days or LCCRT (50.4 Gy in 28 fractions of 1.8 Gy and bolus 5-fluorouracil and leucovorin) and TME surgery 4–6 weeks later. Their results showed that early radiation toxicity was higher in the LCCRT group compared with the SCRT group (18.2% versus 3.2%) as was the severe late toxicity (10.1% versus 7.1%). The actuarial 4-year OS was similar, 67.2% in the SCRT group and 66.2% in the LCCRT group. DFS was 58.4% versus 55.6% and crude incidence of LR was 9 % versus 14.2%. (Bujko, Nowacki et al. 2006).

The only other similar, but smaller (n=84), trial showed similar results but concluded that LCCRT proved more effective compared with SCRT for the advanced rectal cancer (T4 or N2) and for sphincter preserving procedures for lower-lying tumours as denoted by the low rates of LR (Klenova, Georgiev et al. 2007). A recent meta-analysis encompassing both the above trials has confirmed the comparable oncological outcomes of the SCRT and the LCCRT regimens (Sajid, Siddiqui et al. 2010).

Clearly, more evidence is needed to determine whether one neoadjuvant RT regimen is better than the other. The Berlin Rectal Cancer Trial aims to add comprehensive piece of evidence in this area and is currently ongoing (Siegel, Burock et al. 2009). The trial is powered adequately to answer most questions by recruiting 760 patients in two arms: SCRT + TME + adjuvant chemotherapy and LCCRT + TME + adjuvant chemotherapy. The results of the trial will be eagerly awaited.

1.7.2.7 Toxic side effects of RT

An organ or tissue expresses response to radiation damage either as an acute effect or as a late (chronic) effect (Podgorsak 2005).

1. Acute effects: These are manifested soon after exposure to radiation and are characterised by inflammation, oedema, denudation of epithelial and haemopoietic tissue, and haemorrhage.

2. Late effects: These are delayed in onset and in the gastrointestinal tract include, e.g. intestinal fibrosis, atrophy, ulceration, stenosis or obstruction. Late effects may be generic and caused by the absorption of radiation directly in the target tissue, or consequential to acute damage in overlying tissues such as mucosa or the epidermis. The radiation effects of whole body irradiation culminate in the bone marrow syndrome, the gastrointestinal syndrome, and the central nervous system syndrome dependent on the dose of radiation the body is exposed to (Podgorsak 2005).

1.7.2.8 Assessment of histological response to RT: Tumour regression grading

A number of tumour regression grading (TRG) systems have been described in rectal cancer in order to assess tumour response to RT (Vallbohmer, Bollschweiler et al. 2012). All such grading classifications have revolved around assessment for pathological complete response versus partial response and no response. In 1994, a 5-point Mandard's TRG system was proposed to assess tumour response to RT in patients with oesophageal carcinoma with the ability to predict the oncological outcomes (Mandard, Dalibard et al. 1994). Subsequently, the Mandard's grading has been adapted for the assessment of histological response in rectal cancer treated with CRT (Suarez, Vera et al. 2008; Dhadda, Zaitoun et al. 2009). TRG is determined by the histological examination of post CRT resection specimen to assess the degree of pathological response to radiotherapy, 6 - 8 weeks post treatment. Mandard's Five grades have been described as follows (Mandard, Dalibard et al. 1994; Dhadda, Zaitoun et al. 2009) (Figure 1.8):

TRG 1: Complete response with absence of residual cancer and fibrosis extending through the wall.

TRG 2: Presence of residual tumour cells scattered through the fibrosis.

TRG 3: Increase in the number of residual cancer cells, with fibrosis predominant.

TRG 4: Residual cancer outgrowing fibrosis.

TRG 5: Absence of regressive changes.

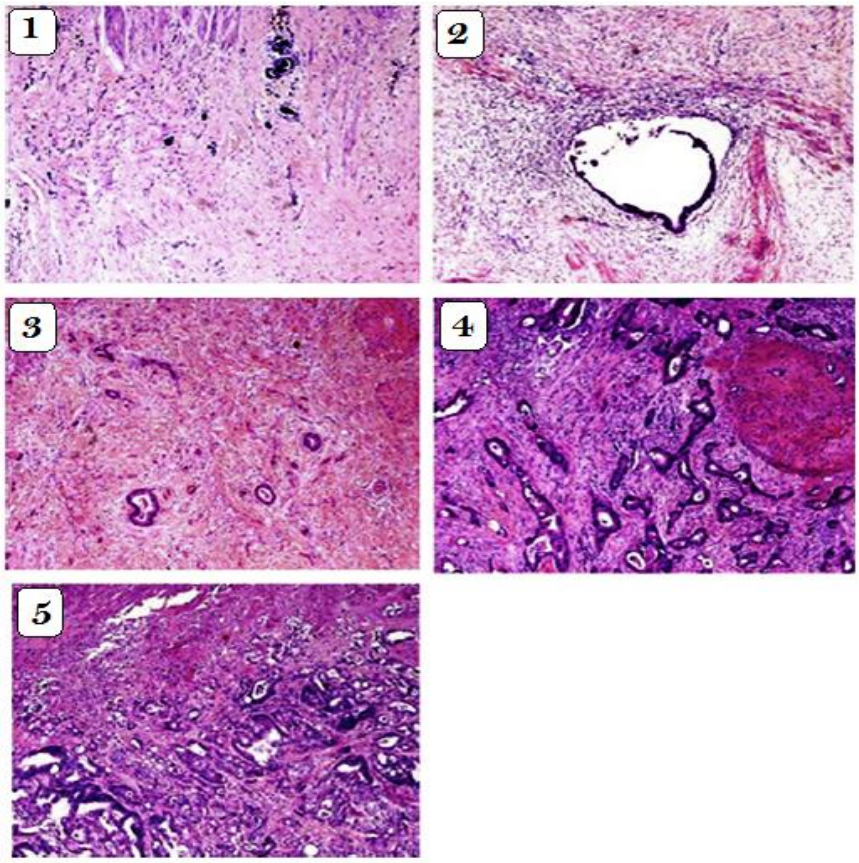

Figure 1.8: Mandard's classification of tumour regression grading. The five categories of TRG are based on histological response of tumour to RT ranging from pathological complete response in TRG 1 to absence of tumour regression in TRG 5 (see text above). Figure key: 1 = TRG 1, 2 = TRG 2, 3 = TRG 3, 4 = TRG 4, 5 = TRG 5. (Adapted from: Dhadda et al. 2009).

TRG 1 and 2 are classified as good responders and TRG 3, 4, 5 are considered poor responders to radiotherapy (Suarez, Vera et al. 2008; Dhadda, Dickinson et al. 2011). Mandard's TRG has been shown to be related to the oncological outcome in rectal cancer. Dhadda et al. (2011) examined the prognostic value of Mandard's TRG system in rectal cancer treated with neoadjuvant CRT (Dhadda, Dickinson et al. 2011). Patients were categorised into responders (TRG 1, 2) and poor responders (TRG 3, 4, 5). They found that of the 158 patients treated with preoperative CRT, 14% tumours were TRG 1, 41% were TRG 2, 31% were TRG 3, 13% were TRG 4 and 1% were TRG 5. The authors

observed improved disease free and overall survival in responders (TRG 1, 2) compared with poor responders (TRG 3, 4, 5). TRG was found to be an independent predictor of disease free survival on multivariate analysis (Dhadda, Dickinson et al. 2011).

Suarez et al. also reported similar results using Mandard's system with good responders (TRG 1, 2) demonstrating statistically significantly improved disease free survival compared with poor responders (TRG-3, 4, 5) (Suarez, Vera et al. 2008). A number of studies have now confirmed the role of Mandard's TRG system in rectal cancer with consistent reports of association with local control and disease free survival (Bouzourene, Bosman et al. 2002; Suarez, Vera et al. 2008; Dhadda, Dickinson et al. 2011).

Given the predictive potential of Mandard's TRG system in rectal cancer, efforts have been made to validate this system in rectal cancer. In order to examine the reproducibility of TRG, Lindebjerg et al. assessed TRG in 100 rectal cancer specimens treated with LCCRT (Lindebjerg, Hansborg et al. 2011). The authors found that the TRG assessment by the two observers did not contain discrepancies by more than one grade. A correlation analysis revealed that the weighted and unweighted kappa values were 0.89 and 0.82, respectively, suggesting high correlation and very low inter-observer variability. The study concluded that the TRG assessment is a highly reproducible grading system in rectal cancer (Lindebjerg, Hansborg et al. 2011).

The predictive potential of TRG for disease free survival has led the investigators to use this grading system as a surrogate of tumour response to CRT for biomarkers validation in rectal cancer. Farkas and colleagues performed immunohistochemical examination for five preselected biomarkers on 69 patients who received LCCRT for locally advanced rectal cancer (Farkas, Pozsgai et al. 2012). The histological response to neoadjuvant treatment was based on Mandard's tumour regression grading. Up to 48% of tumours were assessed as good responders and the remainder were poor responders or radioresistant. The authors found that high expression levels of GHRH-R and Hsp90 were significantly related with poor tumour regression (Farkas, Pozsgai et al. 2012).

It is usual for the surgery to be delayed to 6 - 8 weeks post completion of LCCRT to allow healing and to avoid anastomotic disintegration (Craven, Crellin et al. 2007). Such an interval between CRT and surgery also allows obtaining an optimum tumour regression. Dhadda et al. reported that tumour regression depends on the length of time between the LCCRT and the surgical resection (Dhadda, Zaitoun et al. 2009). In the study of rectal cancer patients treated with RT and concurrent Capecitabine based chemotherapy, they demonstrated that on multiple linear regression analysis the length of the interval to surgery and the tumour volume-halving time were independent predictors of the degree of tumour regression as per Mandard's grading (Dhadda, Zaitoun et al. 2009).

Similarly, a study by Farkas and colleagues demonstrated that based on TRG, there were more responders in the cohort undergoing delayed surgery at 7

weeks post RT compared with those who underwent surgery sooner (63% vs. 37%) (Farkas, Pozsgai et al. 2012). Therefore, best tumour regression is achieved a few weeks following completion of RT at which time surgical resection should be undertaken and tumour regression grading assessed. This is the reason why TRG is not assessed in the patients who receive neoadjuvant short-course RT (SCRT) comprising of five fractions of 5 Gy RT over five consecutive days followed by surgery a week later. Clearly, SCRT regimen does not allow appropriate TRG assessment and in order for any studies involving TRG assessment, patients treated with LCCRT are the only suitable cohort.

1.8 Response of rectal cancer to radiotherapy

1.8.1 Mechanism of action of ionising radiation

Ionising radiation (IR) brings about the desired effect of cell death by causing DNA damage. Whereas DNA may sustain such damage through a direct injury to DNA molecule, in most instances it follows an indirect route through free radical formation via the action of IR on cellular water. The types of DNA damage include DNA base damage, DNA single-strand breaks, and DNA double-strand breaks (DSBs). DSBs represent a minor but highly toxic DNA injury which is rapidly lethal for the cell if not repaired immediately. Following the DNA damage, the cell's fate is either survival or death, as determined by the following sequence of events (Figure 1.9):

a) DNA damage response and cell cycle arrest

b) Repair of damaged DNA - unsuccessful DNA repair leads to

c) Apoptotic cell death

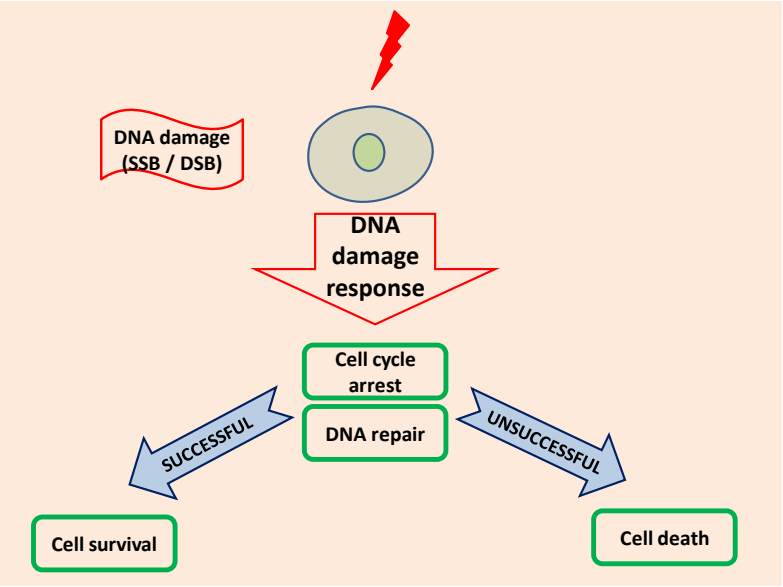

Figure 1.9: Mechanism of radiotherapy induced cell death. IR causes DNA damage in the form of SSB and DSB. Cells upon recognition of DNA damage initiate signal transduction mechanism culminating in cell cycle arrest to allow DNA repair. The ultimate outcome of DDR is cell survival or apoptotic death dependent upon whether or not the cell can successfully repair the DNA damage whilst in the cell cycle arrest.

1.8.2 DNA Damage Response (DDR) and the cell cycle arrest

The cells transit through a cycle between two successive divisions - called the cell cycle. This cycle is of the order of 10 - 20 hours in length and involves the G1, S, G2, and M phases, with DNA replication occurring in the S (synthesis) phase and mitosis in the M phase. It is understood that the cell cycle is controlled by interdependent regulatory mechanisms that bring the DNA to a state competent for duplication and division. The progression of cell through cell cycle is determined by two key sets of molecules; cyclins and the cyclin-dependent kinases (CDKs). CDKs are the main enzymes responsible for cell cycle regulation and require cyclins binding for activation. The complexes of CDK/cyclin are active molecules that cause phosphorylation of the downstream effector proteins to allow cell cycle progression (Tyson, Csikasz-Nagy et al.

2002). The cyclins and CDKs are negatively regulated by CDK inhibitors (CDKIs) at the cell cycle checkpoints (Iliakis, Wang et al. 2003). These checkpoints (G1/S and G2 checkpoints) are biochemical pathways that restrain cell cycle transition and/or induce cell death. These mechanisms ensure that an intact DNA is replicated in the S phase and that the chromosomes segregation is fool proof in the M phase (Tyson, Csikasz-Nagy et al. 2002).

The cells respond to the DNA damage by initiating signal transduction mechanisms and activating the cell cycle checkpoints that result in cell cycle arrest. Both checkpoints initiate protein kinase-based signal transduction cascade to activate downstream effectors that elicit cell cycle arrest. During G1 progression, there is sequential activation of cyclin D1 and cyclin E complexes. Cyclin D1 binds to CDK4, 6 and cyclin E binds to CDK2. Following the exposure of cells to genotoxic agents like IR, the activated p53 induces CDK inhibitor $p21^{WAF1}$. The elevated $p21^{WAF1}$ binds and inactivates cyclin D / CDK4,6 and cyclin E / CDK2 complexes resulting in pRB hypophosphorylation and cell cycle arrest (Pietenpol and Stewart 2002; Schmitt, Paquet et al. 2007). At G2 checkpoint, members of the PI3K family (DNA-PK, ATM / ATR kinases) become activated following the genotoxic insult by IR (Shiloh 2001). DSBs are recognised by the MRN complex (Mre11, Rad50 and Nbs1) which recruit the ataxia telangiectasia mutated (ATM) protein. These proteins can directly phosphorylate p53 and initiate signal transduction pathways that regulate DNA repair and cell cycle progression (Canman and Lim 1998; Shiloh 2001; Pietenpol and Stewart 2002). ATM-dependent signalling induced by DNA damage also results in activation of the CHK1 and CHK2 kinases (Reinhardt and Yaffe 2009). It is understood that following the DNA damage, the

ATM/CHK2 pathway is activated in response to DSBs and the ATR/CHK1 pathway is associated with SSBs or bulky lesions. Both pathways converge on CDC25, a positive regulator of cell cycle progression, which is inhibited by CHK1 or CHK2 mediated phosphorylation (Reinhardt and Yaffe 2009).

The p53 dependent G1 arrest depends upon its gene status. Kastan and colleagues demonstrated that tumour cells with wild type p53 genes (normal human fibroblasts, RKO colorectal cancer cells, and U-20S osteosarcomal cells) exhibited G1 arrest following exposure to IR whereas cells with mutant p53 genes (SW480 colorectal cancer cells and Saos osteosarcoma cells) continued to progress though S phase showing their ability to replicate excessively (Kastan and Kuerbitz 1993).

Key cell cycle regulators and DDR molecules are regulated by the ubiquitin proteasome system. The p53 protein as well as cyclins and CDKIs $p21^{WAF1}$ (CDKI-1) and $p27^{Kip1}$ (CDKI-2) are the major substrates for proteasomal degradation (Kim, Lim et al. 2004). CHK2 promotes stabilisation of the p53 levels. It blocks degradation of p53 by MDM2 (one of the E3 protein ligases) of the ubiquitin proteasome system.

1.8.3 DNA repair

Genotoxic insults like IR cause DNA damage in the form of the SSBs and DSBs. DSBs are the most lethal type of DNA damage because they lead to chromosomal breakage and rearrangement, events that may result in apoptotic

cell death or development of cancer. In order to survive the DNA damage, the cells attempt to repair the damaged DNA for which two major repair mechanisms exist: the non-homologous end joining (NHEJ) and the homologous recombination repair (HRR). The NHEJ constitutes the main DNA DSB repair pathway in mammals and appears to comprise of classical NHEJ and alternative pathways (Rodemann 2009). This pathway is dependent on the presence of the DNA-dependent protein kinase catalytic subunit (DNA-PK_{CS}) and several other downstream proteins (Figure 1.10).

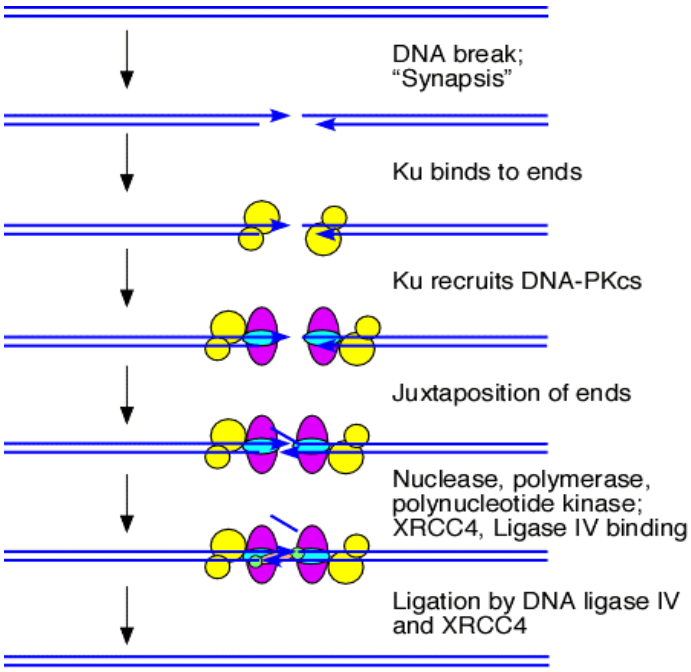

Figure 1.10: The NHEJ DNA-repair pathway. Sequence of events in the repair of DNA DSBs is shown. The Ku70/80 complex binds the broken DNA ends first. DNA-PK_{CS} binds Ku and then functions as a platform for the recruitment of DNA ligase IV and XRCC4, which are ultimately responsible for direct ligation of the two broken DNA ends.

NHEJ occurs primarily in the S phase when no sister chromatid is present. NHEJ functions to join any two ends of exposed DNA, regardless of their individual base sequence. It is a complex process with multiple pathways. The two broken ends of the DNA DSBs destined to be repaired by NHEJ are first recognised by

the Ku70/80 heterodimer which carries a high affinity for DNA ends. Ku is a DNA binding agent. DNA-PK_{CS} locates and binds to the Ku complex at the site of damage. The binding of DNA-PK_{CS} mediates the recruitment of XRCC4 and DNA ligase IV, the proteins responsible for the completion of the ligation reaction (Warmerdam and Kanaar 2010).

Mukherjee and colleagues demonstrated the role of DNA-PK in repairing the DSBs (Mukherjee, McEllin et al. 2009). They showed that EGFR variant III expression enhanced clonogenic survival of glioblastoma cells following RT that was due to the accelerated repair of DSBs. The underlying mechanism was identified to be the elevated activation of DSB repair enzyme DNA-PK_{CS} in EGFR variant III-expressing U87 glioma cells (Mukherjee, McEllin et al. 2009).

In contrast with NHEJ, the HRR uses sister chromatid as a template to faithfully recreate the damaged section and join the ends together properly. It repairs both the DSBs and SSBs. It occurs in S or G2 phase of the cell cycle when sister chromatids are present. It is an error free process. An intact template of DNA in the sister chromatid or homolog is used to repair the damaged DNA. Loss of a cell's ability to undertake HR repair increases radiation sensitivity and the rates of mutation (Dasika, Lin et al. 1999; Rodemann 2009). DSBs are recognised by the MRN complex (Mre11, Rad50 and Nbs1) which recruits the ATM protein involved in both the regulation of HRR and the cell cycle checkpoints (Kelley and Fishel 2008). In yeast, the genes involved in HRR belong to a group called the 'RAD52'. In humans, the genes implicated in the HRR pathway are found to be similar to the yeast RAD52 group of genes and

include, e.g. RAD50, RAD51, RAD52, RAD54 and MRE17 (Thacker 1999). The RAD51 protein is believed to play a central role in HRR, acting in all phases of the pathway (Krejci, Altmannova et al. 2012). Moreover, a link has been proposed between HHR pathway protein RAD51 and BRCA1 and BRCA2, the genes associated with familial breast cancer (Hiom 2000; Krejci, Altmannova et al. 2012), Especially, BRCA2 appears to be principal regulator of the central functions of RAD51 (Krejci, Altmannova et al. 2012).

1.8.4 Apoptosis

Apoptosis is synonym with the term '*programmed cell death'* that is used to describe '*the cell deaths that occur in predictable places and at predictable times during development, to emphasise that the deaths are somewhat programmed into the development plan of the organ'* (Mignotte and Vayssiere 1998). However, the role of apoptosis is not limited to cellular turnover in developmental environment. It is well established that apoptosis serves as a major mechanism for the precise regulation of cell numbers. It provides a defence mechanism to remove the unwanted and potentially dangerous cells including the infected and the tumour cells. Two major cellular pathways have been characterised to describe how apoptotic machinery is activated, functions and is regulated. The classical mitochondrial (intrinsic) pathway is activated by p53 mediated signal transduction, whereas the other route to apoptosis is via death receptor activation (extrinsic pathway).

1.8.4.1 Intrinsic apoptosis

The *intrinsic pathway* is triggered by cellular stresses and cytotoxic stimuli like radiotherapy and chemotherapy. Cells recognise the DNA damage by activation of

the DDR pathways (Section 1.8.2) and activate the intrinsic pathway via activated p53 mediated signalling (Sturm, Rau et al. 2006; Ashkenazi 2008). PUMA and NOXA are among the first proteins up-regulated by the activated p53, which in turn activate BAX and BAK. These pro-apoptotic proteins increase the mitochondrial outer membrane permeabilisation (MMP) causing release of Cytochrome C (Elmore 2007; Ashkenazi 2008). The Cytochrome C binds to the adaptor protein APAF-1 to recruit the initiator pro-Caspase 9. This complex of Cytochrome C, APAF1 and Caspase 9 is called apoptosome. Activated Caspase 9 is responsible for activation of the effector Caspases 3, 6 and 7 which are the executioners of apoptosis (Chang and Yang 2000; Elmore 2007; Ashkenazi 2008). Mitochondria continue to play central role and concurrent with the release of Cytochrome C from the intramembrane space, other factors are also released including apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and SMAC/DIABLO protein to inhibit the inhibitor of apoptosis proteins (IAPs) and thus promoting apoptosis (Mignotte and Vayssiere 1998; Wang 2001; Armstrong 2006). There is evidence that p53 dependent intrinsic pathway is the classical mechanism of radiation induced apoptosis. It has been demonstrated that p53 can interact with BclXL and Bcl-2 to exert its direct apoptogenic function at mitochondria (Fei and El-Deiry 2003). Figure 1.11 illustrates the two apoptotic pathways.

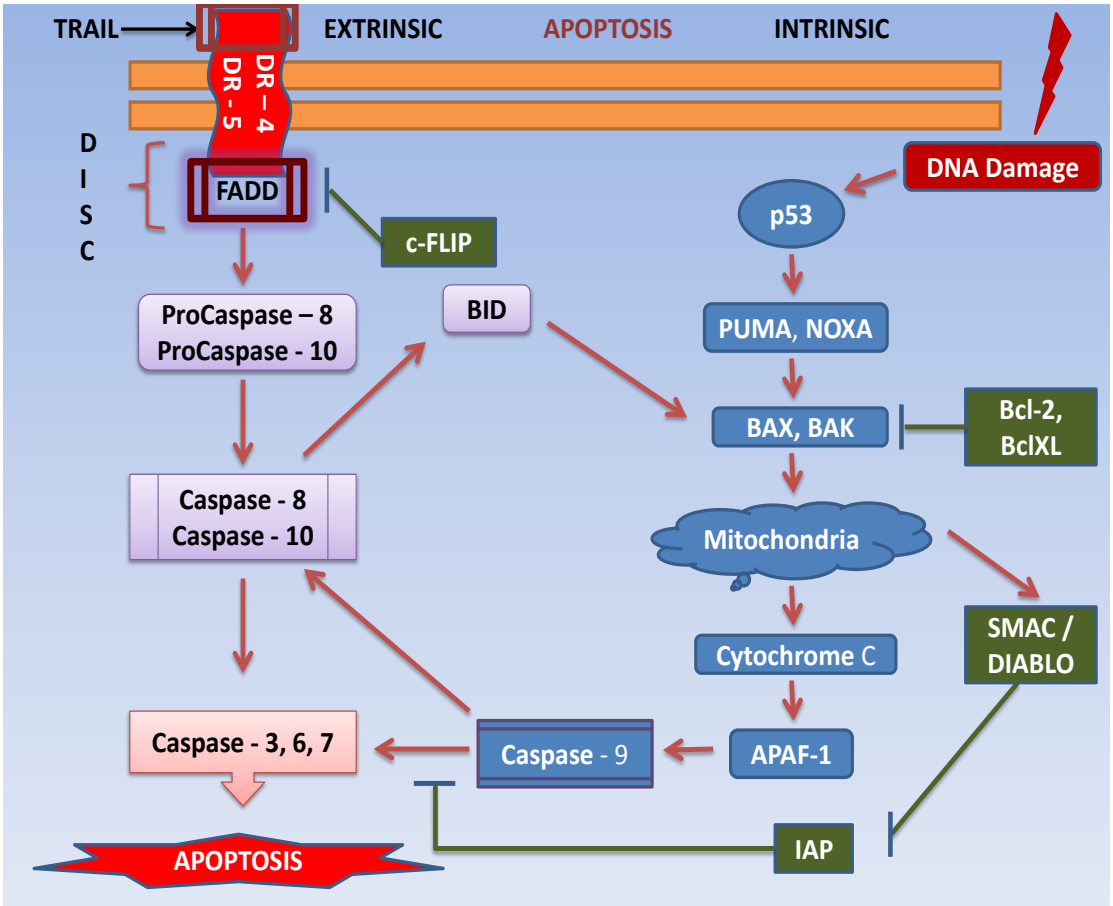

Figure.1.11: Apoptosis pathways. ***Intrinsic pathway:*** Cellular stress e.g. exposure to ionising radiation activates the p53 protein which in turn initiates the intrinsic pathway. The activated p53 up-regulates proapoptotic proteins that increase the permeability of outer mitochondrial membrane to trigger the release of cyctochrome C. A complex of Cytochrome C, APAF-1 and Caspase 9 (apoptosome) is formed. Activated Caspase 9 leads to activation of the effector Caspases 3, 6 and 7 which induce apoptosis. A mitochondrial protein SMAC/DIABLO promotes apoptosis by removing the inhibitory effect of IAP proteins. ***Extrinsic pathway:*** This pathway is mediated by the death receptors. The ligand binding of the death receptors causes their activation. Ligand binding induces recruitment of the adaptor protein Fas-Associated Death Domain (FADD) and the initiator Caspases (proCaspases 8 and 10). A death inducing signalling complex (DISC) is formed comprised of death domains of death receptor and FADD, death effector domains of FADD and proCaspase 8. The DICS is responsible for activation of pro-Caspase 8 to Caspase 8. Caspase-8 activates downstream effector Caspases (Caspases 3, 6, 7) to initiate apoptosis. The regulation of extrinsic pathway is the function of c-FLIP protein. The extrinsic pathway is also linked to intrinsic pathway through Caspase 8 mediated processing of BID. The truncated BID is understood to act on BAX to trigger mitochondrial release of Cytochrome C, forming the final common pathway (Figure adapted from Ashkenazi 2008).

There are tight regulatory mechanisms involving Bcl-2 and IAP protein families (Mignotte and Vayssiere 1998). The IAPs inhibit effector Caspase activation and consists of several members, including XIAP, cIAP1, cIAP2, NAIP, livin, ILP2, BRUCE and survivin (Smolewski and Robak 2011; Fulda and Vucic 2012). Survivin is the smallest and relatively recently identified member of this family and has been linked to radioresistance (Section 1.9.8) (Rodel, Hoffmann et al. 2002). The Bcl-2 family of proteins remains key regulator of intrinsic apoptosis and consists of a number of pro- and anti-apoptotic proteins characterised by the presence of distinctly conserved sequence motifs, called Bcl-2 homology (BH) (Figure 1.12). The key members include pro-apoptotic proteins BAX and BID, and anti-apoptotic proteins Bcl-2 and BclXL (Breckenridge and Xue 2004; Lomonosova and Chinnadurai 2008).

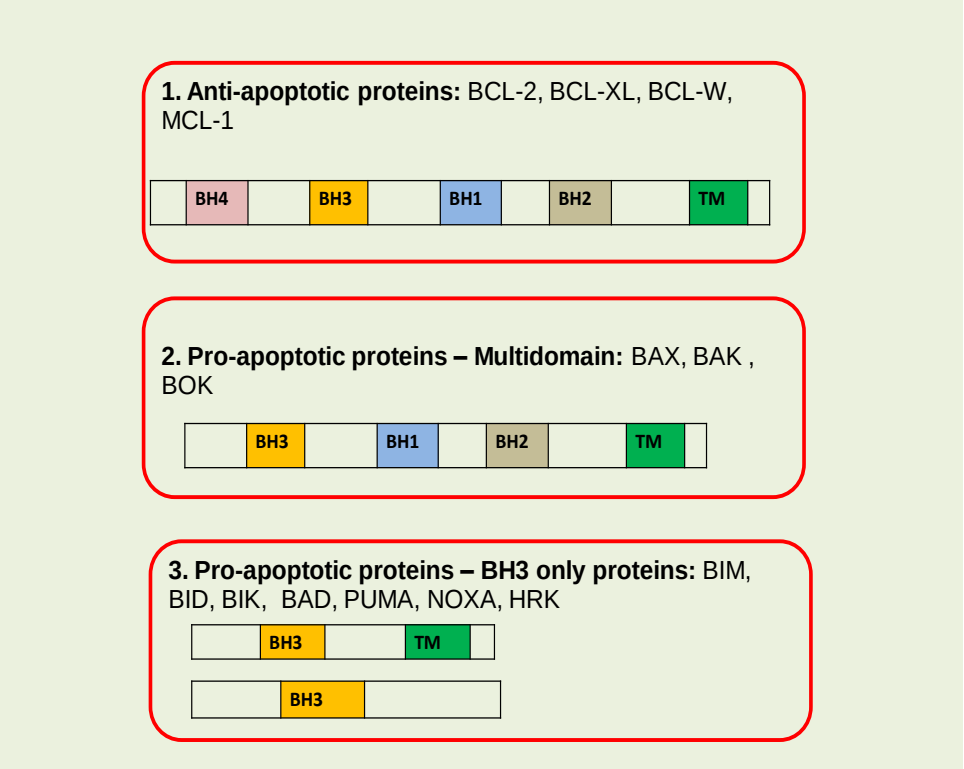

Figure 1.12: The Bcl-2 family of proteins. This family comprises of three types of proteins based on their function and presence of BH domains in their structures. The anti-apoptotic proteins contain all four domains. The BH3 only pro-apoptotic proteins control the pro-apoptotic function and activate the multi-domain pro-apoptotic proteins. BH: Bcl-2 Homology, TM: Trans-membrane

Bcl-2 is the main pro-survival anti-apoptotic protein normally incorporated into the outer mitochondrial membrane. It is understood that Bcl-2 functions by interacting with the pro-apoptotic proteins of Bcl-2 family in an antagonist fashion. It is also suggested that the pro-survival role of anti-apoptotic proteins, Bcl-2 and BclXL, is linked to the BH 1 - 3 domains that form a hydrophobic groove which is proposed to be their functional part (Chan and Yu 2004). Bcl-2 is believed to prevent the release of mitochondrial apoptogenic cytokine Cytochrome C, thereby preventing the activation of Caspases - the executioners of apoptosis. Moreover, Bcl-2 has also been suggested to protect cells from Caspases by dragging them to intracellular membranes (probably the mitochondrial membrane) and by preventing their activation (Mignotte and Vayssiere 1998). The final outcome of the actions of Bcl-2 family members with regards to stimulatory and inhibitory apoptotic function depends on the relative ratios of their pro- or anti- apoptotic members (Mignotte and Vayssiere 1998). An imbalance between the members of the Bcl-2 family of proteins in favour of the anti-apoptotic proteins is commonly observed in malignant disorders, as denoted by over-expression of anti-apoptotic Bcl-2 or BclXL in a number of cancers (Juin, Geneste et al. 2004).

It has been reported that impaired or loss of Bcl-2 expression might be associated with poor prognosis in different cancer types. Some relationship between radioresistance and over-expression of Bcl-2 has been reported in association with colorectal cancer (section 1.9.4), head and neck cancer (Nix, Cawkwell et al. 2005), leukaemia (Kariya, Ogawa et al. 1999), and prostate cancer (Rosser, Tanaka et al. 2004). The association of Bcl-2 expression with radiotherapy response in rectal cancer is discussed in section 1.9.4.

1.8.4.2 Extrinsic apoptosis

The *extrinsic apoptotic pathway* signalling is mediated through death receptors (Figures 1.11 and 1.13). Eight members of the death receptor family have been characterised which are activated by their respective ligands (Figure 1.13) (Ashkenazi and Dixit 1998; French and Tschopp 2003). The death receptors contain an intracellular globular protein interaction domain called a death domain (DD). The activated death receptors recruit an adaptor protein called Fas Associated Death Domain (FADD). The FADD consists of two protein interaction domains namely a death domain and a death effector domain (DED). The DD of FADD binds to DD of death receptors and the DED domain binds to the DED of pro Caspase-8 to form a complex at the receptor called the Death Inducing Signalling Complex (DISC) (Lavrik, Golks et al. 2005). The DISC complexes are formed at Fas, DR4 or DR5 receptors. The DISC formation subsequently leads to self-activation of Caspase 8 through oligomerization of Pro-Caspase 8, promoted by associations of DED domains. Once activated, Caspase 8 activates downstream effector Caspases (Caspases 3, 6, 7) to initiate apoptosis (French and Tschopp 2002). ProCaspase-10 is also activated and forms an active heterotetramer. However, it remains elusive whether Caspase 10 can trigger apoptosis on its own, in response to CD95 or TRAILR1/R2 stimulation, in the absence of Caspase 8 (Lavrik, Golks et al. 2005). The Caspases play a critical role in the execution phase of apoptosis (Cohen 1997). The Caspase 8 and 10 (and Caspase 9 of intrinsic apoptotic pathway) are termed 'initiator Caspases' and the Caspase 3, 6, 7 are called 'effector Caspases' (Cohen 1997; MacFarlane 2003). The death receptor machinery also leads to activation of mitochondrial cascade of apoptotic signalling. Once activated, Caspase 8 causes cleavage of BID (BH3 domain

containing Bcl-2 inhibitory protein) at its amino terminus. The truncated BID (tBID) translocates into the mitochondria and triggers BAX oligomerization and Cytochrome C release to activate the intrinsic pathway, thus connecting the two Caspase activation pathways and amplifying the death receptor apoptotic signal (Srivastava 2001).

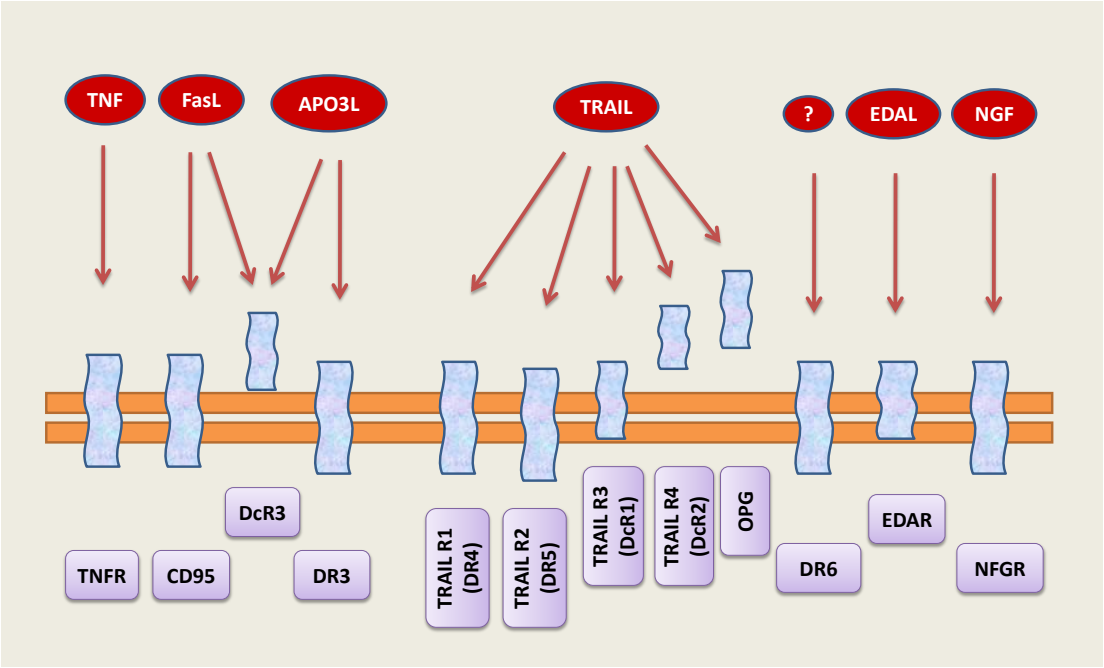

Figure 1.13: Death receptors and death receptor ligands. The death receptors initiate extrinsic apoptosis by binding with their corresponding ligand. These receptors include: TNF receptor 1 (TNFR1; also known as DR1, CD120a, p55), DR2 (also known as CD95, APO-1 and Fas), DR3 (also known as APO-3, LARD, TRAMP), TRAIL receptor 1 (TRAILR1; also known as DR4 and APO-2), TRAILR2 (also known as DR5, KILLER and TRICK2), DR6, Ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR). Adapted from Lavrik et al. (2005).

The death receptor machinery is under tight regulation and there are several levels of modulation. The death ligands also interact with decoy receptors which actively compete with death receptors and lack in intracellular death domain (Lavrik, Golks et al. 2005). In addition, the cellular FLICE-like Inhibitory Proteins (c-FLIP) which possess two DED motifs, without any protease activity, function

by competing with Caspase 8 for binding with the DISC (Thorburn 2004). The regulation of apoptosis is further ensured by downstream inhibitors of apoptosis that inhibit effector Caspase activation (Lavrik, Golks et al. 2005).

DR4 is one of the most important death receptors responsible for the initiation of apoptosis through the extrinsic pathway. DR4 protein is tumour necrosis factor receptor (TNF) superfamily member 10a and the gene which encodes this protein is called TNFRSF10A. DR4 is activated by tumour necrosis factor-related apoptosis inducing ligand (TRAIL, TNFSF10) and is one of the five TRAIL receptors described (Figure 1.13) (Ashkenazi and Dixit 1998; French and Tschopp 2003). It is well established that DR4 mediated apoptotic death is initiated by its ligand binding with TRAIL. In 1995, TRAIL was first identified based on its sequence homology to other members of the TNF superfamily (Wiley, Schooley et al. 1995). DR4 was the first TRAIL receptor to be discovered soon after. While extrinsic apoptosis is the major signalling outcome for TRAIL death receptors, they can also activate survival signals via the transcription factor NFκB, which can up-regulate anti-apoptotic genes (Kimberley and Screaton 2004).

The expression levels of TRAIL and death receptors are associated with response of cancer to anti-neoplastic treatment. Our research group has previously demonstrated a possible association of DR4 with radioresistance in breast cancer. DR4 was found to be under-expressed in three novel radioresistant breast cancer cell sublines MCF-7RR, MDA-MB-231RR and T47DRR on antibody microarray and western blot analyses. The under-

expression of DR4 was subsequently validated in a pilot immunohistochemical study of radioresistant breast cancer specimens (ELFadl, Hodgkinson et al. 2011). Unfortunately, there are no published studies investigating the role of DR4 in radioresistance in rectal cancer.

Despite relative paucity of the direct evidence of TRAIL death receptor pathway regarding its impact on treatment outcome, it offers an attractive target for anticancer therapy. The pro-apoptotic receptor agonists (PARAs) targeting DR4 and DR5 possess selective ability to kill malignant cells without affecting the healthy cells. Emerging clinical results have confirmed that DR4/5 PARAs are relatively well-tolerated and suitable for further investigations (Trarbach, Moehler et al. 2010; Yang, Wilson et al. 2010). Mapatumumab, also called HGS-ETR1, is a fully human immunoglobulin G1 λ agonistic monoclonal antibody to DR4. It specifically binds to DR4 (TRAILR1), competing with TRAIL, to enhance apoptotic signals (Figure 1.14). In-vitro and pre-clinical studies showed that Mapatumumab reduced the viability of multiple types of cancers and induced the activation of several important targets in the apoptotic pathway including Caspase 8, BID, Caspase 9, Caspase 3, and cleavage of PARP (Pukac, Kanakaraj et al. 2005). Further, Mapatumumab treated cancer cells in-vitro enhanced the cytotoxicity of chemotherapeutic agents even in the cell lines that were not sensitive to Mapatumumab monotherapy. Furthermore, the administration of Mapatumumab resulted in rapid tumour regression or repression of tumour growth in pre-established colon, non-small cell lung, and renal tumours in xenograft models (Pukac, Kanakaraj et al. 2005).

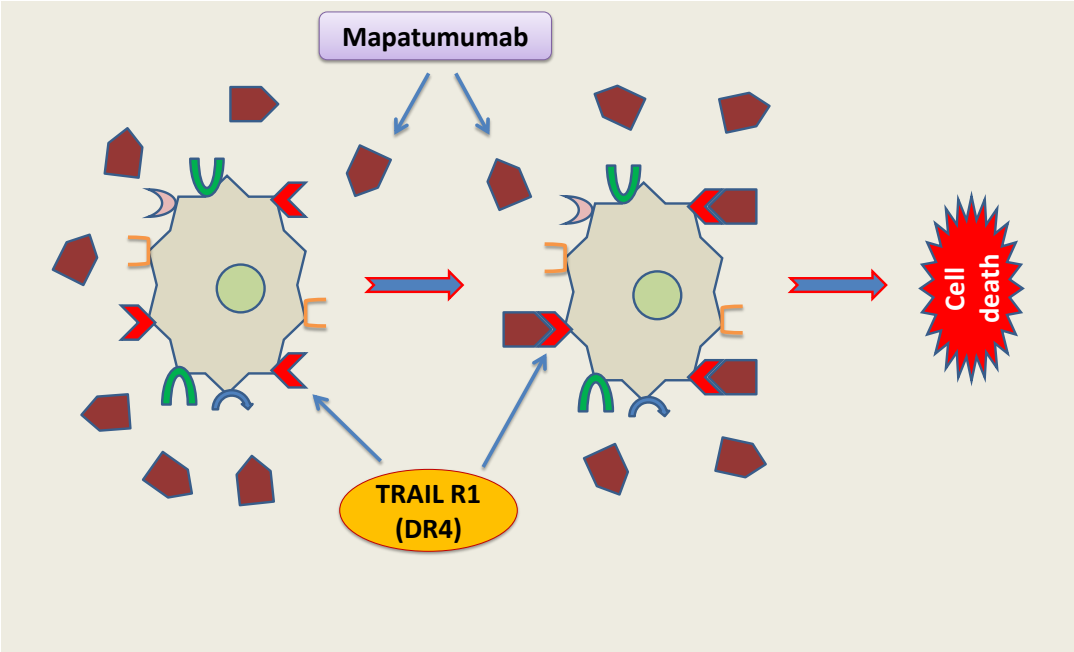

Figure 1.14: Action of Mapatumumab on cells expressing DR4. DR4 (TRAILR1) specific agonistic antibody 'Mapatumumab' promotes apoptosis by selective binding with DR4 (TRAILR-1). Adapted from http://www.hgsi.com/trail-receptor-antibodies-12.html.

Augmenting TRAIL activated DR4/5 mediated apoptosis has been investigated by using recombinant human TRAIL (rhTRAIL). In-vitro treatment of irradiated melanoma cells with rhTRAIL has been shown to substantially enhance the TRAIL mediated apoptosis via up-regulation of DR4 and DR5 (Ivanov, Zhou et al. 2007). Similarly, rhTRAIL and the agonistic antibodies against DR4 and DR5 were observed to enhance the apoptosis in irradiated HeLa cells (Maduro, de Vries et al. 2008). Lapatinib, a dual HER2/EGFR inhibitor, has been reported to enhance the proapoptotic effect of TRAIL and its two receptor agonists, Mapatumumab and Lexatumumab in colorectal cancer (Dolloff, Mayes et al. 2011). Given the potential association of EGFR over-expression with radioresistance (Section 1.9.2), agents like Lapatinib would offer an attractive option in cancer therapeutics owing to dual mode of radiosensitisation by EGFR antagonistic and DR4 agonistic actions, thereby promoting cell death.

The evidence about targeting of DR4 and its ligand TRAIL as anti-cancer treatment is continuing to emerge. Such observations indicate DR4 or TRAIL based agonistic strategies might result in radiosensitisation of cancer. DR4 and TRAIL clearly hold promise and will continue to remain in focus in oncological and pharmaceutical research.

1.9 Prediction of response of rectal cancer to Radiotherapy

As discussed in detail in section 1.7.2, neoadjuvant RT / CRT is offered to patients with locally advanced rectal cancer (T3, T4 tumours), those with nodal disease and those with threatened CRM to achieve resectability, obtain good local control and prevent recurrence (Nagy 2008). Unfortunately, not all patients derive therapeutic benefit in terms of good pathological response. Search for predictors of response to RT / CRT has been an active area of research. Biological markers (biomarkers) would be reliable predictors that can be assayed on the biopsy tissues or in blood prior to radiation treatment.

A biomarker is defined as *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"* (Buyse, Sargent et al. 2010).

This thesis will review the existing work on the biomarkers of response to RT / CRT, summarise the potential of existing biomarkers and will formulate a hypothesis for work undertaken in this thesis.

Potential biomarkers of resistance to radiotherapy in rectal cancer

A comprehensive literature search was made on pubmed / medline using the following search terms: “rectal” “radiotherapy” “chemoradiotherapy” “chemoradiation” “biomarker”. The search revealed 1291 initial articles. The articles were reviewed for relevance to study of biomarkers in relation with radiotherapy response. Studies with end points of tumour regression, pathological response, differential expression, apoptosis, and oncological outcomes were included to draw conclusions about the response to RT / CRT. Other relevant citations were identified from the identified studies. A total of 103 studies were included in the final review (Table 1.9). An exhaustive list of biomarkers with putative association with response to RT / CRT were recorded and summarised in the table 1.9. A panel of biomarkers investigated in more than 10 studies is concluded in the Table 1.10 and summarised in the following sections.

Table 1.9: A review of biomarkers of radioresistance in rectal cancer.
Of the 1291 studies found on initial literature search, 103 were assessed to be relevant and included in the final review.

Biomarkers	Study / Reference	Sample	Number	Methods	Endpoints	Results / correlation with response to RT / CRT
p53	(Adell, Sun et al. 1999)	Pre-treatment biopsy	163	IHC	Local recurrence	Absent p53 protein = significant reduction in local recurrence
Ki-67	(Adell, Zhang et al. 2001)	Pre-treatment biopsy	152	IHC	Local recurrence	Ki-67 expression = increased local recurrence
Circulating cell-free DNA	(Agostini, Pucciarelli et al. 2011)	Plasma pre and post treatment	67	Real-time PCR	TRG	Pre-treatment levels = no association, Post-treatment reduced levels = good tumour regression
MUC2	(Ambrosini-Spaltro, Salvi et al. 2008)	Pre- and post-treatment tissue	32	IHC	TRG	Less than 60% MUC2 staining associated with better tumour regression
Ki-67, p53	(Andrade, Oshima et al. 2011)	Pre-treatment	61	IHC	OS	p53 staining = no difference, Ki-67 under-expression = better survival
Ki-67, Securin	(Avoranta, Korkeila et al. 2011)	Pre- and post-treatment	211	IHC	TRG, survival	Low Ki-67 in post-treatment sample = significant tumour regression. Securin over-expression = reduced DFS
EGFR	(Azria, Bibeau et al. 2005)	Pre-treatment	77	IHC	Loco-regional recurrence, survival	Recurrence rates higher if EGFR staining extent superior to 25%
p53, p21, MLH1, MSH2, MIB-1, TS, EGFR, VEGF	(Bertolini, Bengala et al. 2007)	Pre- and post-treatment	91	IHC	pCR, DFS, OS	MLH+ = higher but insignificant pCR, reduced p21 expression, absent EGFR = better DFS and OS. High MIB-1 expression = reduced OS

GLUT-1	(Brophy, Sheehan et al. 2009)	Pre-treatment	69	IHC	TRG	GLUT-1 under expression = increased tumour regression and good response to CRT
p53, p21, Bcl-2, BAX, Ki-67, Ku-70	(Chang, Jung et al. 2005)	Pre-treatment	130	IHC	Pathological response	BAX expression = higher tumour regression Others = no association
p53	(Chen, Wu et al. 2012)	Pre- and post-treatment	1830	Various: meta-analysis	Various	Wild type p53 / low expression of p53 protein = good response
CEA	(Das, Skibber et al. 2007)	Pre-treatment Serum	562	?	pCR	CEA level>2.5 ng/mL = associated with lower pCR on univariate but not on multivariate analysis
COX-2, Ki-67, Osteopontin, IL6	(Debucquoy, Goethals et al. 2006)	Pre- and post-treatment tissues, serum	30	IHC, serum assays	pCR	Expression of COX-2 increased and Ki-67 decreased post RT. No relation to pCR, Low levels of osteopontin and IL6 = higher pCR.
COX-2, Ki-67, EGFR, VEGF, c-CK18, Carbonic anhydrase IX	(Debucquoy, Goethals et al. 2009)	Pre- and post-treatment tissues	99	Tissue micro-array	Dworak TRG, T down staging	Higher VEGF expression pre-treatment = Low regression grade. No relationship to T stage. No relationship of other proteins to regression or T stage. Ki-67 reduced expression in post-treatment samples. Expression of COX-2 in pre-treatment samples = improved OS
EGFR	(Dvorak, Sitorova et al. 2012)	Pre- and post-treatment tissue	53	IHC	DFS, OS	Higher EGFR post CRT = reduced DFS and OS
p53, COX-2, VEGF, p21, p27, BAX, Bcl-2, APAF-1	(Edden, Wexner et al. 2012)	Pre- and post-treatment tissue	152	IHC	TRG, pCR, T-down staging	Pre-treatment APAF-1 expression = significant regression, Over expression of COX-2 and VEGF = reduced tumour regression
p53	(Elsaleh, Robbins et al. 2000)	Pre-treatment tissue	48	IHC, PCR /SNP	Tumour size	p53 over expression or p53 mutation = No significant differences in tumour size reduction or local failure

PDGFRbeta, c-kit	(Erben, Horisberger et al. 2008)	Pre- and post-treatment	33	PCR	TRG	No correlation between pre-treatment PDGFRbeta and c-kit mRNA expression and tumour regression rate.
Hsp90, sHsp 16.2, p-Akt, GHRG-H, SOUL	(Farkas, Pozsgai et al. 2012)	Pre-treatment tissue	69	IHC	Mandard TRG	High GHRH-H and Hsp90 = minimal or absent regression
BAX, p53, Bcl-2, BclXL	(Fucini, Messerini et al. 2012)	Pre-treatment	67	IHC	T-down staging, OS	BAX expression = Higher down staging and survival, mutant p53, BclXL, Bcl-2 expression = reduced OS
VEGF, COX-2	(Giralt, Navalpotro et al. 2006)	Pre-treatment	81	IHC	Survival	Higher VEGF expression = Reduced DFS
EGFR, VEGF, ERCC1, TS, KRAS, BRAF	(Grimminger, Danenberg et al. 2011)	Pre- and post-treatment	130	Gene analysis	Pathological response	Pre-treatment EGFR, VEGF mRNA expression, KRAS mutation = No response to cetuximab based CRT
p27 kip1	(Gunther, Dimmler et al. 2003)	Pre-treatment	42	IHC	TRG, DFS, OS	p27 kip1 expression and staining intensity = no relationship to tumour regression
HIF-1 alpha, GLUT-1, Bcl-2 and Ki-67	(Havelund, Sorensen et al. 2013)	Pre-, and post-treatment	86	IHC	Expression, pathological response	Decreasing expressions of HIF-1alpha, Bcl-2 and Ki-67 during CRT, No relationship to response
HIF-alpha	(Havelund, Spindler et al. 2012)	Pre-treatment blood	198	Real-time PCR	Mandard TRG	HIF-1 alpha c(*)191T>C CC genotype = higher rate of response in test samples and poor response in validation samples
CD133	(Hongo, Kazama et al. 2012)	Pre-treatment	78	IHC	TRG	Expression of CD133 related to reduced tumour regression in rectal cancer treated with CRT.
SOD2 rs4880, IL13 rs1800925 (SNP)	(Ho-Pun-Cheung, Assenat et al. 2011)	Pre-treatment	71	PCR	Pathological response	SOD2 rs4880, IL13 rs1800925 associated with better tumour response to CRT

Topoisomerase I, TS	(Horisberger, Erben et al. 2009)	Pre- and post-treatment tissue	38	Real-time PCR	T and N down staging	Topisomerase I expression in biopsy = greater response. TS no difference between responders and non-responders
Survivin, Annexin	(Horisberger, Erben et al. 2010)	Pre- and post-treatment	38	Real-time PCR	Down staging, Progression free survival	Expression levels (mRNA) of survivin and annexin A4 and A5 = no correlation with down-staging or progression free survival
MIB, Cyclin E, p21, p27, p53,survivin, Bcl-2, BAX	(Huerta, Hrom et al. 2010)	Pre-treatment	38	Tissue micro-array	Pathological response	Immunoreactivity for MIB, p53, Bcl-2, BAX = significant pathological response. MIB = independent predictor of response on logistic regression analysis
TS, TP, DPD	(Jakob, Liersch et al. 2005)	Pre- /post-treatment	25 and 40	IHC	TRG	High TS expression in biopsy and resection = no response to CRT. No relationship with TP, DPD.
Ki-67, p53, TS	(Jakob, Liersch et al. 2008)	Pre- and post-treatment	22 and 40	IHC	TRG	Low Ki-67 = significant tumour regression, p53 not related to tumour regression
CD133	(Jao, Chen et al. 2012)	Colonic = 157, rectal = 76	76	IHC	TRG	Cytoplasmic CD133 expression = reduced tumour regression
p53, Caspase 8	(Kelley, Coppola et al. 2005)	Pre-treatment biopsy	50	IHC	Pathological response	combined p53 protein staining and caspase 8 negativity = no response
Ki-67, BAX, TS, Bcl-2, Mcm3, grp78, ssDNA, DPD, CD34, VEGF, Nestin, LAT1	(Kikuchi, Mikami et al. 2009)	Pre-treatment biopsy	60	IHC	Dworak TRG	High Ki-67 labelling index, BAX and TS = good response (better tumour regression)
EGFR	(Kim, Kim et al. 2006)	Pre-Treatment	183	IHC	T-down staging	Low EGFR expression = increased down staging

COX-2, EGFR, VEGF, TS, RKIP	(Kim, Kim et al. 2012)	Post-treatment	68	IHC	Recurrence FS, Distant metastasis FE, DFS, OS	VEGF and COX-2 over expression, and RKIP under expression = decreased DMFS, DFS, OS. Combined COX-2+/VEGF+ = reduced DFS. Combined RKIP+/COX-2- and RKIP+/VEGF- = improved DFS
Survivin, COX-2, EGFR, VEGF, TS, Ki-67, p21	(Kim, Chie et al. 2011)	Pre-treatment	54	IHC tissue micro-array	T-down staging	High Survivin expression = less tumour down staging. No relationship of other biomarkers.
p53, Bcl-2, Ki-67	(Kim, Park et al. 2001)	Pre-treatment	23	IHC	Pathological response	p53 mutation and Bcl-2 expression = no difference in pathological response. High Ki-67 labelling index = more complete pathological response
Survivin	(Knutsen, Adell et al. 2004)	Pre-treatment	57 = no RT 41 = RT	IHC	Apoptosis, Recurrence, Survival	Survivin positivity was related to worse survival in all patients. Survival was no different in RT or surgery alone groups.
COX-2, p53	(Kobayashi, Hashiguchi et al. 2007)	Pre- and post-treatment	52	IHC	Rodel TRG	Reduced COX-2 expression = good response. P53 = no correlation to response
TS, TP, DPD	(Kocakova, Svoboda et al. 2007)	Pre- and post-treatment	55	IHC	Pathological response	No correlation of TS and TP mRNA induction post CRT between responders and non-responders
Ku, p53, p21, p16	(Komuro, Watanabe et al. 2005)	Pre-treatment	96	IHC	Pathological response	No relationship for biomarkers expression alone. Combined Ku/p16 and combined Ku, p53, p21, p16 = related to radiosensitivity
Ezrin	(Korkeila, Syrjanen et al. 2011)	Pre- and post-treatment	175	IHC	TRG, DFS, OS	Univariate analysis: Negative Ezrin = Better DFS, DSS. Multivariate analysis = no predictive ability
GLUT-1	(Korkeila, Jaakkola et al. 2011)	Pre- and post-treatment	175	IHC	TRG, DFS, DSS	No significant relationship. Negative GLUT-1 expression = A trend towards longer DFS
Bcl-2, Ki-67, p53, p21, MDM-2	(Kudrimoti, Lee et al. 2007)	Pre-treatment	17	IHC	Pathological response	Bcl-2 expression = complete response. Ki-67, p53, p21(waf1/cip1), and MDM-2 expression = no relationship

CEA	(Lee, Kim et al. 2013)	Pre-treatment	345	Serum assay	pCR, DFS	CEA level>5ng/mL = associated with lower pCR, reduced levels = better DFS
TS, TP, DPD	(Liersch, Langer et al. 2006)	Pre-treatment	40	Real-time PCR	Dworak TRG, downsizing, DFS, OS	Increased TS gene expression = tumour recurrence
CD133	(Lin, Chen et al. 2012)	Pre-/post-treatment	41	IHC	DFS, OS	Increased CD133 expression = reduced DFS, OS
Rsf-1	(Lin, Tian et al. 2012)	Pre-treatment	172	IHC	TRG, DSS, LRFS, MeFS	Higher Rsf-1 expression = reduced tumour regression, DSS, MeFS
CEA	(Lin, Zeng et al. 2010)	Pre-treatment sample	47	Serum assay	pCR	CEA levels > 5ng/ml = reduced pCR rate
p53, p21, p27, Bcl-2	(Lin, Lee et al. 2006)	Pre- and post-treatment	77	IHC	Pathological response	p53 negative and p27 positive staining in pre-treatment biopsy = fair response of tumour
p53	(Lopez-Crapez, Bibeau et al. 2005)	Pre-treatment biopsy	70	IHC, cDNA	TRG, down staging	p53 gene mutations correlated with both nuclear protein over expression and loss of heterozygosity. No correlation between p53 alterations and response to radiotherapy
Fibrinogen	(Lu, Zhu et al. 2011)	Pre-treatment	53	Serum assay	pCR, OS	Serum Fibrinogen > 4g/L (hyperfibrinogenemia) = reduced pCR, reduced OS
Survivin	(McDowell, Smith et al. 2009)	Pre-treatment	36	IHC, TUNEL	TRG	Survivin expression = no correlation with TRG. Increased spontaneous apoptosis = better tumour response
p27, p21, p53, Ki-67, retinoblastoma gene, cyclin D1, and Bcl-2	(Moore, Shia et al. 2004)	Post-treatment	67	IHC	Recurrence free survival (RFS)	Positive p27 expression = reduced RFS. No other proteins related to RFS
p53, cyclin D1, MIB-1 (Ki67), and bcl-2	(Moral, Fdez-Acenero et al. 2009)	Post-treatment	39	IHC	Pathological response	No marker associated with response

CEA	(Moureau-Zabotto, Farnault et al. 2011)	Pre-treatment sample	168	Serum assay	Pathological response, T-down staging	CEA level < 5ng/ml = improved pCR
Caspase 3, 8, and 9, DIABLO, XIAP	(Moussata, Amara et al. 2012)	Cell lines, Pre-treatment biopsy	2 cell lines, 38 patients	WB, IHC	pathological response	Post irradiation radiosensitive SW48 cell line over expressed XIAP compared radioresistant SW480 cell line. Tumour / normal tissue ratio decreased for DIABLO expression and increased for XIAP expression. Other proteins = no correlation
p53, BAX. Bcl-2, MIB1	(Nasierowska-Guttmejer 2001)	Pre- and post-treatment	90	IHC	Pathological response	MIB1 under expression or BAX over expression = total or near-total response. In post treatment specimens: p53, MIB1, BAX and Bcl-2 under-expression = tumour regression
p53, p21, VEGF,TS,MSI	(Negri, Campanini et al. 2008)	Pre-treatment	57	IHC	Pathological response	High TS expression = high pathological response in CRT but not in RT group
p53	(Nehls, Klump et al. 1999)	Pre- and post-treatment	100	IHC	Various	p53 expression = no correlation to histological grade, stage, or survival
p53, Bcl-2, TS	(Okonkwo, Musunuri et al. 2001)	Pre-treatment biopsy	25	IHC	Pathological response	TS expression = increased response. Other proteins = no correlation
CEA	(Park, Lim et al. 2009)	Pre-treatment	352	Serum assay	Pathological response	Pre-treatment CEA level < 3ng/ML = good response
CEA	(Park, Sohn et al. 2006)	Pre-treatment sample	141	Serum assay	Pathological response	CEA level > 5ng/ml = poor tumour response
VEGF	(Peng, Wang et al. 2012)	Post-treatment	116	IHC	Various	Positive VEGF expression = reduced DFS, increased metastasis
CEA	(Perez, Sao Juliao et al. 2009)	Post-treatment sample	170	Serum assay	Stage, relapse, survival	Post-chemoradiotherapy CEA levels <5 ng/ml = increased response, increased DFS, OS. No correlation with initial CEA level or reduction in CEA .

Ki-67, p53, p21, bcl-2, and VEGF	(Qiu, Sirivongs et al. 2000)	Pre-treatment	72	IHC	Pathological response	p21 expression = better response, p53-negative/p21-positive or p21-positive/bcl-2-positive = better response
hPEBP4	(Qiu, Yang et al. 2013)	Pre-treatment biopsy	86	IHC	TRG	hPEBP4 over expression = reduced tumour regression and progression free survival
p53, BAX, p21 WAF1/CIP1, Ki-67, hMSH2	(Rau, Sturm et al. 2003)	Pre- and post-treatment	66	IHC	Survival	Post treatment reduced expression p21 and increased Ki-67 expression = better DFS
CEA	(Restivo, Zorcolo et al. 2013)	Pre-treatment sample	260	Serum assay	Pathological response	CEA < 5ng/dl = higher pathological complete response
PLK1	(Rodel, Keppner et al. 2010)	Pre-treatment biopsy	76	IHC, Micro-array	TRG	Higher PLK1 expression = reduced tumour regression
Survivin	(Rodel, Hoffmann et al. 2005)	Cell lines	2	PCR	Apoptosis	Inhibition of Survivin in RR cell line SW840 increased apoptosis levels upon irradiation
Survivin	(Rodel, Haas et al. 2003)	Cell lines	3	WB, PCR, TUNEL	Differential expression, apoptosis	Higher spontaneous and radiation induced survivin expression in radioresistant cell line (SW480) compared with radiosensitive line (SW48) with inverse relationship to levels of apoptosis.
Survivin, p53, Bcl-2	(Rodel, Hoffmann et al. 2002)	Pre-treatment biopsy	54	IHC	DFS, MfS, LR	Low survivin = improved DFS, reduced recurrence and metastasis
CD133	(Saigusa, Tanaka et al. 2010)	Pre-treatment, cell line	40 = no CRT, 50 = CRT	IHC	Pathological response	CD133 staining in CRT specimens higher than that of non-CRT specimens. CD133 expression in luminal surface and cytoplasm = reduced pathological response
p53, DCC, TS	(Saw, Morgan et al. 2003)	Post-treatment specimen	60	IHC, PCR	Tumour down staging	Absent TS expression = associated with tumour down staging

p53, Bcl-2	(Schwandner, Schiedeck et al. 2000)	Pre-treatment biopsy	160	IHC	Recurrence, DFS	p53 accumulation = higher recurrence, reduced DFS Bcl-2 expression = lower recurrence, longer DFS
Bcl-2, BAX	(Scopa, Vagianos et al. 2001)	Pre-treatment biopsy	35	IHC	Pathological response	Greater Bcl-2 expression or Bcl-2/BAX ratio or reduced BAX expression = radioresistant tumours.
GLUT-1, LDH5, PDK1, HIF-1 alpha	(Shim, Jung et al. 2013)	Pre-treatment	104	IHC	Pathological response, TRG	High GLUT-1 expression = reduced pCR compared / poor response (grade 0, 1)
hTERT	(Shin, Foo et al. 2012)	Cell line, Pre- and post-treatment tissue	1 cell line, 52 patients	PCR, IHC	TRG	Radioresistant SW620 showed up-regulation of hTERT upon irradiation. hTERT IHC + of =< 75% predicted good response (poor correlation)
CD133, COX-2, p53, p21, p27, EGFR	(Shinto, Hashiguchi et al. 2011)	Pre-treatment biopsy	96	IHC	TRG	Positivity for CD133 or cyclooxygenase-2 expression = associated with chemoradioresistance. No correlation of other markers.
HIF-1 alpha	(Shioya, Takahashi et al. 2011)	Post-treatment	50	IHC	Pathological response, TRG	No significant difference between the HIF-1alpha-positive group and HIF-1alpha-negative group for pathological grading and pCR. HIF-1 alpha expression = better recurrence free and metastasis free survival
COX-2, Apoptosis	(Smith, Reynolds et al. 2006)	Pre-treatment biopsy	49	IHC, TUNEL	TRG	COX-2 over expression , reduced apoptosis = moderate to poor tumour regression
EGFR	(Spindler, Nielsen et al. 2006)	Blood, pre-treatment biopsy	77	PCR, IHC	TRG	More major respondents to CRT in GT or TT heterozygotes compared with GG homozygotes
p53	(Spitz, Giacco et al. 1997)	Pre-treatment	42	IHC	Pathological response	Aberrant p53 protein accumulation = reduced pathological response, higher chance of residual cancer in lymph nodes of surgical specimen

Survivin	(Sprenger, Rodel et al. 2011)	Pre- and post-treatment	116	IHC	Pathological stage, DFS, OS	High survivin pre- and post-treatment = high pathological stage.
p53, p21, apoptosis	(Suzuki, Sadahiro et al. 2004)	Pre-treatment biopsy	93	IHC	TRG, degree of tumour shrinkage	Combined p53-, p21+ and apoptosis + = highest degree of tumour shrinkage (41.5% +/- 8.5%) compared with one or two of these. No difference in tumour regression.
p53, p21, Ki-67, apoptosis	(Suzuki, Sadahiro et al. 2013)	Pre-treatment, at 7 days during CRT	101	IHC	TRG, degree of tumour shrinkage	p21 + on biopsy and at 7 days into CRT treatment = greater tumour regression and tumour shrinkage
Survivin	(Takasu, Shimada et al. 2013)	Pre-treatment biopsy	43	IHC	TRG (Pathological response)	High survivin expression = reduced pathological response
p53, Survivin, Ki-67	(Terzi, Canda et al. 2008)	Pre-treatment biopsy	37	IHC	Pathological response	No relationship for biomarkers expression with histological response post CRT.
SKP2 (E3 Ubiquitin ligase)	(Tian, Chen et al. 2013)	Pre-treatment biopsy	172	IHC	TRG, Recurrence, Nodal status	High SKP2 expression = advanced nodal disease, low tumour regression, reduced recurrence free survival
EGFR, VEGF, HIF-1	(Toiyama, Inoue et al. 2010)	Pre-treatment biopsy	40	Real-time PCR	TRG, DFS	Lower gene expression of EGFR, VEGF and HIF-1 = responder to CRT. Only VEGF expression related to DFS: lower VEGF = lower DFS.
CEA	(Wallin, Rothenberger et al. 2013)	Pre-treatment sample	267	Serum assay	Pathological response	Low pre-treatment CEA levels = higher pathological complete response
CEA	(Yan, Wang et al. 2011)	Pre-treatment	98	Serum assay	TRG	CEA levels <3.0 ng/dl= better tumour regression
CEA	(Yeo, Kim et al. 2013)	Pre-treatment sample	201	Serum assay	Down-staging, TRG	CEA levels <5.0 ng/dl= better tumour regression and down staging. No survival benefit on multivariate analysis.

Hydroxy-methyl glutaryl-coenzyme A synthase 2	(Yeo, Kim et al. 2012)	Cell lines (colon), Pre-treatment biopsy	12 cell lines, 45 rectal cancer biopsies	2D GE, WB, IHC	Expression difference, TRG, down-staging	Expression of hydroxymethylglutaryl-coenzyme A synthase 2 was significantly correlated with intrinsic radiation resistance of 12 cancer cells and TRG / down staging in rectal cancer
CEA	(Yoon, Kim et al. 2007)	Pre-treatment biopsy	351	Serum assay	Dworak TRG, down staging	Pre-treatment CEA levels = independent predictor of complete tumour regression
VEGF	(Zlobec, Steele et al. 2005)	Pre-treatment biopsy	59	IHC	Down-staging, pathological response	VEGF expression = significant association with non-responders
VEGF, Bcl-2, p21, p53, and APAF-1	(Zlobec, Steele et al. 2005)	Pre-treatment biopsy	62	IHC	Pathological response	Absent / reduced expression of VEGF, Bcl-2 = complete pathological response
EGFR, VEGF, Bcl-2, p53, and APAF-1	(Zlobec, Vuong et al. 2008)	Pre-treatment biopsy	?	IHC	Pathological response	Loss of VEGF and positive EGFR = independent predictor of pathological complete response

Table 1.10: Summary of Biomarkers investigated in 10 or more studies				
Biomarkers	**Number of studies**	**Association of response to RT**		**Conclusion**
		Positive	**No**	
p53	33	11 + 1 meta-analysis	22	The positive association studies and the meta-analysis conclude that the wild type p53 or p53 protein under-expression is related to radiosensitivity
Bcl-2	17	6	11	There is conflicting evidence from 6 small studies, half of those suggest Bcl-2 expression is related to better response of rectal cancer to RT, whilst others conclude that reduced expression is predictive of better response. More studies are needed to help determine the usefulness of this biomarker.
Ki-67	17	8	9	Conflicting evidence as both Ki-67 expression and reduced expression are related to greater tumour regression. Unlikely to provide predictive ability about the response to RT.
$p21^{WAF1}$	15	6	9	The conflicting association with reduced or over-expression from the 6 studies, and another 9 studies failing to prove any association between $p21^{WAF1}$ and response to RT / CRT mean that it is unlikely to serve as predictor of response.
VEGF	14	10	4	Consistent evidence that VEGF expression is related to reduced tumour regression, less response, reduced DFS, OS and increased likelihood of metastasis. Anti-VEGF treatment and combined Anti- EGFR / anti-VEGF treatment are a subject of current trials.
TS	13	5	8	Relevant only if 5-FU based CRT. Conflicting association from the 5 studies. Unlikely to serve as a predictor of response.
EGFR	12	8	4	Consistent evidence that EGFR expression is related to reduced tumour regression, down-staging, DFS, and OS, and increased likelihood of metastasis. Anti- EGFR and combined Anti- EGFR / anti-VEGF treatment are part of trials.
Survivin	11	6	5	Consistent evidence that survivin expression related to reduced tumour regression, down-staging, and OS. Survivin suppression is an active area of research.
CEA	11	9	2	All 9 positive association studies but one concluded that low / normal pre-operative CEA levels predict better pathological response to RT / CRT.

1.9.1 p53

The tumour suppressor gene p53 is one of the first tumour suppressor genes discovered and, with its protein product, remains one of the most extensively studied biomarkers. The primary functions of p53 include cell cycle regulation, DDR (Section 1.8.2), and stimulation and regulation of apoptosis (Section 1.8.3) (Berardi, Maccaroni et al. 2009). A wild-type (functional) p53 protein has the capacity to both activate and repress gene transcription in order to exert its function in response to genotoxic stress (e.g. radiotherapy). It is a short-lived protein which is stabilised and activated by a wide range of cellular stresses (Fei and El-Deiry 2003). The mutations of p53 gene produce an inactive protein which accumulates in tumour cells and has been reported to be expressed in up to 70% of rectal cancers. Such cellular abnormality provides a selective growth advantage for cancer cells inhibiting a physiological check point during the cell cycle progression (Berardi, Maccaroni et al. 2009).

The literature search revealed 33 original articles and one meta-analysis investigating the relationship of p53 gene and its protein product with radiotherapy response. Of those, 11 studies found positive association between p53 status and radiation response of tumours and the remainder failed to find such an association (table 1.10). The largest (n=160) of the studies showing positive association was undertaken by Schwander and colleagues in 2000. The authors found that 39% of rectal cancers treated with RT / CRT showed p53 nuclear accumulation that was associated with higher tumour progression (local or distant recurrence) and poorer DFS (Schwandner, Schiedeck et al. 2000). Adell et al. investigated the relationship between the expression of nuclear p53 protein and the outcome in rectal carcinoma in 163 patients

randomised to either preoperative SCRT and surgery or surgery alone (Adell, Sun et al. 1999). The expression of p53 protein was demonstrated in 41% of the tumours (either arm). The p53 negative patients treated with preoperative radiotherapy demonstrated a significant reduction in rates of local failure (1/47) compared with the non-irradiated p53 negative patients (13/49) (P = 0.0008) (Adell, Sun et al. 1999).

Aberrant p53 protein accumulation on immunohistochemical staining correlate inversely with a complete pathological response (Kelley, Coppola et al. 2005) and overall survival (Fucini, Messerini et al. 2012), and directly with an increased likelihood of residual cancer in the lymph nodes of surgical specimen (Spitz, Giacco et al. 1997). Combined expression of p53 and at least one other biomarker has been demonstrated to correlate to response of tumours to RT (Suzuki, Sadahiro et al. 2004; Kelley, Coppola et al. 2005; Lin, Lee et al. 2006). The above findings, in a total of 11 studies, demonstrated that the wild type p53 may be associated with favourable response to RT / CRT as summarised in table 1.10. However, there is a body of evidence proving absence of such association between p53 status and response to RT / CRT (22 studies, table 1.10).

In 2012, Chen et al. reported a landmark meta-analysis concluding that p53 status was associated with pathological response to RT in rectal cancer (Chen, Wu et al. 2012). Thirty studies including 1,830 cases who received neoadjuvant RT / CRT were included in the meta-analysis. Wild-type p53 status (low expression of p53 protein and/or wild-type p53 gene) was associated with

pathologic response (Good response: risk ratio [RR] = 1.30; 95% confidence intervals [CI] = 1.14–1.49; p,0.001; Complete response: RR = 1.65; 95% CI = 1.19–2.30; p = 0.003; Poor response: RR = 0.85; 95% CI = 0.75 0.96; p = 0.007). In further stratified analyses, authors found that this association remained for sub-groups of good and poor response in RT setting, and good and complete response in CRT setting. Furthermore, the association between response and the presence of p53 gene mutations was stronger than that between response and protein positivity (Chen, Wu et al. 2012).

However, the results of the meta-analysis should be viewed with some caution as a few inconsistencies were observed whilst undertaking above literature review (table 1.9). It was noted that the meta-analysis included studies that found no association of p53 with response to RT/CRT when studied alone, but showed promise when a combined expression with other biomarkers was observed (Kelley, Coppola et al. 2005; Komuro, Watanabe et al. 2005). Similarly, the meta-analysis included studies that were counted and used twice for data synthesis if both the p53 gene and protein product were analysed in the same study (Elsaleh, Robbins et al. 2000; Suzuki, Sadahiro et al. 2004; Lopez-Crapez, Bibeau et al. 2005). The authors did not provide an explanation as to how such issues were addressed during data synthesis that might have introduced a statistical bias. Such studies were counted one each in this thesis.

The evidence to date suggests p53 status has potential to serve as a predictor of response to RT / CRT in rectal cancer, however, further robust evidence sourced from large, well-controlled studies and carefully performed pooled

analysis is certainly required. Future meta-analysis, in particular, should address the shortcomings of existing meta-analysis to help draw firm conclusion from level-I evidence. Nevertheless, p53 remains a promising maker and should continue to be actively researched.

1.9.2 EGFR

Epidermal growth factor receptor (EGFR) is a member of transmembrane tyrosine kinase superfamily. It is a glycoprotein receptor for the members of epidermal growth factor (EGF) family. It consists of an external ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. EGFR activates downstream signalling pathways involved in cell proliferation and survival, in response to the binding of structurally related ligands such as EGF and transforming growth factor α (TGFα). Binding of the protein to a ligand induces receptor dimerization followed by tyrosine autophosphorylation. As a result, proteins attach to the newly phosphorylated tyrosine culminating in activation of a signalling cascade, mediated through two major EGFR-dependent pathways, the PI3K-AKT and the Ras-MAPK pathways (Marquardt, Rodel et al. 2009). This in turn results in activation of transcription factors and regulation of cellular responses including cell proliferation, inhibition of apoptosis, cell differentiation, angiogenesis, cell migration, adhesion and invasion (Siena, Sartore-Bianchi et al. 2009). The EGFR activity is related to the poor radiation response in several cancer types and is reported to be abnormally expressed in 60% to 80% of the colorectal cancers (Wadlow and Ryan 2010).

From literature review (8 positive association and 4 no association studies), there is consistent evidence that EGFR expression is related to reduced tumour regression, down-staging, DFS and OS and, increased likelihood of development of metastasis. Kim et al. investigated the immunohistochemical expression levels of EGFR in predicting tumour down-staging in 183 patients with locally advanced rectal cancer treated with preoperative CRT. The EGFR expression in pre-treatment biopsy specimens was determined from the intensity and extent of staining. A high level of EGFR expression was found to be a significant predictor of decreased tumour down-staging on multivariate analysis (Kim, Kim et al. 2006). Giralt et al. (2002) performed immunohistochemical analysis of EGFR on pre-treatment biopsies from rectal cancer patients treated with neoadjuvant RT (Giralt, Eraso et al. 2002). EGFR positive tumours were found to be associated with poor pathological response and DFS (Giralt, Eraso et al. 2002). EGFR over-expression has also been found to be an independent predictor of reduced OS in patients treated with RT (Kopp, Rothbauer et al. 2003).

EGFR inhibitors have been developed as a molecular targeted strategy in the treatment of cancer and have been one of the most researched molecular targeted treatments (Sartor 2004; Krempien, Muenter et al. 2005; Vokes and Chu 2006). Two classes of anti-EGFR agents have been developed. These include monoclonal anti EGFR antibodies Cetuximab and Panitumumab that bind to the extracellular domain of EGFR and inhibit ligand-mediated activation of downstream signalling cascades. The second class is the tyrosine kinase inhibitors. Gefitinib (Iressa) and Erlotinib (Tarceva) are the small molecules that competitively inhibit ATP binding to the tyrosine kinase domain of EGFR,

thereby preventing phosphorylation of downstream signalling proteins (Harari 2004; Mendelsohn and Baselga 2006; Wadlow and Ryan 2010).

Cetuximab and Panitumumab have been shown to possess promising efficacy in the treatment of metastatic CRC indicating that the EGFR pathway is a biologic target for anticancer treatments (Wong 2005; Siena, Sartore-Bianchi et al. 2009). Phase-I/II trials have shown some encouraging results in achieving pathological down-staging (Machiels, Sempoux et al. 2007). Similarly, Iressa has been shown to potentiate radiotherapy in a human colorectal cancer xenograft model (Williams, Telfer et al. 2002). The radiosensitising effect was observed to be more apparent when radiation was administered in a fractionated protocol suggesting an anti-proliferative effect of Iressa on tumour cell repopulation between radiotherapy fractions (Williams, Telfer et al. 2002). However, a recent review summarising the results of 13 phase I/II trials concluded that there is not enough evidence to recommend Cetuximab combined with fluoropyrimidine-based CRT in rectal cancer. It has been suggested that better understanding of the mechanisms involved in combinations of CRT might allow more effective combination of such agents (Glynne-Jones, Mawdsley et al. 2010).

1.9.3 VEGF

The vascular endothelial growth factor (VEGF) protein is a glycosylated mitogen that specifically acts on endothelial cells and is involved in mediating increased vascular permeability, angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis (Lazarus and Keshet

2011). Angiogenesis is required for tumour growth and malignant progression, and VEGF is a crucial regulator of this process. Characteristically, the blood vessels in neoplastic lesion consist of a disorganised architecture that contributes to intermittent or chronic hypoxia within the tumour.

VEGF expression has been associated with radioresistance and poor oncological outcomes in rectal cancer patients. Edden et al. carried out immunohistochemical evaluation of VEGF on pre-treatment biopsy specimens in rectal cancer patients treated with neoadjuvant CRT and found that VEGF expression correlated with reduced tumour regression (Edden, Wexner et al. 2012).

VEGF expression has been demonstrated to be an independent predictor of distant metastasis and DFS in preoperatively irradiated stage III rectal cancer (Peng, Wang et al. 2012). Higher tumour regression has been reported in tumours with significantly lower gene expression levels of VEGF on quantitative real-time PCR performed on pre-treatment biopsy specimens (Toiyama, Inoue et al. 2010). Moreover, both the loss of VEGF and positive EGFR demonstrated independent predictive value for complete pathological response in patients treated with pre-operative high dose brachytherapy. Simultaneous positive VEGF and negative EGFR status was consistent with radioresistance (Zlobec, Vuong et al. 2008).

The anti-VEGF antibody Bevacizumab has been developed as a molecular targeted treatment strategy based on the principle that inhibition of VEGF over-activity leads to a more normalised tumour vasculature, thereby resulting in greater tumour penetration of anticancer agents, including enhanced susceptibility to RT. The Anti-VEGF treatment has antivascular effects and hence can cause killing of the cancer cells indirectly by damaging tumour blood vessels (Willett, Kozin et al. 2006).

VEGF targeted treatment has been used with promising results in patients with metastatic CRC for which Bevacizumab has received the FDA approval (Hurwitz, Fehrenbacher et al. 2004; Wadlow and Ryan 2010). Efforts have been directed at evaluating the use of Bevacizumab in non-metastatic, locally advanced rectal cancer treated with preoperative CRT. In doing so, Willett et al. (2004) first provided the direct evidence of the effect of Bevacizumab on rectal cancer vasculature (Willett, Kozin et al. 2006). They demonstrated a significant decline in tumour blood perfusion and blood volume, and significant decrease in tumour microvessel density (Willett, Boucher et al. 2004). A recently reported phase II study assessed safety and activity of neoadjuvant Bevacizumab, Capecitabine and standard radiotherapy in locally advanced rectal cancer (Gasparini, Torino et al. 2012). The pathological complete response was achieved in 14% (TRG-1), and intermediate response (TRG-2, 3) in 51% of patients. Significant tumour and lymph nodes down-staging was observed in 35% and 37%, respectively. The therapy was safe and well tolerated. (Gasparini, Torino et al. 2012). (Gasparini, Torino et al. 2012). Another similar phase II study reported similar safety profile and efficacy with some early improvement in disease free survival (Spigel, Bendell et al. 2012).

From literature review (10 positive association and 4 no association studies), there is consistent evidence that VEGF expression is related to reduced tumour regression, response to RT, DFS and OS, and increased likelihood of development of metastasis. The recent developments in VEGF targeted treatments are promising. However, more feasibility and efficacy studies are required to determine whether anti VEGF agents carry clear clinical value in rectal cancer.

1.9.4 Bcl-2

Bcl-2 belongs to Bcl-2 family of proteins and plays an essential role in regulation of intrinsic apoptosis (Section 1.8.4). Bcl-2 is a key pro-survival anti-apoptotic protein and it is understood that Bcl-2 functions by interacting with the pro-apoptotic proteins of Bcl-2 family in an antagonist fashion (Chan and Yu 2004).

Due to its key position in intrinsic apoptotic pathway (route to cell death by the actions of IR), Bcl-2 has been investigated for a possible link with radioresistance. The literature search found 17 studies investigating the role of Bcl-2 in predicting response to RT / CRT. Of those there were 6 positive association studies. Unfortunately, the results of those studies were somewhat conflicting. Bcl-2 expression has been shown to predict significant tumour regression (Qiu, Sirivongs et al. 2000; Kudrimoti, Lee et al. 2007; Huerta, Hrom et al. 2010), and longer DFS and reduced recurrence (Schwandner, Schiedeck et al. 2000). In contrast, Zlobec and colleagues found that a reduced Bcl-2 expression was related to better tumour regression (Zlobec, Steele et al. 2005).

Fucini and colleagues recently found that Bcl-2 expression in pre-treatment biopsies of 67 patients with rectal cancer treated with CRT was predictive of reduced overall survival (Fucini, Messerini et al. 2012). Similarly, its reduced expression combined with that of p53, BAX and MIB is linked with improved pathological response (Nasierowska-Guttmejer 2001). Moreover, it is reported that an elevated Bcl-2/BAX ratio in tissue specimens suggested increased tumour resistance to RT (Scopa, Vagianos et al. 2001). Such observations signify the paucity of evidence from these small studies that fails to establish a clear link between Bcl-2 expression and response to RT.

Admittedly, more evidence leading to pooled analysis is needed to help determine the usefulness of this biomarker. If future evidence shows promising association of Bcl-2 with radioresistance, the early evidence regarding targeting of this protein can be extrapolated to rectal cancer. For instance, G3139 is the first oligonucleotide to demonstrate an antisense effect in human tumours by down-regulation of the target Bcl-2 protein (Chan and Yu 2004). Therefore Bcl-2 inhibition might become a suitable therapy to enhance apoptosis in our attempts to potentiate the radiotherapy response.

1.9.5 Ki-67

Ki-67 is a marker of cellular proliferation and activity. It is required for the cell cycle process to occur. The Ki-67 protein is present during all active phases of the cell cycle but is absent from resting cells (G0 phase). It is well characterised at the molecular level and extensively used as a proliferation marker, however, the functional significance still remains unclear. Nevertheless, it would appear

that Ki-67 protein expression is an absolute requirement for progression through the cell cycle (Scholzen and Gerdes 2000; Kuremsky, Tepper et al. 2009).

Literature review revealed 16 studies, of those 6 supported the role of Ki-67 in predicting response to RT / CRT, and a further two reported altered expression post-radiotherapy. Four studies demonstrated that reduced Ki-67 expression was correlated to good response due to greater tumour regression (Kim, Park et al. 2001; Jakob, Liersch et al. 2008; Kikuchi, Mikami et al. 2009; Avoranta, Korkeila et al. 2011). The largest (n=211) of those investigated the Ki-67 expression in pre- and post-treatment samples of rectal cancer patients treated with CRT. The authors found that a reduced Ki-67 expression in post-treatment sample was associated with significant tumour regression (Avoranta, Korkeila et al. 2011). One study reported that Ki-67 expression resulted in higher tumour recurrence (Adell, Zhang et al. 2001). Moreover, it is understood that a reduced expression is linked with better DFS (Rau, Sturm et al. 2003) and OS (Andrade, Oshima et al. 2011). However, 9 studies failed to prove an association between Ki-67 expression and response to RT / CRT. The evidence available to date would suggest that Ki-67 is unlikely to serve as a predictor of response to RT.

1.9.6 $p21^{WAF1}$

The protein $p21^{WAF1}$ is a cyclin-dependent kinase inhibitor which is important in cell cycle regulation in response to genotoxic stress. It is a major transcriptional target of p53. Based on the localisation, $p21^{WAF1}$ protein executes various functions in the cell. Following the exposure of the cells to genotoxic agents like radiation, the activated p53 induces $p21^{WAF1}$. The elevated $p21^{WAF1}$ binds and

inactivates cyclin D / CDK4,6 and cyclin E / CDK2 complexes resulting in pRB hypophosphorylation and cell cycle arrest.

The literature review revealed 15 studies, of those only 6 supported the role of $p21^{WAF1}$ in predicting response to RT / CRT. Most of the studies were hetergenous and looked at a variety of endpoints. A reduced $p21^{WAF1}$ expression resulted in better DFS (Rau, Sturm et al. 2003; Bertolini, Bengala et al. 2007) and OS (Bertolini, Bengala et al. 2007). $p21^{WAF1}$ expression has been reported to result in favourable pathological response and tumour shrinkage when analysed alone (Qiu, Sirivongs et al. 2000; Suzuki, Sadahiro et al. 2013) or in combination with p53 and ku (Suzuki, Sadahiro et al. 2004). The conflicting association with reduced or over-expression from the 6 studies, and another 9 studies failing to prove any association between $p21^{WAF1}$ and response to RT / CRT indicate that it is unlikely to serve as a predictor of radiotherapy response.

1.9.7 Thymidylate Synthase (TS)

Thymidylate Synthase serves as the primary target of 5-FU and therefore is of relevance in 5-FU based CRT in rectal cancer. TS over-expression is associated with 5-FU resistance and poor prognosis in CRC (Kuremsky, Tepper et al. 2009). TS catalyses the conversion of dUMP into dTMP for subsequent incorporation into the DNA. High TS protein levels lead to increased production of dTMP and DNA synthesis and are associated with resistance to 5-FU (Jessup and Loda 1998).

Literature review revealed 13 studies, of those only 5 supported the role of TS in predicting response to RT / CRT. Most of the studies concluded that TS expression predicted good response. One study each reported that a higher TS gene expression resulted in tumour recurrence and that absent TS expression was associated with tumour down-staging. The conflicting conclusions from 5 studies, and another 8 studies failing to prove any association between TS and response to RT / CRT suggest that TS would not be a useful marker to predict radiotherapy response.

1.9.8 Survivin

Survivin is a member of inhibitor of apoptosis protein family (Section 1.8.4). Survivin inhibits apoptosis by blocking the activation of Caspase-3 and 7, and regulates cell cycle in G2/M phase (Berardi, Maccaroni et al. 2009). It is one of the newer members of the IAP family and has been met with a lot of enthusiasm with promising early results about its association with radioresistance.

Initial work on this protein has been the effort of Rodel and colleagues who, in 2002, reported the relationship of this newly discovered biomarker with radioresistance in rectal cancer patients treated with CRT (Rodel, Hoffmann et al. 2002). The expression of survivin, p53, Bcl-2 and the apoptotic index was evaluated by immunohistochemistry on pre-treatment biopsies of 54 patients with locally advanced rectal cancer. Their results showed that survivin expression inversely correlated with the apoptotic index. Low survivin expression was significantly related to an improved DFS and to a reduced risk for distant metastases and local failure (Rodel, Hoffmann et al. 2002). Further,

survivin expression has been demonstrated to be related to lower level of apoptosis in radioresistant colorectal cancer cells SW-480 compared with the radiosensitive SW-48 cells (Rodel, Haas et al. 2003), which was enhanced by the inhibition of survivin expression by survivin SiRNA providing further evidence that survivin expression affects apoptosis and hence impacts tumour cells response to RT (Rodel, Hoffmann et al. 2005). In patients treated with neoadjuvant RT / CRT, a high survivin expression has been shown to be related to reduced pathological response (Takasu, Shimada et al. 2013), reduced tumour down-staging (Kim, Chie et al. 2011), worse overall survival (Knutsen, Adell et al. 2004), and high pathological stage (Sprenger, Rodel et al. 2011).

The applicability of survivin-driven strategies in clinical practice is currently under investigation. The survivin inhibitors may present novel molecular antagonists to enhance the efficacy of RT as radiosensitisers. YM155 is a small molecule inhibitor of survivin that functions in the cell cycle as a transcriptional inhibitor (Kelly, Lopez-Chavez et al. 2011). YM155 has demonstrated potent anti-proliferative activity against various haematological and solid cancers, including CRC. Preclinical studies have revealed its ability to serve as a radiosensitising and chemosensitising agent or as a monotherapy (Satoh, Okamoto et al. 2009; Kelly, Lopez-Chavez et al. 2011). The work on this potential biomarker is encouraging and holds promise for the future (Kelly, Lopez-Chavez et al. 2011).

1.9.9 Biomarkers – Summary

As appears from the table 1.9, a wealth of evidence on biomarkers discovery is available with heightened interest in the last few years. Further to the discussion of biomarkers above, only a few have shown some promise to predict the response of RT / CRT in rectal cancer. However, the search continues for a promising biomarker that is sensitive, specific and is measurable. The work presented in this thesis is aimed at discovery of biomarkers that would be helpful in predicting which tumours are radioresistant or radiosensitive.

1.10 Biomarker discovery

Various global techniques exist to identify novel biomarkers associated with the response to anticancer treatment. These include genomics, transcriptomics and proteomics. Biomarker discovery in this thesis will employ proteomics approach.

1.10.1 Proteomics

Proteins are regarded as the fundamental molecules that maintain the cellular physiology. In humans, more than 90% of the genome serves no known purpose. It is well known that not all DNA is transcribed into RNA and there is no direct correlation between the mRNA levels and the protein expression (Hocquette 2005). Further, proteins themselves undergo post-translational modifications which affect their stability, localisation and function (Aldred, Grant et al. 2004). One gene can produce several different proteins. It is suggested that some 10% of genes encode more than 90% of the proteome. The proteome is the entire set of proteins expressed by a genome, cell, tissue or

organism. It refers to the set of expressed proteins in a given type of cells or an organism at a given time under defined conditions.

Proteomics is the study of the structure and functions of proteins and the diverse biological functions that they perform. Proteomics refers to the comprehensive study of proteins including detection, identification, measurement of protein concentration, detection and characterisation of modifications, and characterisation of protein-protein interaction and regulation (Chung, Levy et al. 2007). Proteomic approaches target the identification of all genome protein products and a mapping of their interactions and expression profiles (Bradshaw and Burlingame 2005). It is now well understood that true cellular events are governed and predicted by proteins. Proteomics provides the ultimate end point of a gene of interest in the form of protein expression analysis. This discovery approach holds great promise for the identification of disease markers and thus provides important drug targets in anti-cancer treatment.

Proteomics provides powerful tools for identifying factors associated with resistance to anticancer therapy because they facilitate the simultaneous analysis of the whole proteome. Comparative proteomics experiments identify the proteins with differential levels between two samples at different disease stages or treatment conditions (Hong, Jiang et al. 2006; Zhang and Liu 2007). Proteomics represents a very active area of research in recent times due mainly to the applicability of proteomic markers and clinical implications of molecular targeted treatments. The applications and aims of proteomics encompass

several avenues (Hong, Jiang et al. 2006). This approach aims to catalogue and characterise the full protein complement in the genome, and to compare the levels of protein expression under different conditions (control versus pathological / experimental / post-treatment conditions).

Proteomics approach is considered a powerful technique for biomarker discovery, however, certain methodological limitations should be remembered. No single method could analyse the whole proteome in a single experiment. Therefore a range of complementary proteomic approaches may be required in order to cover the whole proteome. From a practical perspective, proteomic methods are mainly the screening tools which can detect differential expression of proteins. Hence, the identification of proteins by proteomics methods requires further confirmation by other independent methodologies (Section 1.11). This would set the foundation for targeted confirmatory studies e.g. technical confirmation with western blotting and clinical validation with immunohistochemistry on tumour specimens. Furthermore, some of the conventional proteomic methods possess a few inherent technical limitations e.g. requirement of protein abundance in specimen, separation and identification of hydrophobic membrane proteins, and the proteins with extreme mass or molecular weight. However, newer proteomics techniques and methodologies of high throughput proteomic profiling have helped overcome most of these limitations. Fractionation of cell lysates may also help enrich proteins with low abundance (Chung, Levy et al. 2007; Zhang and Liu 2007).

Current proteomics approaches are broadly classified into three sets of methodologies (Smith, Lind et al. 2006; Zhang and Liu 2007; Soreide, Nedrebo et al. 2009; Hodgkinson, Eagle et al. 2010)

1. Gel-based mass spectrometry methods (for example two-dimensional polyacrylamide gel electrophoresis with matrix assisted laser desorption ionisation time of flight mass spectrometry; 2D-PAGE/MALDI-TOF-MS)
2. Gel-free MS methods (liquid chromatography with electrospray ionisation MS;LC/ESI-MS)
3. Microarray-based methods (antibody microarray)

Antibody Microarray (AbMa) is one of the newest and high throughput proteomics technique and will be employed in the current study.

1.10.1.1 Antibody microarray

The basic concept of microarray technology was introduced in 1990s by Ekins et al (Ekins, Chu et al. 1990) in the form of ambient analyte model which states:

"*microspot assays that rely on the immobilisation of interacting elements on a few square microns should, in principle, be capable of detecting analytes with a higher sensitivity than conventional macroscopic immunoassays*".

Based on above principle, the DNA microarray technology evolved as the first practical application of the microarray model (Ekins, Chu et al. 1990; Angenendt 2005). Antibody microarray is a high throughput proteomics technique that is built on the microarray technology, originally introduced as cDNA technology (Ekins and Chu 1999). Since its inception, microarray technology has been taken with enthusiasm for its potential of producing rapid, high throughput data and ability to overcome some of the problems associated with the conventional

proteomic assays. The microarray technology has seen widespread use in the form of DNA microarray, tissue microarray, protein microarray and antibody microarray (Ekins and Chu 1999; Elrick, Walgren et al. 2006; Hong, Jiang et al. 2006; Watson, Lind et al. 2007; Hu, Xie et al. 2011).

AbMa is a novel high throughput proteomic technique which provides a powerful technology for analysing expression of hundreds of proteins simultaneously. It represents a forward-phase type of protein array in which capture molecules i.e. antibodies for target proteins are immobilised onto a glass slide (Chung, Levy et al. 2007). To do so, a high precision robot is used to print hundreds of monoclonal antibodies at a high density onto the glass slide. Microarrays consist of large numbers of molecules distributed in rows in a very small space with spot sizes <250 µm. Microarrays permit scientists to simultaneously characterise complex analyte solutions with regard to many features (Angenendt 2005). Protein extracts from two different samples are differentially labelled with fluorescent dyes (e.g control sample with Cy3 and treated sample with Cy5 dye). A fluorescence-based detection procedure is used. Covalently immobilised antibodies are used to capture fluorescently labelled antigens as a result of an antigen-antibody reaction (Figure 1.15).

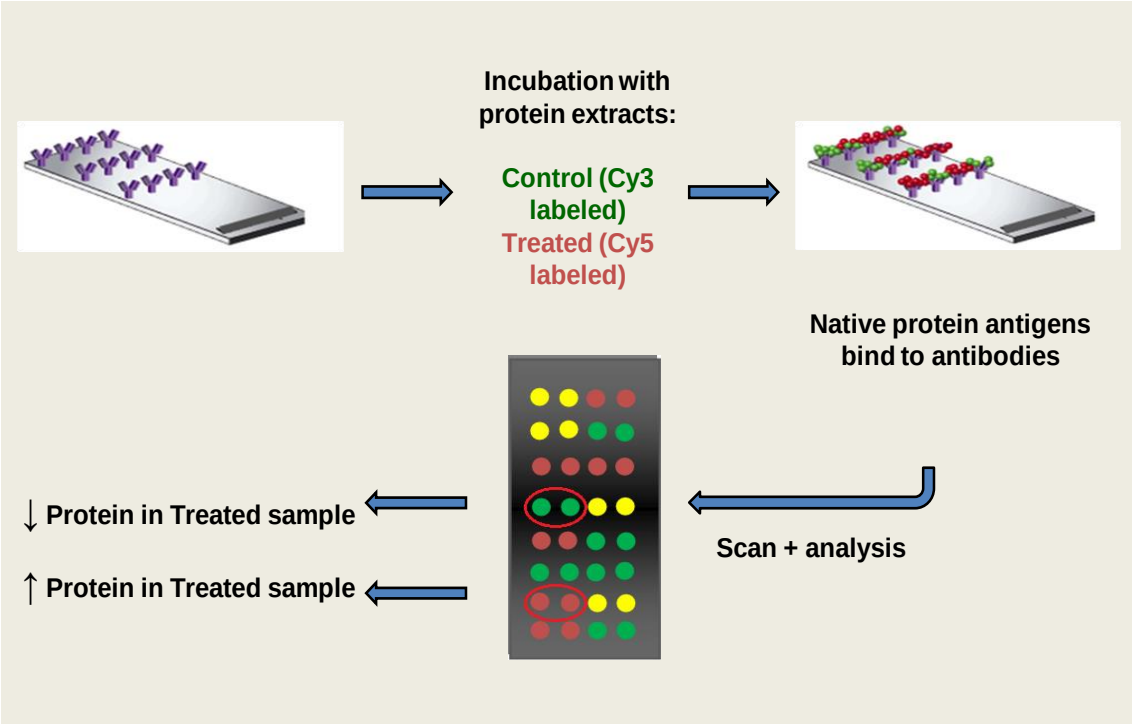

Figure 1.15: A schematic diagram of antibody microarray experiment. A nitrocellulose coated glass slide is arrayed with hundreds of monoclonal and polyclonal antibodies and is hybridised with protein lysates from the Control (Cy3 labelled) and Treated (Cy5 labelled) samples. The antigen-antibody reaction is allowed to complete before slide is scanned and array analysed using the Genepix pro software. The relative ratios of red and green fluorescence at each spot determine the expression levels of different proteins. **Yellow spots:** Equal expression, **Green spots:** Protein down-regulated in treated sample, **Red spots:** Protein up-regulated in treated sample.

Expression levels of specific proteins in each sample are determined by the relative ratios of red and green fluorescence at each spot (Smith, Lind et al. 2006). Yellow spots denote equal amount of a protein in lysates from each sample. Green spots show the protein is in abundance in Cy3 labelled control sample, i.e. down-regulated in treated sample. Red spots show that the protein is in abundance in Cy5 labelled treated sample, and therefore up-regulated in that sample.

The antibody microarray technique has gained widespread popularity and has been shown to produce highly reliable and reproducible results that can be obtained in a short period of time compared with the conventional proteomics tools (Angenendt 2005; Smith, Lind et al. 2006; Scaife, Hodgkinson et al. 2011). The clinical applications of this novel proteomic tool include biomarkers discovery for diagnosis, prognosis, and treatment response, characterisation of signalling and protein pathways, and modifications associated with disease development and progression (Angenendt 2005; Sanchez-Carbayo 2006). It is also preferred over conventional proteomics tools for its compatibility with lower sample volume and antibody concentration requirements, higher format versatility, and reproducibility (Sanchez-Carbayo 2006).

AbMa slides can be custom made in labs by spotting antibodies of interest onto a glass slide, however, commercially available kits with pre-spotted antibodies are more commonly used. Sreekumar et al. used custom made protein microarrays to study novel radiation regulated proteins in LoVo colon cancer cell line (Sreekumar, Nyati et al. 2001). They prepared an AbMa slide consisting of a total of 146 antibodies against proteins involved in stress response, cell cycle progression, and apoptosis. The antibodies were diluted in PBS and spotted on poly-lysine coated or superaldehyde-modified glass slides using a high precision robotic arrayer, replicated ≥ 10 times on each slide (Sreekumar, Nyati et al. 2001). There are a number of commercially available AbMa kits available with pre-spotted antibodies. For example Panorama XPRESS^{TM} profiler 725 kit (Section 2.4.2) has been reported in a recent study by Hodgkinson et al. (2012) aimed at discovery of biomarkers associated with chemoresistance in breast cancer tissues (Hodgkinson, ELFadl et al. 2012).

Smith et al. (2006) used Panorama Cell Signalling Antibody Microarray kit (Section 2.4.2) to investigate proteins related to chemotherapy resistance in chemoresistant breast cancer cell line. This kit contained 224 antibodies representing a variety of pathways, including apoptotic and cell signalling pathways (Smith, Watson et al. 2006). Moreover, AbMa has also been used to compare protein expression levels in the sera of primary breast cancer patients and healthy controls in an attempt to discover biomarkers which can serve as a diagnostic tool (Bohm, Keller et al. 2011).

In summary, antibody microarray analysis is a promising new technique. Antibody microarray technology may offer several advantages over conventional proteomic technologies in terms of ease of use and rapid quantitative data generation, especially with the availability of slides containing several hundred antibodies. The analysis of low abundance and membrane-associated proteins may be more suited to an AbMa approach which can detect protein levels as low as a few nanograms per ml, ensuring that important biomarkers are not missed because of technical limitations. The antibody microarray technology offers a high throughput approach to study protein expression. Although the study is usually limited to the number of antibodies printed on the slide, several different types of antibody kits are now available commercially with different antibody composition, some containing over 700 antibodies. Moreover, researchers can create their own array slide by spotting the antibodies of interest, using a high precision robot, according to individual requirements.

1.11 Biomarker validation

Study of biomarkers conventionally involves discovery, confirmation and validation phases. Biomarker discovery is usually the outcome of screening tools (e.g. Antibody Microarray) that often complement each other. The identification of proteins by screening proteomics is further verified by targeted confirmatory studies e.g. technical confirmation with western blotting or clinical validation with immunohistochemistry on tumour specimens (Sullivan and Chung 2008). Such verification pre-requisite ensures only the proteins with potential clinical relevance are given further consideration. The need for this is due to known problems with such tools, e.g. screening tool might detect proteins with altered expression because of experimental conditions e.g., stress response proteins or may get 'false discovery' simply by chance when simultaneously analysing hundreds of markers (Hodgkinson, ElFadl et al. 2011). The proteomic pipeline of biomarkers discovery is illustrated in figure 1.16.

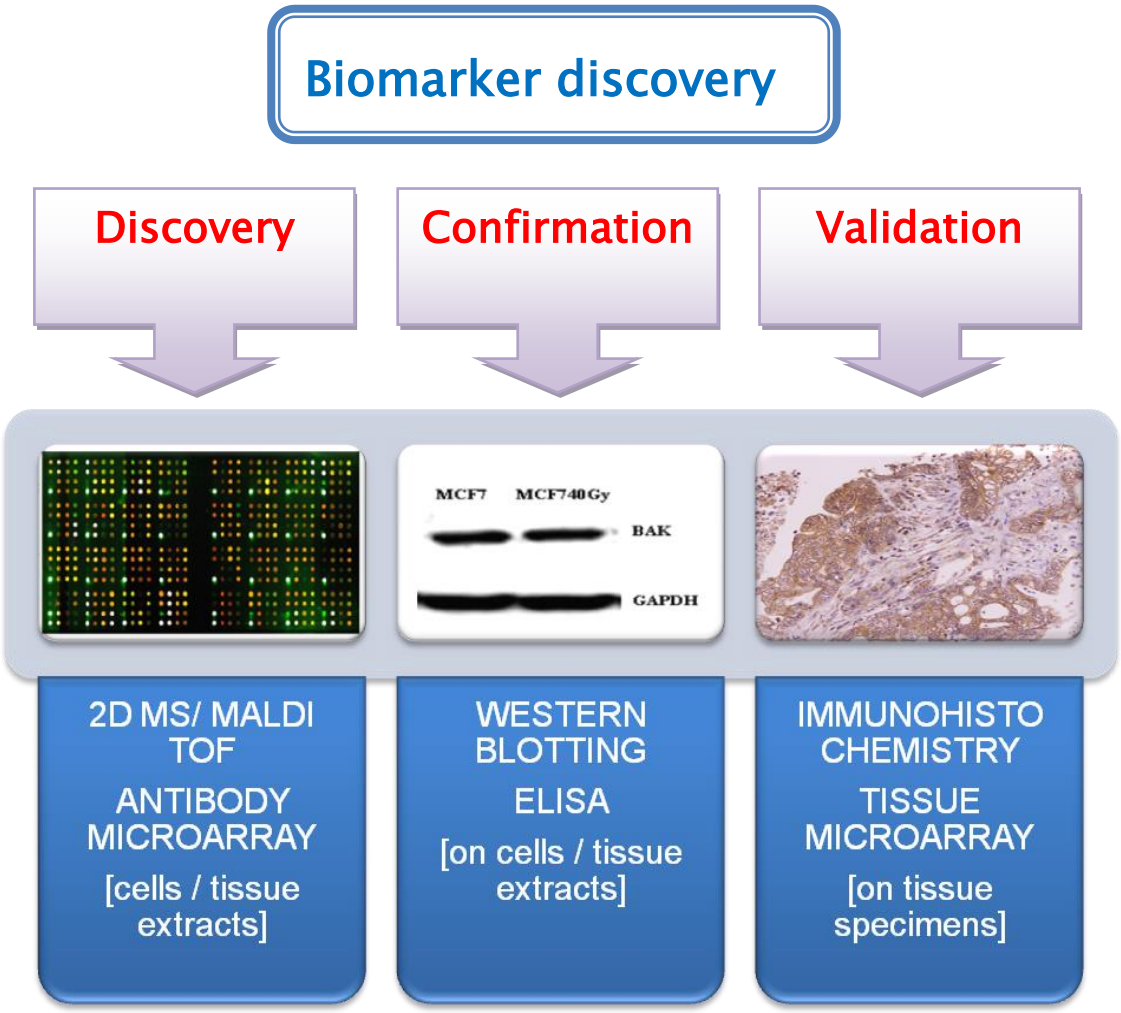

Figure 1.16: The proteomic model of biomarker discovery pipeline

1.12 Hypothesis and Aims

Neoadjuvant radiotherapy provides local control of disease in rectal cancer, however, the ability to predict response to radiotherapy is limited. There has been a drive to establish means to determine the radiotherapy outcome. As discussed above, the effect of radiotherapy on tumour cells may be determined by biomarker expression analysis in pre-treatment biopsy or post-treatment specimens. In particular, predictive biomarkers may be studied from biopsy tissue before treatment commences. A wealth of evidence now exists in this area, however, no single biomarker has shown the promise to reliably predict such response.

We hypothesised that the identification of proteomic markers of response to radiotherapy in rectal cancer might enable clinicians to tailor therapy according to the predicted response. Such an approach would allow patients with radioresistant tumours to avoid ineffective radiotherapy which indeed is a toxic treatment modality as discussed in section 1.7.2.7. Such an approach would avoid undue delay of a number of weeks normally associated with RT and post radiotherapy interval. Radioresistant tumours may be allowed to proceed to 'straight to surgery' if deemed operable. The objective of this research was to identify protein biomarkers through protein expression analysis that would help identify radioresistant rectal cancers so that a tailored approach in management of such patients may be adopted to achieve best possible oncological outcomes.

The main aims of this thesis are outlined below:

- To establish novel radioresistant (RR) rectal cancer cell line model from pre-established cell lines using a fractionated irradiation protocol
- To identify putative protein biomarkers of response to radiotherapy using antibody microarray based proteomics platform
- To identify a panel of common differentially expressed proteins related to radioresistance for the validation phase of biomarker discovery pipeline
- To identify a suitable series of rectal cancer patients treated with neoadjuvant long-course chemoradiotherapy for biomarkers validation
- To undertake immunohistochemical analysis of archival rectal cancer biopsy tissues to validate the differential expression of biomarkers identified by antibody microarray

CHAPTER-2: Materials and Methods

2.1 Materials and methods

2.1.1 Study design

This research involved an experimental in-vitro project using rectal cancer cell lines and a pilot immunohistochemical study using archival rectal cancer pre-treatment biopsies conducted under supervision of Dr Lynn Cawkwell, senior lecturer, Cancer Biology Proteomics Group, University of Hull, and Mr Iain Andrew Hunter, consultant colorectal surgeon, Castle Hill Hospital. All cell cultures and proteomic experiments were performed at Daisy Research Laboratory, Daisy Building, Castle Hill Hospital, University of Hull. Irradiation of cells was undertaken at Queen's Centre for Oncology, Castle Hill Hospital, Cottingham, in collaboration with a team of radiation physicists; Prof Andy Beavis, Dr Gary Liney and Dr Matthew Bush.

2.1.2 Rectal cancer cell lines

There are a number of commercially available rectal cancer cell lines, of both human and animal origin (http://www.phe-culturecollections.org.uk). This research involved human cell lines and tissues only. In-vitro experiments were performed on SW-837 and HRA-19 rectal adenocarcinoma cell lines and their radio-resistant sub-clones developed as part of this project. The cell lines were purchased from European Collection of Cell Cultures (ECACC) in frozen state.

2.1.3 Inclusion criteria for cell lines

Scientific literature depicts several controversies surrounding the status of the commercially available cell lines (Freshney 2005; Capes-Davis, Theodosopoulos et al. 2010; Masters 2010). A rectal cell line HRT-18 has been considered the same as the colonic cell line HCT-15 before the evidence emerged that both cell lines, and in fact another two, originated from the same patient and contained the same genetic material (Vermeulen, Chen et al. 1998). It remains unclear whether they represent the same tissue type. The cell lines might possibly have originated from synchronous lesions in colon and rectum, however, there is no evidence to support that. In addition, quality in-vitro studies have been carried out on cell lines claimed to be rectal cancer cell lines (Rodel, Hoffmann et al. 2005) which are, in fact, the colon cancer cell lines (Leibovitz, Stinson et al. 1976). Furthermore, it is understood that there is a significant degree of cross contamination in the laboratories and that a named cell line may actually contain a different tissue cells or, in extreme circumstances, a different species cells (Capes-Davis, Theodosopoulos et al. 2010).

In view of above controversies surrounding the quality of the commercially available cell lines, strict quality inclusion criteria were applied to ensure that all the experiments in this research produce high quality, valid and representative results. Based on above, the first two of four inclusion criteria were defined. The remaining two criteria were based on the following. As discussed in section 1.5.4, it has been proposed that colorectal cancers of left side (which includes rectal cancers) are less likely to originate from microsatellite instability pathway (Coggins, Cawkwell et al. 2005; Mehigan, Ashman et al. 2006; Soreide, Nedrebo et al. 2009). Therefore rectal cancer cell lines with proficient MMR

gene would most reliably represent the typical rectal cancer. The MMR deficient cell lines might not be representative i.e. they would be atypical. The fourth criterion was related to the p53 status of the cell lines. In view of the extensive studies to establish the role of p53 in radioresistance (Section 1.9.1), there is a possibility that p53 status might correlate to radioresistance (Samowitz, Holden et al. 2001; Mehigan, Ashman et al. 2006; Soreide, Nedrebo et al. 2009; Nyiraneza, Jouret-Mourin et al. 2011).

Based on above, following criteria were defined for inclusion of cell lines:

1. Definitive origin from rectal adenocarcinoma

2. Evidence of laboratory testing for absence of cross contamination

3. MMR proficient

4. Known p53 status (wild type / mutated)

Based on the above criteria, SW-837, SW-1463 and HRA-19 cell lines were selected from the ECACC from the available 6 options. Those not considered included: a) HRT-18: As discussed above, it may be same as HCT-15 i.e. of possible colonic origin b) HRT-19 and HRA-16: Not enough literature about their growth and molecular characteristics. Of the three selected cell lines, SW-1463 did not grow successfully in our lab despite the culturing conditions provided as per ECACC's recommendation and was finally discontinued. Thus the two cell lines used in these experiments were SW-837 and HRA-19 (table 2.1). There is evidence that they originated from rectal cancer (Leibovitz, Stinson et al. 1976; Kirkland and Bailey 1986) and that there is no cross contamination (Capes-

Davis, Theodosopoulos et al. 2010). Table 2.1 gives the detailed features of selected cell lines with their p53 and MMR status.

Table 2.1: Characteristics of rectal cancer cell lines. Two rectal adenocarcinoma cell lines were selected based on the selection criteria (see text above). Both SW-837 and HRA-19 cell lines were p53 mutant and MMR gene proficient.

Cell line, Catalog #	Origin and characteristics	p53 status	MMR status	Growth type / requirements
SW-837 (91031104)	53-year old Caucasian male Grade IV rectal adenocarcinoma (Leibovitz, Stinson et al. 1976)	P53 mutant (Nigro, Baker et al. 1989; Liu and Bodmer 2006)	MMR proficient (Lengauer, Kinzler et al. 1997)	Adherent type cells Passage sub-confluent cells 1:3 to 1:6
HRA-19 (10012802)	Primary rectal adenocarcinoma in a 66-year old male. Well differentiated Dukes' stage B (Kirkland and Bailey 1986)	P53 mutant (Liu and Bodmer 2006)	MMR proficient (Wheeler, Beck et al. 1999)	Adherent type cells Passage sub-confluent cells 1:5 to 1:6 (Kirkland and Bailey 1986)

2.1.4 Cell culture

Cells were grown in tissue flasks in their respective medium as per depositor's or supplier's recommendations. Cells were either grown in a T25 ($25cm^2$) or a T75 ($75cm^2$) tissue flask suspended in 5 – 7 ml or 7 – 10 ml culture medium, respectively. Cells were grown in an incubator at culturing conditions of 37^0C temperature, 95% oxygen and 5% CO_2. Medium was changed every 48 – 72 hours, on an average three times a week. This was always performed in a class II cell culture hood observing standard safety precautions. Culture hood was

pre-cleaned with 1% Virkon (w/v) solution and sprayed with 70% Alcohol (v/v) and routinely disinfected with ultraviolet radiation.

Both the cell lines in these experiments consisted of adherent epithelial cells, therefore to allow medium change and passage, cells were treated with 0.25% Trypsin (TrypLE^{TM} EXPRESS, Invitrogen, UK) to facilitate detachment from the base of flask. To achieve that, all medium from a flask was removed, 3-4 ml of Trypsin added and the flask returned back to incubator for three minutes for Trypsin to act. After 3 minutes, cells were examined under the microscope, and observed for detachment. HRA-19 cells were found to be excessively adherent and needed treatment with trypsin for 6-8 minutes. Detachment of cells was helped by gentle tapping of flask. Once the cells were detached, 7ml of culture medium was added to flask to neutralise trypsin. The resultant cell suspension was then transferred into a 25ml screw capped tube and centrifuged at 400 x *g* for 3-4 minutes. The supernatant was discarded and resultant cell pellet was re-suspended in appropriate volume of medium according to the requirement on the day i.e. change of medium or passage. When cells reached 80% confluence, they were usually split into three further flasks. Each split represented a passage and passage numbers were allotted accordingly.

2.1.5 Culture medium

SW-837 rectal adenocarcinoma cell line was cultured in RPMI-1640 (# R1383, Sigma-Aldrich UK). This medium was developed by Moore and colleagues at Roswell Park Memorial Institute, hence the acronym RPMI (Moore, Mount et al. 1963). The formulation is based on the RPMI-1630 series of media utilising a

bicarbonate buffering system and alterations in the amounts of amino acids and vitamins. RPMI-1640 medium has been used for the culture of human normal and neoplastic leukocytes and a wide range of other tissue cell types. RPMI-1640, when properly supplemented, has demonstrated wide applicability for supporting growth of many types of cultured cells. RPMI-1640 provides balanced energy source to serve as carbon skeleton for anabolic processes as well as protein production and nucleic acid metabolism while limiting toxic ammonia build-up. It is used with 5% CO_2 to maintain the pH (Moore, Mount et al. 1963; Sigma-Aldrich 2012).

HRA-19 cells were routinely cultured in high glucose Dulbecco's Modified Eagle's medium (DMEM) medium, as recommended by the depositor of cell line (Kirkland and Bailey 1986). DMEM is among the most widely used modifications of Eagle's Medium. DMEM is a modification of Basal Medium Eagle that contains a four-fold higher concentration of amino acids and vitamins, as well as additional supplementary components (Sigma-Aldrich 2012). The original DMEM formula contains 1000 mg/L of glucose and was first reported for culturing embryonic mouse cells. A further alteration with 4500 mg/L glucose (high glucose DMEM) has proved to be optimal for cultivation of certain cell type, the formulation used for culturing HRA-19 cells in this research.

2.1.6 Cell banking

Cells were stocked every few passages to maintain a local cell bank. Cells upon reaching ~ 80% confluence were trypsinised as described above and suspended in 10% freezing medium. Freezing medium was constituted by adding 5ml of 5% DMSO to 45ml of usual reconstituted medium for the

particular cell line. DMSO was added to prevent crystal formation in cell suspension at ultra-low storage temperatures. Following trypsinisation, a cell pellet was obtained in the usual way as described above. One ml of pre-warmed freezing medium was then slowly added, drop by drop, to re-suspend the cells. Resultant suspension was then transferred to a 1.5ml cryovial which was stored in -80 freezer or in liquid nitrogen.

Following protocol was followed to thaw the cells to use them from frozen state. Cryovial was transferred to a water bath from -80 freezer, in a sealed polyethylene bag to slowly warm at 37^0 C. Once fully thawed, the contents were then transferred to a 25 ml screw capped tube and 9ml of pre-warmed culture medium was slowly added, drop by drop. The suspension was mixed by gentle pippeting before centrifuging at 400 x *g* for 3-4 minutes. Following that, the supernatant was discarded and the resultant cell pellet was then suspended in 7-10 ml of culture medium before transferring to a tissue flask. Tissue flask was then returned to incubator and examined for cellular attachment to base of flask in 24 hours by which time most of cell attachment would normally have occurred. Culture medium was then routinely changed every 48 to 72 hours as described above.

2.2 Development of novel radioresistant rectal cancer cell lines

Radioresistant novel rectal cancer cell lines were developed from rectal cancer cell lines. Radioresistance was produced by treating the cells with clinically relevant doses of RT. The protocol to induce radioresistance, developed by Cancer Biology Proteomics Group, University of Hull, was followed (Smith, Qutob et al. 2009). It was hypothesised that the treatment of cells with

fractionated radiation would leave a small fraction of surviving cells that might be resistant to RT (Figure 2.1).

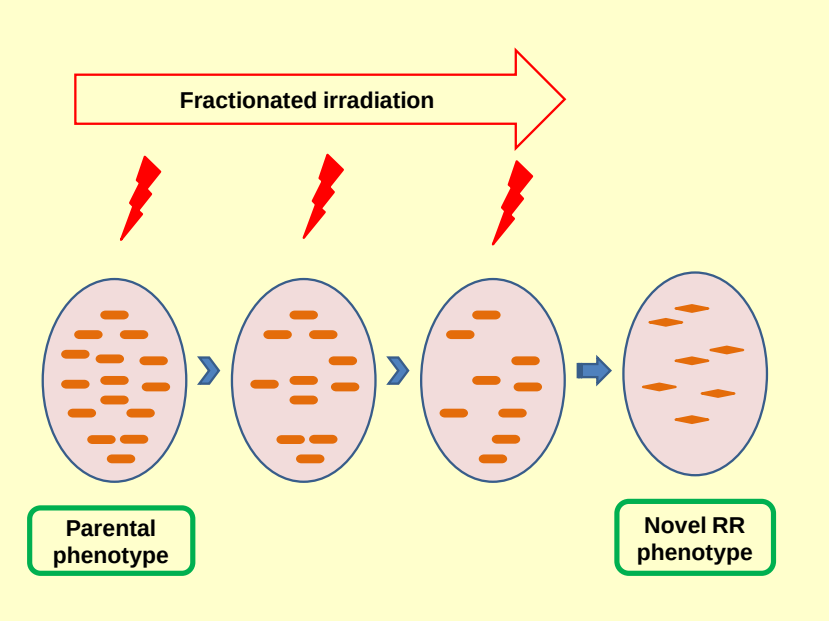

Figure 2.1: Principle of inducing radioresistance. The parental cells treated with fractionated dose of RT over time are left with a small fraction of surviving cells that can survive the further doses of RT by developing radioresistant phenotype.

2.2.1 Irradiation protocol

Cells were treated with RT at the Queen's Centre for Oncology, Castle Hill Hospital in collaboration with a team of radiation physicists; Prof Andy Beavis, Dr Gary Liney and Dr Matthew Bush. The experimental set-up and sequence are described below:

2.2.1.1 Experimental model: Phantom

A phantom was used which consisted of a glass jar and housed a cradle into which the screw-capped vials could be held securely in position (Figure 2.2). The phantom was filled with water when in use and the glass jar had flat sides so that the irradiated volume of water was a simple cuboid. Water filled

phantom containing two dummy vials was imaged on a CT scanner to enable an accurate radiotherapy delivery to be planned using a pair of parallel opposed beams.

2.2.1.2 Irradiation

The cells were irradiated using a 6 MV photon beam from a Varian Linear Accelerator. The phantom housing the screw capped vials containing cells in their respective culture medium was positioned in front of Linear Accelerator as shown in figures 2.2 and 2.3. Irradiation consisted of X-ray beams at gantry angles of 90° and 270° with a 8 cm x 8 cm field size and each delivering 54 monitor units (MU) in order to produce a uniform dose to the centre of the vial of 1 Gray (Gy). This delivery was incremented according to the specific dose required on each occasion.

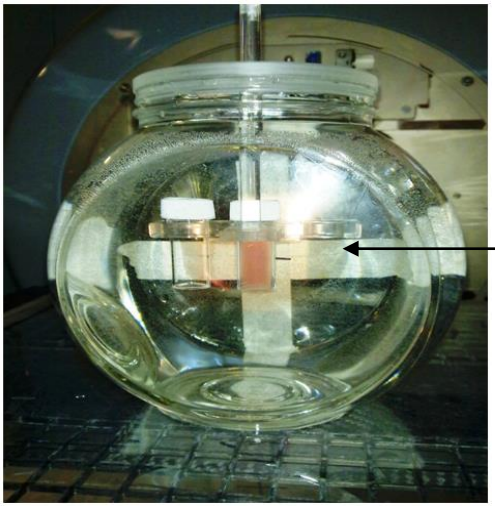

Figure 2.2: Phantom for irradiation of cells. A water filled glass jar (phantom) containing a cradle into which two vials are slotted. Arrows shows the vial containing SW-837 cells suspended in RPMI medium. The reference vial contains equal amount of water.

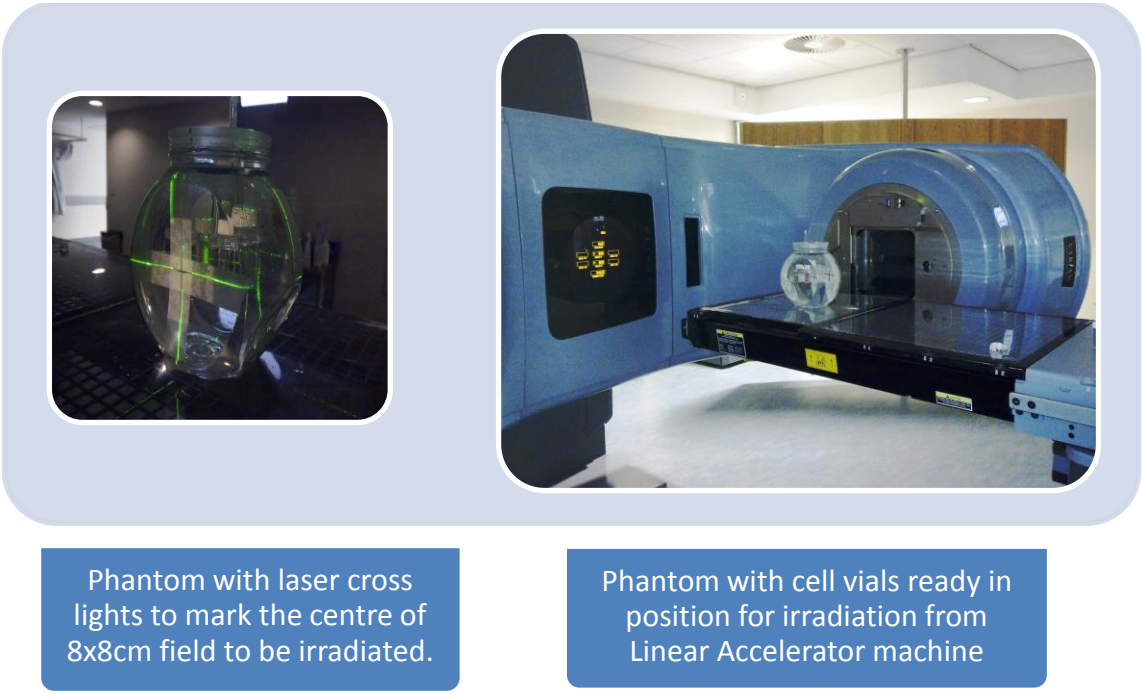

Figure 2.3: Phantom and Linear accelerator

2.2.2 Cell Counting

Cell counting in the labs is routinely carried out using a counting chamber. In this experiment, a haemocytometer was used which was originally designed to count blood cells, hence the name, but is widely used to count any type of cells. Cells were harvested using trypsin and re-suspended in 6ml medium. Of this, 25µL was taken and mixed with 25µL 0.4% (w/v) trypan blue giving 1:1 concentration. 25µL of this suspension was applied to a haemocytometer under a glass cover slip. Cells were counted under microscope using a hand-held counter. Cells were counted in 5 squares, four corner squares and the central square. Average was calculated and the number of cells per ml counted as per following formula:

$[(1+2+3+4+5)/5 \times 2] \times 10^4$ = cells/ ml

2.2.3 Modified Colony Counting Assay

Modified colony counting assay, modified from clonogenic assay (Blumenthal 2005; Blumenthal 2005a) was carried out as previously described (Smith, Qutob et al. 2009). This assay measures cell survival based on the ability of the cells to establish a single colony following a cytotoxic insult. It determines the ability of a cell to proliferate indefinitely which shows its reproductive ability. A cell able to retain its reproductive ability will form a large colony or a clone and is called clonogenic (Blumenthal 2005a). A single colony is defined as a group of 25 to 50 cells (Blumenthal 2005a; Xu, Gao et al. 2008). It is important, however, to adopt a consistent approach in defining a colony in a single experiment to produce consistent and reliable results. In these experiments, a colony was defined as group of 50 or more cells. This assay is applicable to measure cell survival following any cytotoxic insult which includes exposure to ionising radiation as well as chemotherapeutic agents. Therefore, this assay can be employed to identify dose of radiotherapy which will kill the majority of a cell population while at the same time leave enough resistant cells to form a novel radioresistant derivative population.

2.2.4 Modified Colony Counting Assay for radiotherapy resistance

2.2.4.1 Assay set up

Modified colony counting assay was carried out to assess cell survival following exposure to radiation, as previously described (Franken, Rodermond et al. 2006; Smith, Qutob et al. 2009). In order to perform this assay, $1x10^6$ cells, in a 7ml polypropylene vial, were irradiated with single fractions of 2, 4, 6, 8 and 10Gy. Post irradiation, sample of cells treated with each dose of RT, along with

control cells receiving no RT (0 Gy), were incubated for 12-14 days in 6 well plates which offers 9.40 cm^2 growth area / well.

2.2.4.2 Cell preparation and dosage

Cells upon reaching more than 80% confluence in a T75 tissue flask were detached using trypsin and re-suspended in 6ml of respective supplemented culture medium. A cell count was taken using haemocytometer as described in section 2.2.2. A sample of one million cells was then transferred into each of six 7-ml polypropylene vials. Volume in each vial was supplemented with respective culture medium to obtain a uniform volume of 5ml in each vial. Therefore each vial contained one million cells in 5ml of culture medium. These vials were pre-labelled with the required dose of radiation to be given. The single doses for modified colony counting assay were 0 (control), 2, 4, 6, 8 and 10 Gy of x-rays (Table 2.2).

Table 2.2: Radiation dosage for the irradiation protocol. Fifty-four MU delivered from each gantry angle (90^0 and 270^0) equalled one Gy dose of RT. A monitor unit (MU) is a measure of machine output of a linear accelerator.

Radiation dose (Gy)	**X-rays delivered at 90^0 (MU)**	**X-rays delivered at 270^0 (MU)**
1	54	54
2	108	108
4	216	216
6	324	324
8	432	432
10	540	540

2.2.4.3 Cell transportation

Cells were transported from daisy research lab, Castle Hill Hospital to Queen's centre for oncology for irradiation in a polyethylene bag in a protective box in

order to minimise any direct impact to vials, spillage, contamination, and the exposure to low outside temperature. Care was taken not to prepare cells too soon before irradiation would commence to keep their time outside incubator to a minimum (aiming to prepare within an hour before irradiation).

2.2.4.4 Seeding density and incubation

After single dose irradiation with the required doses, all six vials were returned to the lab. Three samples of cells containing 1000 cells each, from each vial were then transferred to 6-well plates to have three replicates from each vial. Thus a seeding concentration of 1000 cells per well was obtained for all irradiated and the control samples. Total volume of cell suspension per well was supplemented with 3-5 ml of respective culture medium (section 2.1.5). Cells were cultured for 12-14 days for colonies to become established.

2.2.4.5 Completion of assay: Colony fixation and staining

After incubation for 12 to 14 days, all medium was removed and the cells fixed with ice cold methanol : acetic acid solution (3:1 v/v), 3ml solution per well for 5 minutes. After 5 minutes, the fixations solution was discarded and the cells were then left to air dry for few hours (or overnight). Following fixation, cells were stained with 3ml per well of 0.005% crystal violet stain for 5 minutes. The crystal violet solution was then discarded and residual stain was removed in slowly running tap water in a sink. The plates were allowed to air dry for a few hours to overnight.

2.2.4.6 Colony counts

High resolution images of each 6-well plates, and each well separately, were taken using an 18 megapixel digital SLR camera with optical mega lens (Canon EOS 550D). The images were exported to a computer connected to a colour printer to print a full page colour image without further processing or zooming. A well from 6-well plate from each set of experiments was examined under a light microscope and a colony of 50 cells was identified. This was then correlated with the corresponding image and a colony on the print image was defined. Colonies of 50 cells or more were deemed to represent surviving cells from the original cell line. The colonies were counted from the printed images of each well for all experiments. The counts were taken by two investigators independently and inter-observer limits of agreement were checked by plotting Bland-Altman plots (section 2.2.4.7). For further calculation of plating efficiencies and surviving fractions, the average counts of two observers were used (section 2.2.4.8).

2.2.4.7 Inter-observer limits of agreement: Bland-Altman plot

To analyse limits of agreement between colony counts of two observers, Bland-Altman plots were created using Analyse-it® - Statistics software for Microsoft Excel. Bland and Altman introduced the Bland-Altman plot to describe agreement between two quantitative measurements. There is no p-value available to describe this agreement but rather a quality control concept. The difference of the paired two measurements is plotted against the mean of the two measurements. 95% of the data points lying within the ± 2sd of the mean difference represent strong agreement between measurements.

2.2.4.8 Plating efficiency and surviving fraction

Plating efficiency (PE) and surviving fraction (SF) were calculated from colony counts as per following formulas (Blumenthal 2005a).

PE = Number of colonies counted / number of cells plated x 100

SF= PE of treated sample / PE of control x 100

2.2.4.9 Dose response curve (DRC)

Dose response curves were generated for each parent and resistant cell line. The loss of the ability of the cells to retain their colony forming potential can be displayed on a log-linear DRC. SF were plotted on a logarithmic Y-axis against corresponding radiation dose on linear X-axis to generate log-linear DRC. The colony counting assays were performed twice for each parental cell line and its RR derivative. From each experiment, three technical replicates were obtained for each dose of RT and control groups (section 2.2.4.4). Therefore, for each cell line and its RR derivative, six replicates of colony counts were obtained from two independent experiments. The mean SF for each dose / each cell line was calculated and DRCs were plotted. Comparative DRCs were plotted for comparison of the SFs of parent cell lines and their respective novel RR derivatives.

2.2.5 Induction of Radioresistance: Fractionated irradiation protocol

In order to induce radioresistance by fractionated irradiation into a fresh sample of each cell line, SW-837 and HRA-19 parental cell lines, sub-lethal doses of RT were selected from the DRCs of respective parental cells which were guided by

the clinically relevant fractions for long course RT (1.8 Gy per day, 9 Gy per week). At the beginning of these experiments, it was aimed to achieve a total dose of radiation which mimics the long course RT (45 – 50.4 Gy). Each cell line was irradiated weekly or fortnightly as per repopulation of cells, to achieve 70-80% confluence before the next dose. This regimen was continued until a total targeted dose was reached. At the completion of fractionated irradiation, dose response curves were generated in a similar fashion and plotted against the respective parent cell line. A statistical test of significance was applied to confirm that significant radioresistance had been induced.

2.3 Statistical analysis

Independent sample *student's t test* was used to detect significant difference in surviving fraction between the parental and radioresistant cells using statistical package SPSS^{TM} version 17 for Windows$^{®}$ (SPSS Inc., Chicago, IL, USA). A p-value of <0.05 was considered statistically significant.

2.4 Biomarker discovery

Proteomics experiments using antibody microarray technique was used to study differentially expressed proteins between parental and radioresistant cell lines.

2.4.1 Antibody Microarrays

There are a number of commercially available antibody microarray (AbMa) kits and the choice is determined by the molecular pathway or a protein of interest in a given study. There are several suppliers of antibody and protein microarray kits including Sigma-Aldrich, Life Technologies, and Cell Signalling amongst a long list (Nature 2006). Our research group has previously optimised the array

kits from Sigma-Aldrich that was therefore the source of kits in the work presented in this thesis. Table 2.3 shows some of the array kits that are named after their molecular profile (Sigma-Aldrich 2012). The Panorama Antibody Microarray XPRESS profiler 725 contains a comprehensive (although not complete) range of antibodies to proteins linked with apoptosis, cell signalling, cell cycle, and cell adhesion and proliferation. Because these pathways could play a role in modulating response of anti-cancer treatment, this kit was selected for use in our experiments.

Table 2.3: Commercially available Antibody Microarray kits. The antibody microarray kits that are commercially available. The kits contain antibodies representing important cellular pathways and can be chosen according to the researcher's requirements. (http://www.sigmaaldrich.com/life-science/cell-biology/cell-biology-products.html?TablePage=14842165).

Catalog #	Antibody Microarray kit	Number of antibodies spotted
CSAA1	Panorama® Antibody Microarray: Cell signalling	224
GRAA2	Panorama® Antibody Microarray: Gene regulation	112
MPAA3	Panorama® Antibody Microarray: MAPK & PKC pathways	84
PPAA4	Panorama® Antibody Array: p53 pathways	112
NBAA5	Panorama® Antibody Array: Neurobiology	224
XP725	Panorama® Antibody Microarray: XPRESS profiler 725	725

2.4.2 The Sigma-Aldrich Panorama XPRESS™ profiler 725 kit

The Panorama Antibody Microarray – XPRESS™ profiler 725 Kit was purchased from Sigma-Aldrich UK (Catalog # XP725). The arrays consist of polyclonal and monoclonal antibodies spotted in duplicate on nitrocellulose-

coated glass slide. The antibodies are species nonspecific and recognise human, mouse and rat proteins. The antibodies are distributed in 32 sub-arrays each containing duplicate spots of 23 antibodies, as well as duplicate positive control spots for Cy3 and Cy5 dyes (a monoclonal antibody that recognizes Cy3 and Cy5), and several negative controls. Each spot is 0.3 mm in diameter and distance between spots is 0.5 mm. The distance between sub-grids is ~1.4 mm. Typical thickness of nitrocellulose coating is 9 µm. The array can be used for comparing protein expression profiles of two samples (test versus reference samples) each labelled with a different fluorescent dye (Cy3 or Cy5). A fluorescent detection procedure is used and fluorescent signal intensity for each sample is recorded individually at the wavelength corresponding to the dye label of the sample.

This kit detects a wide variety of proteins representing a broad range of biological pathways and molecular processes, including but not limited to cell stress, cell cycle, signalling transduction, apoptosis, neurobiology, and gene regulation. Results obtained using the array should be further evaluated by other methods such as immunoblotting assays, ELISA, or IHC. Figure 2.4 illustrates an overview of the AbMa protocol.

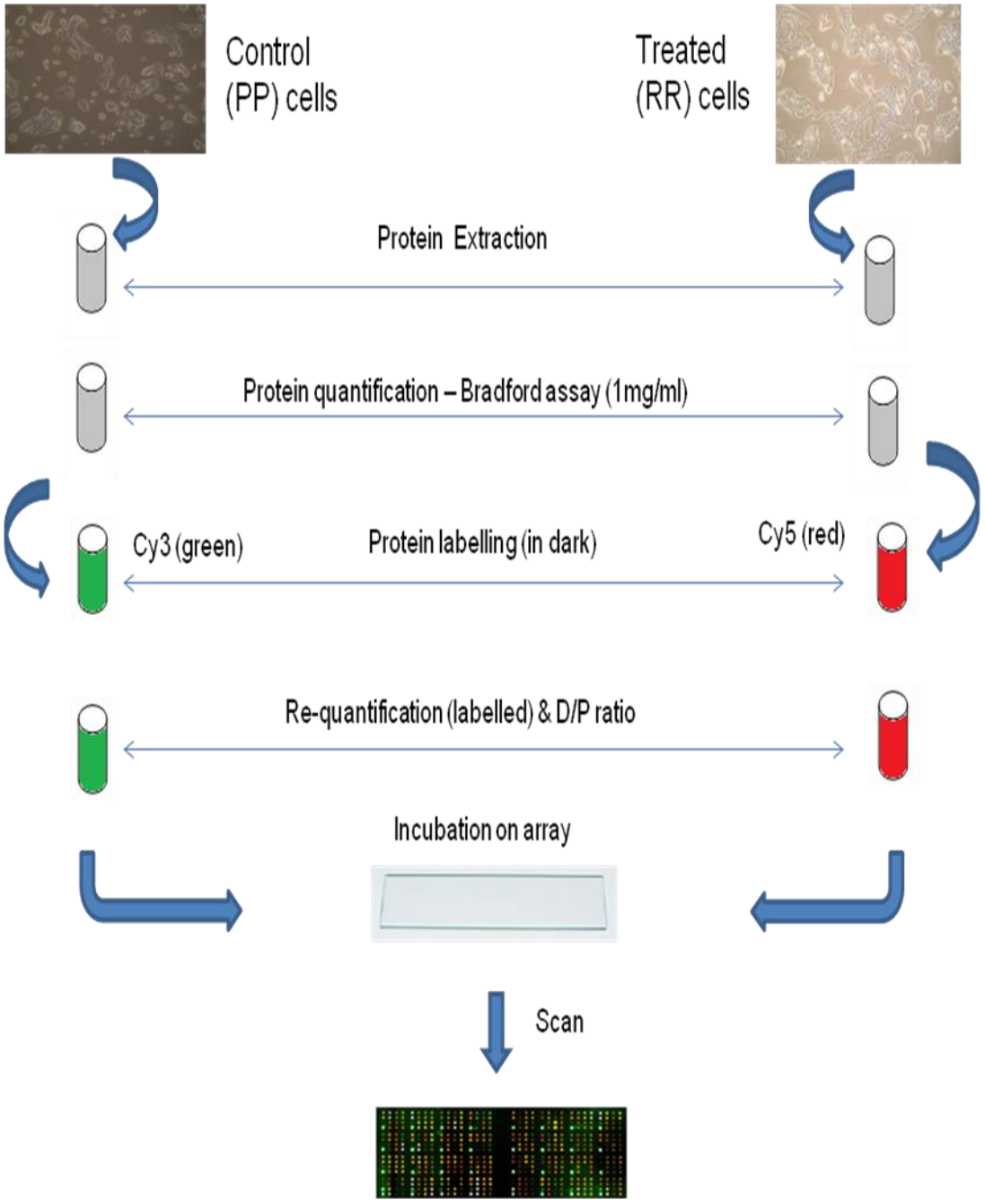

Figure 2.4: A schematic illustration of the antibody microarray protocol. Total proteins from PN and RR samples were extracted and quantified using Bradford method. The samples were labelled with Cy3 and Cy5 dyes, re-quantified and D:P ratios were determined. Antibody microarray slide was hybridised with protein lysates from the PN (Cy3 labelled) and RR (Cy5 labelled) samples. The slide was scanned and data analysed using the Genepix pro software. The relative ratios of red and green fluorescence at each spot determined the expression levels of different proteins.

2.4.3 Protein extraction

In order to perform antibody microarray, total proteins from both parental and radioresistant cells were extracted closely following the clonogenic assays. To do so, PN and RR cells were cultured under usual conditions and in parallel. Cells were considered ready for extraction when they reached around 70 - 80% confluence in a T75 cm^2 flask. Cells were harvested from three flasks for each cell line using a cell scraper. This was carefully undertaken to eliminate the play of other environmental factors in the differential expression of proteins as it is known that stress proteins are expressed upon exposure to different stressful conditions (handling, trypsinisation, temperature, CO2 levels etc). The yield of cells per flask was ~ 1 x 10^6. The aim was to obtain a final protein concentration of 1mg /ml in each sample. Cells were washed twice in 5 ml cold PBS before re-suspension in 1ml PBS and transfer into a 1.5ml microcentrifuge tube. Cells were centrifuged at 10,000 xg and re-suspended in 1 ml of buffer A. Reagents for Buffer A were supplied with the kit and prepared as follows: Ten ml of extraction/labelling buffer (# E0655, Sigma-Aldrich UK) was aliquoted into a vial and 50 µl of protease inhibitor cocktail (catalog number P4495, Sigma-Aldrich UK), 100 µl of phosphatase inhibitor cocktail II (catalog number P5726, Sigma-Aldrich UK), and 1.2 µl of Benzonase working solution were added. The solution was kept on ice at all times. Benzonase working solution was made from adding 18µl of extraction/labelling buffer with 2 µl Benzonase (catalog Number B8309, Sigma-Aldrich UK).

Cell suspension with 1m buffer A, was mixed and placed on an end-over-end rotator in a cold room for 5 minutes. Following that, the cell suspension was

centrifuged for 2 minutes at 10,000 xg and supernatant transferred to a fresh 1.5 ml microcentrifuge tube. It was aimed to obtain the protein extract visibly clear and that it was not cloudy or viscous. Whenever this was encountered additional quantity of Benzonase, which is a potent DNase enzyme, was added to Buffer A to ensure DNA degradation. Small particles were eliminated by a rapid centrifugation just before the labelling procedure. The protein extracts were kept on ice, when the AbMa experiment was performed the same day, or stored in -80^{0}C freezer, if AbMa experiment was planned for a later date.

2.4.4 Protein quantification

For protein quantification Bradford Assay was performed to assess quantity of proteins in the extracts using Bradford reagent (Catalog # B 6916, Sigma-Aldrich, UK). The reagent consists of Brilliant Blue G in phosphoric acid and ethanol. The procedure is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution (Bradford 1976). The protein dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present.

The 96 well plate protocol of the assay was followed which allows rapid assay of multiple protein samples, while using a small sample volume (5 µl). The Bradford reagent, which is normally kept in fridge, was taken out of fridge half an hour before starting experiment to acclimatize to room temperature. The protein standards were prepared using BSA standard diluted with double distilled water at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4

mg/ml. To ensure the concentration of each sample was within this range (0.1-1.4 mg/ml) unknown protein samples were prepared at 1:5 and 1:10 dilutions with Buffer A (see section 2.4.2.2). Five µl of each BSA standard was added to top two rows of a 96-well plate (to obtain replicates) and 5 µl of each dilution for both the control (PN) and treated (RR) protein samples was also added to the plate in triplicates (Table 2.4). Double distilled water was added to the blank wells in the top two rows. Finally, 250 µl of Bradford reagent was added to each well.

Table 2.4: Bradford assay for protein quantification. Unknown protein concentration of the test sample was determined by Bradford assay using BSA standard known protein concentrations. The BSA protein standards were diluted to obtain concentrations ranging from 0.1 to 1.4 mg/ml. Five µl of each concentration was added to separate wells in a 96-well plate. The protein extracts of unknown samples (PN and RR) were also added to separate wells in the 96-well plate at a volume of 5 µl. Bradford Reagent was added to each well containing standards and samples (250 µl per well). The 96-well plate was then mixed for 30 seconds on the spectrophotometer and incubated at room temperature for 5 minutes before the absorbance was measured at 595 nm.

Wells	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Standard	Blank	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Standard	Blank	0.1	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Control Sample	PN Sample	PN Sample	PN Sample	PN 1:5	PN 1:5	PN 1:5	PN 1:10	PN 1:10	PN 1:10
Treated sample	RR Sample	RR Sample	RR Sample	RR 1:5	RR 1:5	RR 1:5	RR 1:10	RR 1:10	RR 1:10

The samples were mixed for 30 seconds on the spectrophotometer before the plate was incubated at room temperature for 5 minutes. The absorbance was then measured at 595nm using a plate reader.

A standard curve was plotted for the BSA standards and the unknown concentrations of the protein samples were calculated. Samples with protein concentrations 1.0 mg/ml were used for fluorescent labelling. Whenever higher concentration was obtained samples were diluted using Buffer A solution.

2.4.5 Protein labelling with fluorescent dyes

This part of experiment was performed in the dark as it involved photosensitive fluorescent dyes Cy3 and Cy5. One ml of extract at 1 mg/ml concentration was used to label with Cy3 (control PN sample) or Cy5 (RR sample). One ml of the control sample (PN) was added to Cy3 vial and 1ml of the treated (RR) sample was added to the Cy5 vial. The vials were capped and contents thoroughly mixed using a vortex mixer. Care was taken to avoid pipetting which can introduce the foaming of the sample, possible sample loss, and more sample handling etc. The labelled samples were incubated in the dark at room temperature for 30 minutes. During this period, both the samples were mixed using vortex every 10 minutes.

The labelled protein samples were removed from free Cy3/Cy5 dye using the SigmaSpin post-reaction clean-up columns (Catalog # S0185, Sigma-Aldrich UK). The columns were centrifuged in a microcentrifuge for 2 minutes at 750 xg (3200 rpm) following which the eluate in the collection tube was discarded. Columns were then ready to use and were placed in new collection tubes. To load labelled protein samples, 150 µl of labelled sample was loaded onto the centre of the column and centrifuged at 750xg for 4 minutes. The eluate was retained and columns were discarded. The eluate was labelled protein sample

after removal of unbound dye and the post-labelling protein concentration was determined using the Bradford Assay as described in section 2.4.4 Labelled protein was stored at -80°C until hybridisation.

2.4.6 Determination of dye to protein molar ratio (D:P ratio)

For best results, it is recommended that only labelled preparations with dye:protein (D:P) ratio >2 be used. Therefore D:P ratio was determined once the dye was bound to protein samples. The labelled samples were diluted for this step; typically 15 µl of labelled sample diluted with 135 µl ddH_2O in separate tube was mixed. The cuvette was washed thoroughly with ddH2O between each use. Using a pipette, 100 µl of labelled protein sample was added to cuvette. The absorbance of Cy3 and Cy5 labelled samples was measured at excitation wavelengths of 552nm and 650nm, respectively, using a Libra S11 Spectrophotometer. These absorbance values were used to calculate the dye to protein molar (D:P) ratio as per following calculations:

Cy3 concentration (µM) = (A552 / 0.15) x 10

Cy5 concentration (µM) = (A650 / 0.25) x 10

10 = dilution factor, Y (mg/ml) = protein concentration after labelling (obtained after 2nd Bradford Assay)

Protein concentration (µM) = (Y / 60,000) x 1,000,0000

D:P ratio = Cy3 or Cy5 concentration / Protein concentration of sample

The D:P ratio was used to assess the labelling efficiency and samples with D:P ratios greater than 2 were incubated.

2.4.7 Hybridisation:

Following determination of D:P ratios, the Cy3 and Cy5 labelled PN and RR samples from each cell line in an experiment were co-incubated onto array slide, a step called hybridisation. Sigma-Aldrich, the manufacturer of Panorama XPRESS profiler 725 kit, recommend a pre-requisite of D:P ratio of more than 2, below which the sensitivity of array reduces. It may still work, however, the incubation time in that case is prolonged to 45 minutes (instead of 30 minutes) and a higher background may be observed. For array incubation, equal amount of proteins from each sample were calculated aiming to load 50 - 150 µg of proteins from each sample. The volume of sample for incubation was calculated as per following formula:

Required concentration / Available concentration

In a tube, equal amounts of Cy3 and Cy5 labelled samples were mixed with 5ml of array incubation buffer (Catalog # A9602, Sigma-Aldrich UK) and mixed by inverting the tube. The mixture was added to well 1 of the incubation tray (quadriPERM Cell Culture Vessel) supplied in the kit. The microarray slide was washed briefly by dipping in PBS before it was immersed into labelled samples in well 1 of the quadriPERM Cell Culture Vessel. The incubation tray was covered with its lid and protected from exposure to light by covering with aluminium foil. Tray was incubated for 30 minutes at room temperature on an orbital shaker (shaking with a frequency of ~30 rpm).

During this 30 minute period, wash buffer was prepared from Phosphate Buffered Saline, pH 7.4, with Tween 20 (Catalog # P3563, Sigma-Aldrich UK). Wash buffer solution was filtered through a 0.45 µm filter. Five ml of Washing

Buffer was added to wells 2, 3 and 4 of the vessel and the microarray slide was subsequently washed on an orbital shaker for 5 minutes in each well. Washing Buffer was removed from well 4 and 5ml of distilled water was added for a final wash for 2 minutes. The microarray slide was removed from incubation tray and allowed to air-dry completely for30 minutes in the dark.

2.4.8 Scanning microarray slide

The microarray slides were scanned as soon as they dried using a GenePix Personal 4100A Microarray Scanner (Axon Instruments) and Genepix Pro software. The scanner used 532 nm and 635 nm lasers for the excitation of Cy3 and Cy5 dyes, respectively. The laser photomultiplier tube (PMT) gains were manually set for each dye to minimise saturated spots (which appear white) and minimise the background. The pixel size was set to 5 µM which is best for spots less than 100 µM in diameter. After the preview scan, histogram was reviewed for the count ratio. Ideal count ratio is 1 which indicates an equal visibility of each Cy dye. The count ratio of between 0.82 and 1.18 are acceptable. Once optimised with above settings and satisfactory preview image, region of interest was highlighted for high resolution scanning. The resultant scan was then saved as a TIFF file.

2.4.9 Analysis

Primary analyses were performed with the GenePix Pro software. The antibody location grid file ".GAL" file was used to apply the spot location map. Spot location grid was positioned over the detectable region of the image and the

software was allowed to automatically align the map to the image, before all spots were manually checked for alignment. Any features which were not correctly aligned/sized, were altered manually using the feature manipulation tool.

The images of scanned microarrays were linked to the protein print array list. Results files were generated, which included feature name, individual dye intensities at each feature and the dye ratio (635/532) at each feature. Negative controls were flagged and results saved as ".GPR" file and saved to Acuity software.

The images were analysed using Acuity (version 4.0) software for the identification of differentially expressed proteins. Antibody microarrays were normalised on the basis of the Lowess method which moves the data into normal distribution. In order to correct for the quality of the microarray experiment data was initially filtered based upon physical qualities such as the presence or absence of features or the signal to noise ratio. This was done by running a wizard in Acuity software, which undertakes a complicated selection based upon signal to noise and background fluorescence etc. Quality control was applied to the spots, and only spots with the following features were included:

- Spots with only small percentage of saturated pixels.
- Spots that were not flagged bad, not found or absent.
- Spots with relatively uniform intensity and uniform background.

- Spots that were detectable above background.

The evaluation page of query wizard reported the percentage of features that matched the query. A substance match value was produced and a minimum substance match value of 90% was the threshold set for valid results (Hodgkinson, ElFadl et al. 2011).

The initial dataset produced log ratios for protein expression. In order to convert this log ratio into a workable number following formula was applied to detect a fold change in differential expression of proteins:

$y = \log_2 x$

Therefore $x = 2^y$

Proteins demonstrating a ≥ 1.8-fold change in differential expression between PN and RR were deemed significant (Hodgkinson, ElFadl et al. 2011). A protein with expression fold change of ≥ 1.5 may be taken into consideration when such differential expression was complemented by significant change (≥ 1.8-fold) in the other cell line (Hodgkinson, ElFadl et al. 2011). Figure 2.5 is a schematic diagram of the final image of array obtained showing different types of spots corresponding to type of fluorescence obtained. It also gives the interpretation of different spots as follows:

1. Blank / black spot: Negative control
2. Yellow spot: Either positive control (cy3/5 dye) or equal amount of a protein in test and control samples
3. Red spot: Protein over-expressed in test sample
4. Green spot: Protein under-expressed in test sample

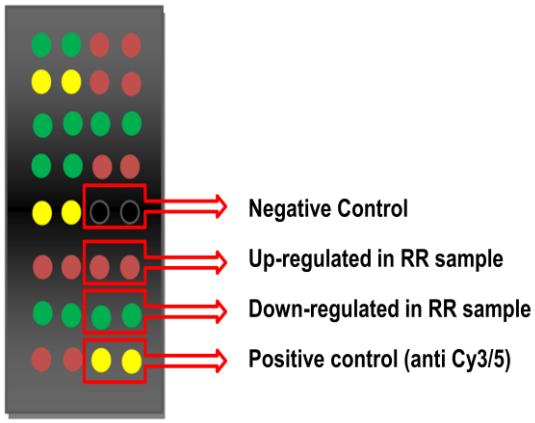

Figure 2.5: Interpretation of spots on antibody microarray slide. The slide contains 1536 spots consisting of 725 antibodies (spotted in duplicate) with positive and negative controls. Four different types of spots are illustrated with their interpretation.

2.5 Biomarker Validation

Biomarkers discovered with screening proteomics were taken to validation phase of biomarkers discovery pipeline using immunohistochemistry on archival series of pre-treatment rectal cancer specimens obtained endoscopically at Castle Hill Hospital. Local research ethics committee approval for the use of archival rectal cancer tissues was obtained (Reference 09/00/181 Hull and East Riding Research Ethics Committee).

2.5.1 Definition of radiotherapy response

Differential expression of biomarkers by IHC on rectal cancer tissue was assessed by examining the expression difference between two groups of patients as per the tumour response to RT. For the purpose of assessing response to RT in this study, tumour regression grading system was followed (section 1.7.4.1).

2.5.2 Tumour regression grading

Tumour regression grading is based on the degree of pathological response to radiotherapy which is assessed on surgical resection specimen, performed 6 weeks post long course CRT (section 1.7.2.1). This grading system has been validated and is being followed by the colorectal cancer multidisciplinary team at the Castle Hill Hospital. TRG is based on the histological examination of post radiotherapy resection specimen. Prior to November 2009, a three point TRG grading system and after that Mandard's five grading system was in use by the pathologists at this hospital (Table 2.5) (Mandard, Dalibard et al. 1994; Dhadda, Zaitoun et al. 2009).

Table 2.5: Tumour regression grading systems. Different TRG systems in use by the colorectal multi-disciplinary team over the study period. Note that TRG 1 on 3-point grading system represented as wide spectrum of response to RT and therefore these tumours were excluded from the current study.

Mandard's 5-point TRG system		**3-point TRG system**	
Grade	**Description**	**Grade**	**Description**
TRG 1	Complete response with absence of residual cancer and fibrosis extending through the wall	**TRG 2**	Complete pathological response
TRG 2	Presence of residual tumour cells scattered through the fibrosis	**TRG 1**	Intermediate tumour regression
TRG 3	Increase in the number of residual cancer cells, with fibrosis predominant		
TRG 4	Residual cancer outgrowing fibrosis		
TRG 5	Absence of regressive changes	**TRG 0**	No tumour regression

Grade-2 of three grading system was deemed equivalent to Mandard's TRG 1 and considered to be responder, whereas grade-0 was deemed equivalent to Mandard's TRG-5 and thus considered non-responder. Grade-1 represented a

wide degree of response (equivalent to Mandard's TRG 2, 3, 4) and these patients were not suitable for response assessment in current study (personal communication: Dr AW MacDonald, senior colorectal pathologist, Castle Hill Hospital, November 2010).

2.5.3 Case selection for Immunohistochemistry

A cohort of patients who received LCCRT for rectal cancer was identified and categorised into Good-Response and Poor-Response groups based on the tumour histological response to RT as assessed by the TRG systems (Table 2.6, Section 2.5.2). The histology reports of cases assessed by 3-point TRG system were reviewed prior to their inclusion. The selection criteria and group dynamics are further discussed in chapter 5.

Table 2.6: Classification of response to radiotherapy - Study groups: Tumour regression post radiotherapy in rectal cancer is based on TRG. For IHC analyses, two groups of patients were defined - good responders included complete or near complete histological regression and the poor responders included minimal or absent regression.

Good-Response group	**Poor-Response group**
Mandard TRG: 1, 2	Mandard TRG: 3, 4, 5
3-point TRG grade: 2	3-point TRG grade: 0

2.5.4 Immunohistochemistry

Immunohistochemistry (IHC) is essentially a histological method for localising specific antigens in tissues or cells based on antigen-antibody recognition in archival formalin fixed, paraffin embedded (FFPE) tissues. It relies on the specificity provided by the binding of an antibody with its antigen at a light

microscopic level. IHC combines histological, immunological and biochemical techniques to identify discrete tissue components i.e. the molecular targets of interest. This is accomplished by the interaction of target antigens with specific antibodies tagged with a visible label. Cellular distribution and localisation of the target of interest can be mapped and compared between the two groups exposed to different conditions e.g. Control versus intervention (treatment) group.

2.5.4.1 Preparation of slides for Immunohistochemistry

Pre-treatment rectal cancer specimens of patients treated with LCCRT were obtained from the pathology lab at the Hull Royal Infirmary for these experiments. The specimens were FFPE archival tissue blocks. Three 4 μm sections per case were cut onto Superfrost Plus microscope slides. Slides were allowed to air dry overnight at 37^0C. The slides were pre-labelled in pencil with the corresponding identification number and the antibody (with its dilution factor) to be tested.

2.5.4.2 De-waxing and Rehydration

The slides were arranged in a metal slide rack for further treatment. The slides were dewaxed using a pre-warmed (50^0C) solution of Histo-clear™ (National Diagnostics, UK) for 10 minutes. Slides were then briefly dipped (for 10 seconds - 5 or 6 dips) successively into another two pots containing cold Histo-clear™. Following that, the slides were re-hydrated by dipping for 10 seconds each in three solutions of 100% ethanol and then rinsed for one minute under running tap water. The aim of this step was to rehydrate the cells to a state as near as

possible to their in vivo condition. The endogenous peroxidase of red blood cells was blocked by placing the slides in 400ml of methanol to which 8ml of 30% (w/v) hydrogen peroxide was added. The slides were left in this solution for 20 minutes before rinsing under running tap water for 1 minute.

2.5.4.3 Antigen site retrieval

The retrieval buffer was boiled in a pressure cooker. Approximately 1500 ml of double distilled water was added to a pressure cooker and mixed with 15ml of Antigen Unmasking Solution (1:100) (#H-3300, Vector UK). The solution was heated to boil. When boiling vigorously, the slides were added. Antigenic site retrieval by this procedure is accomplished by boiling under full pressure at 103 Kpa (Cawkwell, Gray et al. 1999). After three minutes the pressure cooker was cooled and the slides quickly transferred to Tris-Buffered Saline (TBS - 0.05 mol/l Tris HCL, 0.15mol/l NaCl, pH 7.6) to prevent any drying.

2.5.4.4 Prevention of nonspecific binding

The immunohistochemical reactions were performed using the Sequenza system. The cooled slides were assembled with cover plates and TBS solution, and placed in a Sequenza slide holder ensuring no air bubbles were formed. Each reservoir was filled with TBS solution and left for five minutes to prevent dehydration. One hundred µl (3 drops) pre-diluted blocking serum (normal horse serum) from Quick Kit (Vector #PK-7800) was added to the middle of each slide reservoir at the junction with cover-plate for 10 minutes to block any non-specific binding sites. The slides were rinsed in TBS for five minutes.

2.5.4.5 Antibody incubation

At this stage, 100 µl of diluted primary antibody was added (Section 5.2.2.1). The antibody was diluted in TBS containing dilute blocking serum. For this purpose, 1.5% dilution of pre-diluted blocking serum with TBS was made. One hundred µl of TBS containing dilute blocking serum was added to negative (control) slide. Therefore, the negative control had antibody omitted from it. Slides were covered and incubated at room temperature for 2 hours.

Following 2 hours incubation, slides were rinsed in TBS twice for 5 minutes. Hundred µl (3 drops) prediluted biotinylated pan-specific universal secondary antibody (Quick kit, Vector UK) was added, slides were covered and left for 20 minutes. Following that slides were rinsed in TBS for 5 minutes and 100 µl (3 drops) ready to use streptavidin/peroxidase complex reagent (from Quick Kit) was added for 10 minutes. Slides were rinsed in TBS for 5 minutes.

2.5.4.6 Slide staining, antibody detection and visualisation

The slides were removed from the Sequenza and replaced in the rack into a pot containing fresh TBS. A solution of 400ml of TBS, 3ml DAB (3,3'-diaminobenzidine tetrahydrochloride, catalog # D5905, Sigma-Aldrich UK) and 15 drops of hydrogen peroxide (30% w/v) was prepared and the slides immersed in it for 5 - 15 minutes (up to a maximum of 30 minutes). Slides were examined under microscope for appearance of brown colour. If left longer than 30 minutes the DAB would precipitate in clumps onto slides. As soon as sufficient brown colouration of tissues was obtained, the slides were rinsed in running tap water for 2 minutes to terminate the reaction. To enhance the

staining the slides were then immersed in a solution of 0.5% w/v copper sulphate solution for five minutes and then rinsed in running tap water for 30 seconds.

2.5.4.7 Counterstaining with Harris Haematoxylin

In order to counterstain cellular nuclei blue, Harris Haematoxylin stain was used. The slides were dipped in Harris Haematoxylin (#HHS32, Sigma Aldrich, UK) for 20 seconds and rinsed under tap water for 30 seconds. The haematoxylin stain was then differentiated by dipping the slides in acid alcohol ten times and rinsing again under running tap water for 30 seconds.

2.5.4.8 Mounting Slides

The slides were taken through dehydration stage prior to mounting with cover slips. The tissue sections were dehydrated by immersing for 10 seconds in each of three different pots containing 100% ethanol (industrial methylated spirit). The slides were then dipped in three changes of Histoclear II solution and then mounted by applying a cover slip with Histomount (#HS-103, National Diagnostics). The slides were then left overnight to air dry at room temperature.

2.5.4.9 Scoring

The slides were examined by an experienced observer (Lynn Cawkwell) independently for the staining intensity of primary antibody as per predefined criteria for that antibody. The observer was blinded to the group of cases the slide belonged to. Ideally, the author would have undertaken second scoring to

reduce the bias normally associated with one observer's findings. However, due to lack of training opportunities and lack of other trained staff in the lab who could perform the above examination, it was decided that staining scoring would be reported by Dr Lynn Cawkwell who is fully trained and highly experienced in IHC.

CHAPTER-3: Development of novel radioresistant rectal cancer cell lines

Aims:

- To determine inherent relative radiosensitivity of SW-837 and HRA-19 rectal adenocarcinoma cell lines
- To establish novel radioresistant rectal cancer cell sub-lines from parental SW-837 and HRA-19 cell lines using fractionated irradiation protocol
- To confirm significant radioresistance between SW-837 and HRA-19 cell lines and their novel radioresistant sub-lines by performing clonogenic assays

3.1 Introduction

The treatment options in rectal cancer are multimodal and as discussed in section 1.7.4, RT is an essential treatment modality for rectal cancer with proven efficacy. However, it is known that not all patients receiving RT derive therapeutic benefit. All tumours may not respond to RT in a similar and predictable way. In rectal cancer, the response to RT is variable with up to 55% of patients deriving good pathological response (Dhadda, Zaitoun et al. 2009). As discussed in section 1.9, it is currently not possible to reliably predict which tumours will respond favourably to RT. A recurrent cancer signifies the challenge of ineffective treatment due to tumour resistance. The biomarker studies hold promise to predict the biological behaviour of a tumour.

Cancer cell lines offer a useful resource for the study of biomarkers for a number of reasons. Cell lines are cost effective, easy to use, provide an unlimited supply of material and bypass ethical concerns associated with the use of patient tissue. They are a pure population of cells which is valuable since it provides a consistent sample and reproducible results. The cell lines are widely popular in academic and industry research and are being used in testing drug metabolism, antibody production, study of genes and proteins function, and study of treatment response amongst many others (Kaur and Dufour 2012). There are, however, certain limitations of cell lines that should be considered whilst planning in-vitro studies. It is understood that the cell lines might undergo phenotypic alterations to better adapt to growth in cultures. Cells may demonstrate altered expression of some proteins and in extreme circumstances might acquire a molecular phenotype quite different from the respective cells in-

vivo (Pan, Kumar et al. 2009; Kaur and Dufour 2012). Due to such limitations, the results from cell line analyses are subjected to targeted confirmatory studies.

In-vitro cell line models have been developed to study molecular characteristics altered in response to ionising radiation. Feng and colleagues established a radioresistant cell line CNE2-IR from parental cell line CNE2 by treating the cells with a total of 55 Gy radiation in five fractions (11 Gy a fraction) of sublethal ionising radiation (Feng, Yi et al. 2010). For the confirmation of radioresistant phenotype, clonogenic survival assays were performed (Feng, Yi et al. 2010). Similarly, Lin et al. (2010) developed an *in vitro* RR cell line model of head and neck cancer using two cell lines (OECM1 and KB). Two RR subclone cell lines were established after completion of fractionated irradiation to 60 Gy. The RR sublines were subjected to clonogenic survival assays to authenticate their radioresistance (Lin, Chang et al. 2010).

In this chapter, it is aimed to establish novel RR cell line model from rectal cancer cell lines by using a fractionated irradiation protocol. Such a RR model would then be used to investigate cellular mechanisms of radioresistance by studying the biomarker profile using proteomics techniques.

3.2 Materials and methods

3.2.1 Experimental model and colony counting assay

The protocol for cell culture, irradiation and clonogenic assay was followed as described in section 2.2. Briefly, rectal adenocarcinoma cell lines SW-837 and

HRA-19 were grown in their respective culture media. In order to determine inherent radiosensitivity of parental cell lines, the modified colony counting (clonogenic) assays were performed. One million cells were suspended in 5ml of their respective culture medium in a 7ml vial, suspended in a water filled phantom. The cells were treated with ionising radiation from a linear accelerator with single doses of 0, 2, 4, 6, 8 and 10 Gy. Post irradiation, the cells from each dose were seeded in 6-well plates at a density of 1000 cells per well. The cells were grown for 12 – 14 days before they were fixed, stained and colony counts were taken from the printed image taken by a digital SLR camera. A colony was defined as a cluster of 50 or more cells. The colony counts were performed by two independent observers. Inter-observer variability was tested by plotting the Bland-Altman plots. The surviving fractions were calculated from the platting efficiencies and dose response cures (DRC) were plotted. A total of 6-replicates were obtained from two independent experiments. Clonogenic assays were also performed on novel RR cell sub-lines after those were established and DRCs of PN and respective RR cells were compared.

3.2.2 Induction of radioresistance: Fractionated irradiation protocol

Once the inherent radiosensitivity of SW-837 and HRA-19 was established, a fractionated irradiation protocol was followed to induce radioresistance into parental cells, as described in section 2.2.5. Briefly, a sub-lethal dose per fraction (8 Gy for SW-837 cells and 4 Gy for HRA-19 cells) was selected from the respective DRCs. A fresh sample of each cell line reaching 70 – 80% confluence and containing ~ 1million cells was chosen for fractionated irradiation. After each fraction, cells were grown in tissue flask to 70 – 80%

confluence before the next fraction was given. Hence, the time interval between fractions was 1-2 weeks for SW-837 and 2-3 weeks for HRA-19 cells.

After the cells had received half the required total dose of radiation (24 Gy), they were passaged into two tissue flasks, one to continue to receive fractionated irradiation and the other was banked in -80^0 freezer as a back-up. The fractionated irradiation was aimed at delivering a final total RT dose of 48Gy in keeping with clinical LCCRT regimen. Once that dose was reached, irradiation was stopped and the final surviving cells were deemed radioresistant having survived the fractionated irradiation over a number of weeks. Figure 3.1 summarises the above protocol of establishment of radioresistant cell lines.

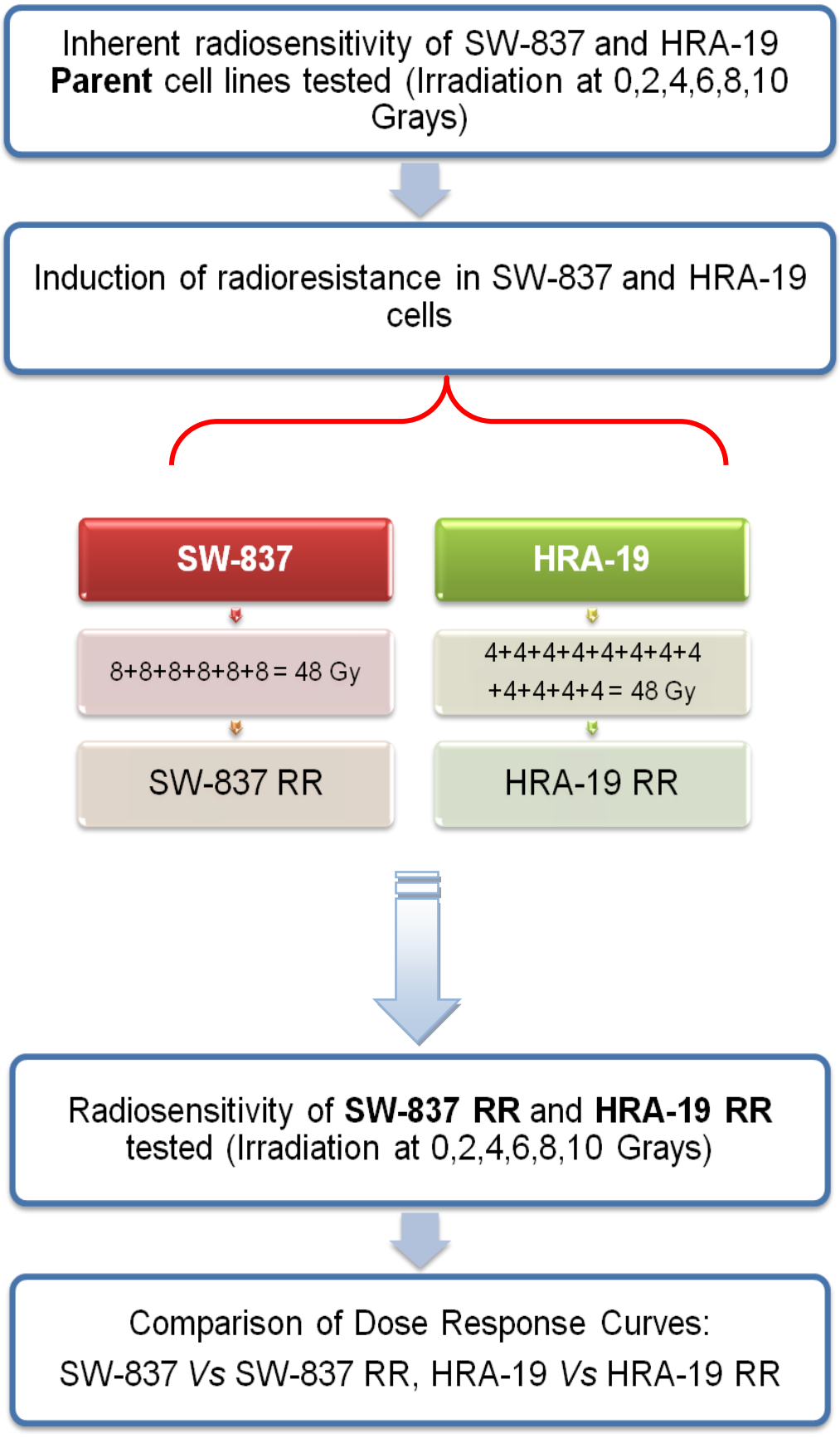

Figure 3.1: Protocol for the development of radioresistant cell lines

3.2.3 Comparison of radiosensitivities: PN versus RR cells

After the RR cell sub-lines were established, modified colony counting assays were performed on them as described above and DRCs were generated. The DRCs of RR cells were compared with those of respective PN cells. Fold changes in the surviving fractions between the PN and RR cells were calculated. Independent sample *student's t test* was used to compare the radio-sensitivities of PN and RR cells using SPSS v.17 for windows.

3.3 Results

3.3.1 Optimisation of the protocol of colony counting assay

The protocol for colony counting assay involved staining colonies with Eosin and capturing images of 6-well plates using densitometer. The images thus produced were in black & white owing to inability of the densitometer to capture colour images. It was noted that the resolution of the printed images was not sufficient enough for counting the smallest size colonies (a bunch of 50 cells) due to less pixels. Moreover, it was observed that there were hardly any surviving colonies of parental SW-837 cells at higher doses of radiation (10 Gy) under light microscope, whereas the densitometer captured image showed a few spots that would qualify as countable colonies. Careful microscopic evaluation suggested that such erroneous observations were likely due to artefacts (possibly dried water marks and non-stained airborne particles during colonies staining and drying stages) (Figure 3.2). All such particles appeared as dark spots, and looked very similar to the spots of colonies that could result in inaccurate colony count. To troubleshoot above difficulties, several adjustments to protocol were tried. As a direct solution, colony counting was attempted under microscopic examination. However, it was not possible to count colonies

under microscope due to large area of a well. Similar problem was encountered when a laser capture of the well was attempted. Staining with crystal violet improved the colour intensity. In order to obtain colour image, a 7-megapixel camera was used which successfully captured a colour image but did not have enough pixels to allow a quality print. Eventually, a high resolution 18 megapixel digital SLR camera with optical mega lens was used that produced the image exactly how it appeared under microscope. The images taken by SLR camera from several different wells were correlated with direct microscopic examination of corresponding wells and it was concluded that the SLR camera was able to exclude non-stained artefacts and produced high resolution colour image that was reliable for accurate colony counting (Figure 3.2).

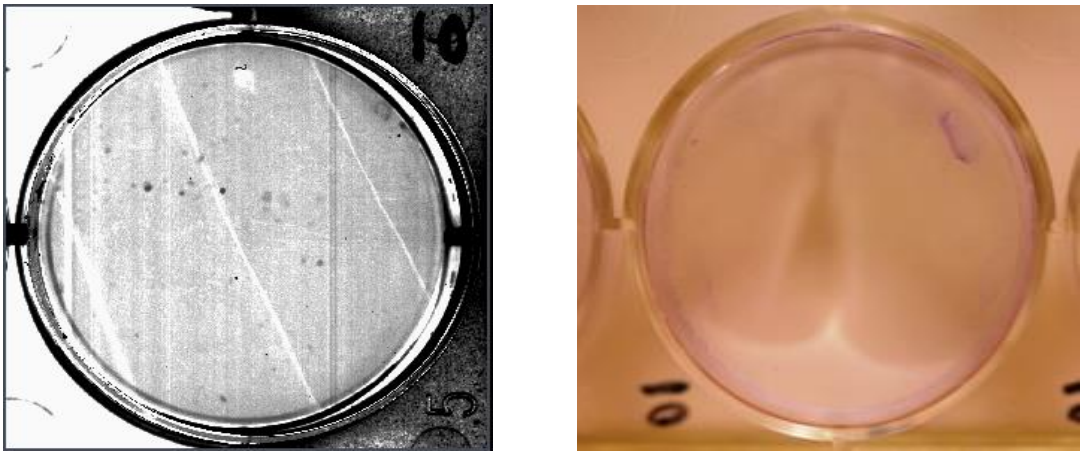

Figure 3.2: Comparison of Densitometer (Left) and SLR camera (Right) based images of a well from 6-well plates exposed to 10 Gy radiation. The image on the left showed artefacts including dust / airborne particles and water marks that appeared black & white making it difficult to distinguish those from true colonies. It showed a few spots that would be erroneously counted as colonies whereas at the same radiation dose no countable colonies were imaged by the SLR camera, both the observations were confirmed by direct microscopic examination.

3.3.2 Inherent radiosensitivity of parental SW-837 and HRA-19 cell line

Figure 3.3 shows a colony as it appeared in the print image of modified colony counting assay. When exposed to single doses of X-rays for modified colony counting assay, the SW-837 cells produced a dose dependent response in colony formation. As evident from data presented in table 3.1, there was a variable response to different doses of radiation. Of the 1000 cells plated and grown for 12 to 14 days, only less than half (446) cells retained the clonogenic ability to form colonies in control (untreated, 0 Gy) sample. Therefore the plating efficiency of untreated cells was 44.6. Upon treatment with radiation, only a quarter of cells produced colonies when a lowest dose (2Gy) was given.

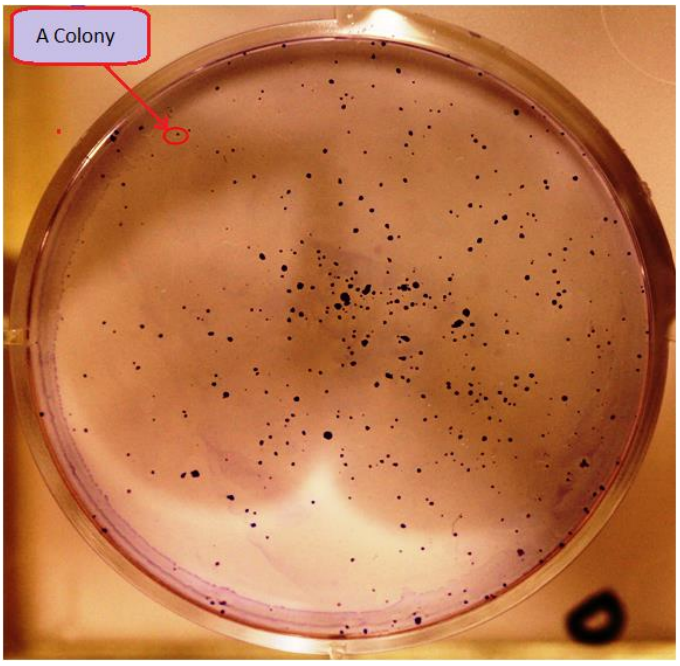

Figure 3.3: A colony of cells. A colony was defined as a cluster of cells consisting of a minimum of 50 cells under the light microscope (at 10x magnification). The colony was correlated with the print image that was taken as reference size for colony counting.

Similarly, the calculated surviving fraction demonstrated an inverse relationship to radiation dose. Figure 3.4 is a log-linear dose response curve showing SF plotted against the radiation dose. As the radiation dose was increased, a progressive decline in surviving fraction was observed at 2 to 10 Gy. It was noticed that most cells were killed by small doses of RT as appears from surviving fraction of 19% and 7% at RT dose of 4 and 6 Gy, respectively. At 8 Gy only 0.8% of cells demonstrated survival whereas almost all cells were killed at 10 Gy. Therefore it was considered that a radiotherapy dose of 8 GY represented the sub-lethal dose which was selected for induction of radioresistance in this cell line.

Table 3.1: SW-837 PN Modified colony counting assay. Mean of two experiments, six replicates. PE: Plating efficiency, SF: Surviving fraction

Rad Dose (Gy)	Colony counts (6-replicates at each dose)						Av. colony counts	PE	SF
	1st experiment			2nd experiment					
0	566	604	501	328	341	339	446	44.6	1
2	266	263	250	332	205	346	276	27.6	0.618
4	91	79	104	119	75	70	89	8.9	0.199
6	61	39	38	26	31	11	33	3.3	0.073
8	1	5	2	7	9	4	4	0.4	0.008
10	0	1	1	0	0	3	1	0.1	0.001

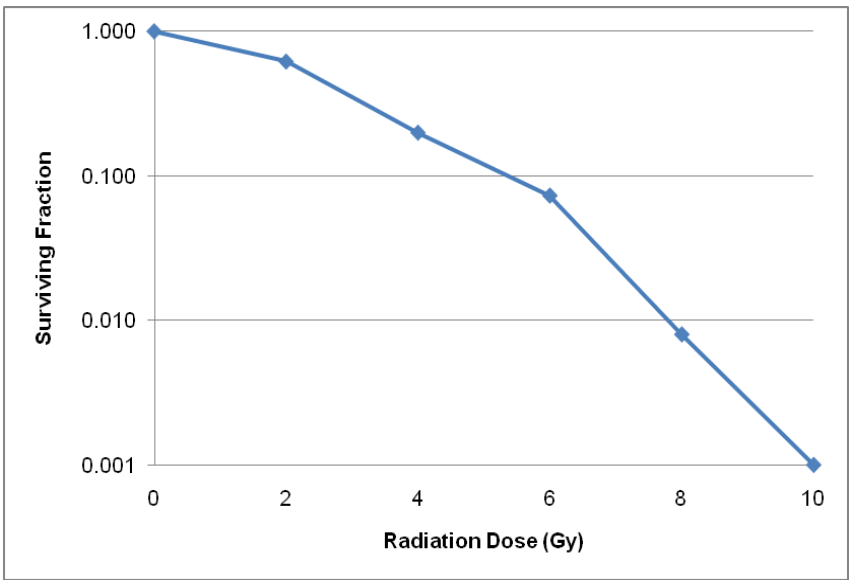

Figure 3.4: Dose response curve: SW-837 PN. Surviving fraction is plotted on a logarithmic Y-axis against the radiation dose on a linear X-axis. Each data point is a mean of 6 replicates from two independent experiments. The surviving fraction of SW-837 cells showed an inverse relationship with the dose of radiotherapy, which approached zero at dose of 10 Gy.

For HRA-19 cells, the modified colony counting assay revealed that of the 1000 cells plated per well, an average of 78 colonies were formed yielding a plating efficiency of 7.8. Similar to SW-837 cells, a dose dependent response to radiotherapy was observed with reduction in colony formation from doses 2 to 10 Gy. However, the HRA-19 cells were generally more sensitive to RT compared with SW-837 cells as evidenced in their reduced colony formation and plating efficiency. A substantial reduction in colony formation was observed at doses of 4, 6, 8 and 10 Gy with only 8, 2, 1 and 0 colonies formed, respectively (Table 3.2, Figure 3.5). In contrast to SW-837 cells, when the sub-lethal doses of 6 and 8 Gy were used for fractionated irradiation, all cells died. The highest sub-lethal dose at which some cells survived after irradiation was 4 Gy which was chosen for fractionated irradiation. In general, HRA-19 cell line demonstrated characteristics different from SW-837. HRA-19 cells were noted

to be generally slow grower and displayed substantially lower plating efficiency compared with SW-837 cells.

Table 3.2: HRA-19 PN Modified colony counting assay: Mean of two experiments, six replicates. PE: Plating efficiency, SF: Surviving fraction.

Rad Dose (Gy)	Colony counts (6-replicates at each dose)						Av. colony counts	PE	SF
	1st experiment			2nd experiment					
0	54	64	53	95	96	104	78	7.8	1.000
2	35	29	27	51	42	46	38	3.9	0.516
4	5	9	12	10	2	7	8	0.8	0.105
6	5	3	3	0	1	1	2	0.2	0.034
8	2	2	2	0	1	0	1	0.1	0.017
10	0	0	0	0	0	0	0	0.0	0.000

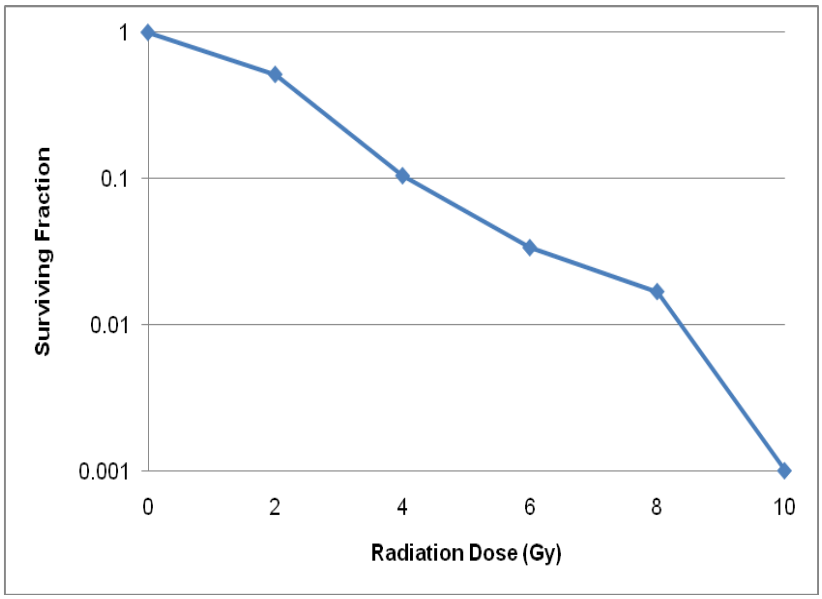

Figure 3.5: Dose response curve: HRA-19 PN. Each data point is a mean of 6 replicates from two independent experiments. The surviving fraction reduced as the radiation dose was incremented. No survival was demonstrated at 10 Gy dose.

3.3.3 Establishment of radioresistant (RR) cell lines

A sample each of SW-837 and HRA-19 cells received fractionated radiation to a final total dose of 48 Gy in 8 Gy and 4 Gy fractions, respectively. The total intended dose of 48 Gy was completed over 3 and 6 months, respectively. The novel RR derivatives assumed slightly different morphology with accelerated growth pattern compared with their PN counterparts. Figure 3.6 provides morphological appearances of one of the RR cell sub-line compared with its PN cell line and figure 3.7 compares the colony formation of the same cell line. As can be seen from figure 3.7, there were more colony formation for RR cells at each dose of RT compared with the PN cells.

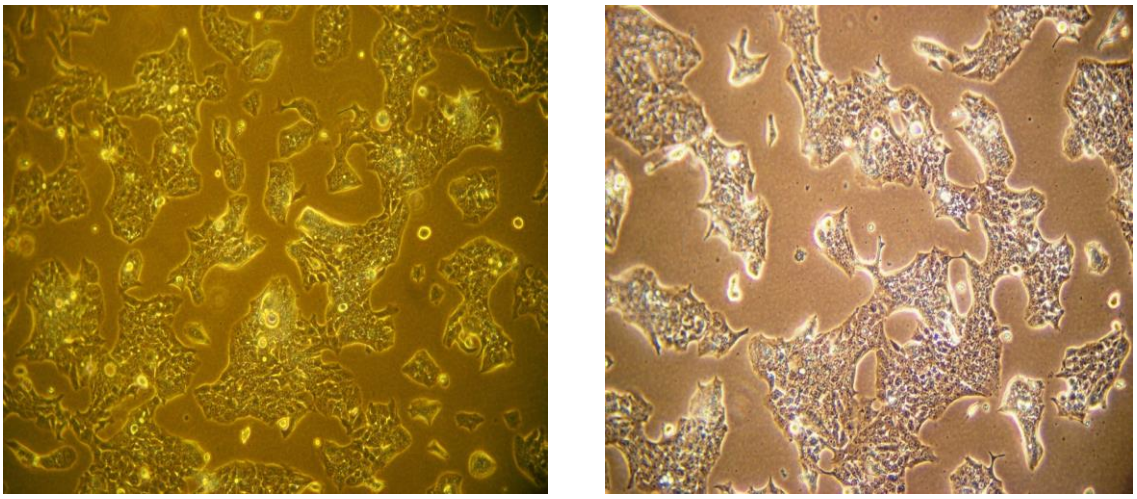

Figure 3.6: Microscopic appearance of PN & RR cells. A light microscopic photograph of SW-837 PN (LEFT) and SW-837 RR cells (RIGHT) at 10x magnification. The cells grow in clusters and are adherent to container wall. The microscopic appearance of RR cells assumed slightly appreciable difference in morphology in becoming smaller, more spindle shaped cells.

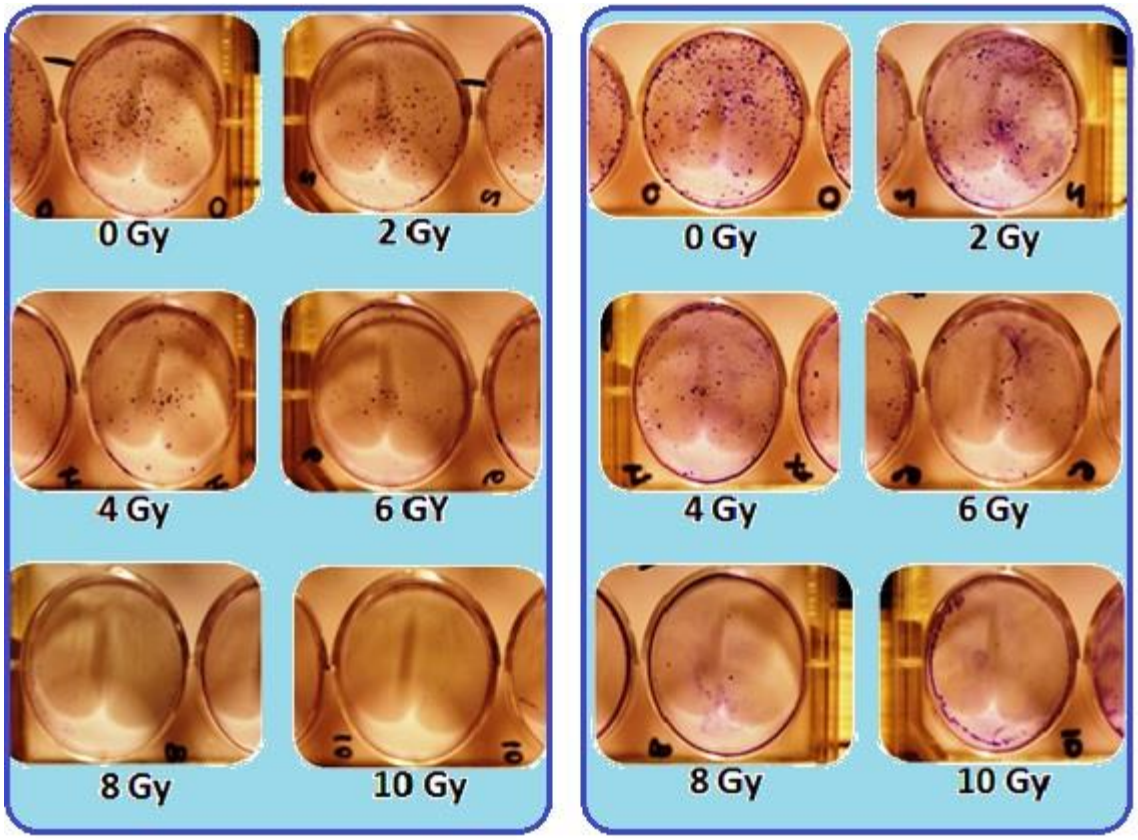

Figure 3.7: Modified colony counting assay: LEFT - PN cells, RIGHT - RR cells. Photographs of SW-837 PN versus SW-837 RR at different radiation doses. Cells were fixed with methanol:acetic acid solution and stained with 0.005% crystal violet. These high resolution images were taken with an 18 megapixel digital SLR camera with optical mega lens. Note the difference in number of colonies formed at each dose of RT between PN and RR cells.

3.3.4 Radioresistance of SW-837 RR and HRA-19 RR sub-lines

For SW-837 RR cells, the modified colony counting assay showed that nearly half of control cells seeded into 6-well plates gave rise to colony formation, the count ranged between 402 and 629 across six replicates (Tables 3.3). The RR cells displayed improved plating efficiency (49.3%) which might be due to its enhanced ability to withstand various environmental stresses owing to presumed radioresistant phenotype. The DRC of SW-837 RR cells demonstrated better survival compared with their PN counterparts at each dose of RT (Figure 3.8). Although a progressive decline in survival was observed with

increasing radiotherapy dose, a significant survival (3.4%) was observed at 10 Gy confirming that higher dose of RT might be required to achieve total killing of the novel RR sub-line.

Table 3.3: SW-837 RR Modified colony counting assay. PE: Plating efficiency, SF: Surviving fraction

Rad Dose (Gy)	Colony counts (6-replicates at each dose)						Av. colony counts	PE	SF
	1st experiment			2nd experiment					
0	413	402	482	442	629	592	493	49.3	1
2	313	329	336	310	340	322	324	32.4	0.657
4	182	167	174	148	170	138	162	16.2	0.328
6	76	50	60	94	96	78	75	7.5	0.152
8	21	23	22	30	22	45	27	2.7	0.054
10	12	10	11	20	17	25	16	1.6	0.034

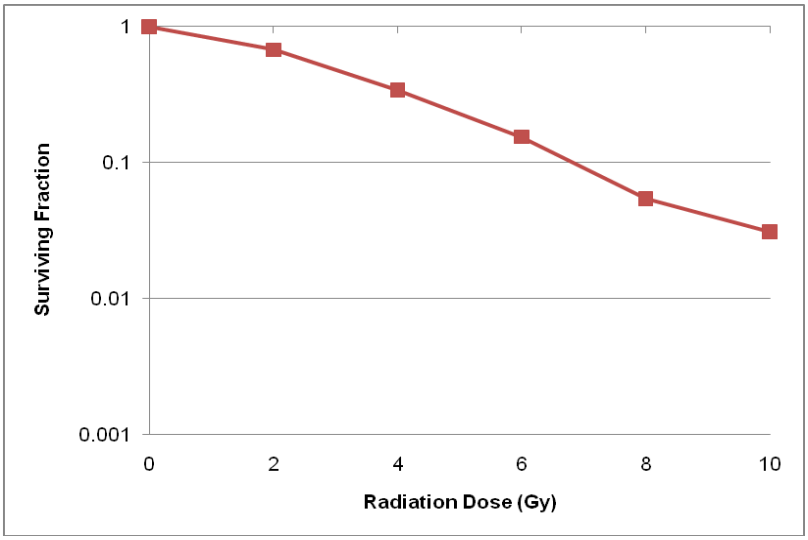

Figure 3.8: Dose response curve: SW-837 RR. Each data point is a mean of 6 replicates from two independent experiments. The SW-837 RR cells demonstrated better survival as compared with their PN counterparts. Although a progressive decline in survival was observed with increasing radiotherapy dose, 3.4% cells survived at 10 Gy.

Likewise, the HRA-19 RR cells demonstrated significantly better clonogenic ability (Tables 3.4). Control cells demonstrated an improved plating efficiency of 11 compared with PN cells and retained some colony formation at the highest dose (10 Gy) of X-rays. The DRC of these cells showed less pronounced decline in surviving fractions to incremental dose of radiotherapy (Figure 3.9). The surviving fraction at 10 Gy was 5.9% compared to zero survival of PN cells.

Table 3.4: HRA-19 RR Modified colony counting assay

Rad Dose (Gy)	Colony counts (6-replicates at each dose)						Av. colony counts	PE	SF
	1st experiment			2nd experiment					
0	64	51	54	168	173	152	110	11	1
2	27	31	32	109	123	105	71	7.1	0.612
4	21	18	11	54	47	44	33	3.3	0.294
6	5	10	8	18	23	13	13	1.3	0.124
8	4	5	7	12	12	8	8	0.8	0.08
10	1	10	3	6	3	6	5	0.5	0.059

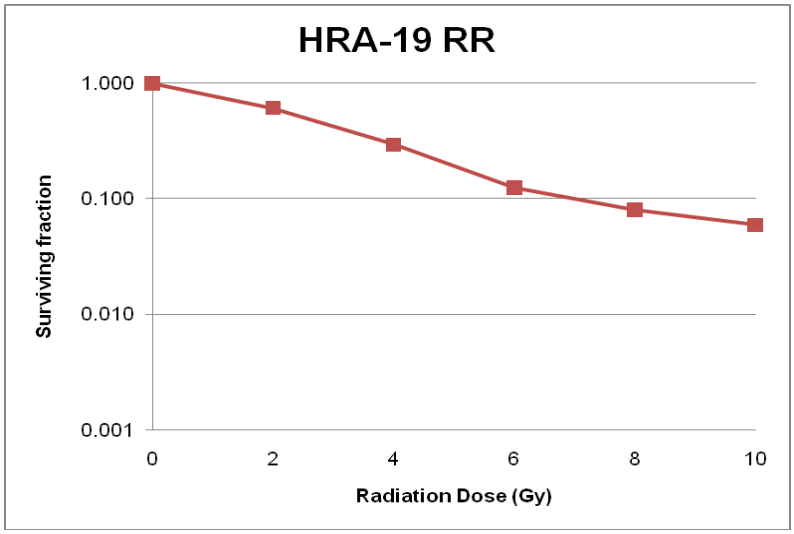

Figure. 3.9: Dose response curve: HRA-19 RR. Each data point is a mean of 6 replicates from two independent experiments. The HRA-19 RR cells demonstrated better survival as compared with their PN counterparts. Although a progressive decline in survival was observed with increasing radiotherapy dose, 5.9% cells survived at 10 Gy.

3.3.5 Bland-Altman plots for inter-observer variability

The Bland-Altman plot represents the limits of agreement between two observers for colony counts. The difference of the paired two measurements (Y-axis) is plotted against the mean of the two measurements. 95% of the data points lying within the ± 2sd of the mean difference provide 95% limits (strong) agreement between the two measurements.

The Bland-Altman plot for SW-837 PN and SW-837 RR cells showed that the bias (the deviation of average difference between the two observers' count from line of no difference) between the two observers was low (-0.7 and 5.0 respectively). More than 95% of values were within the 95% limits of agreement (Figures 3.10). Therefore there was strong agreement in colony counts of two observers.

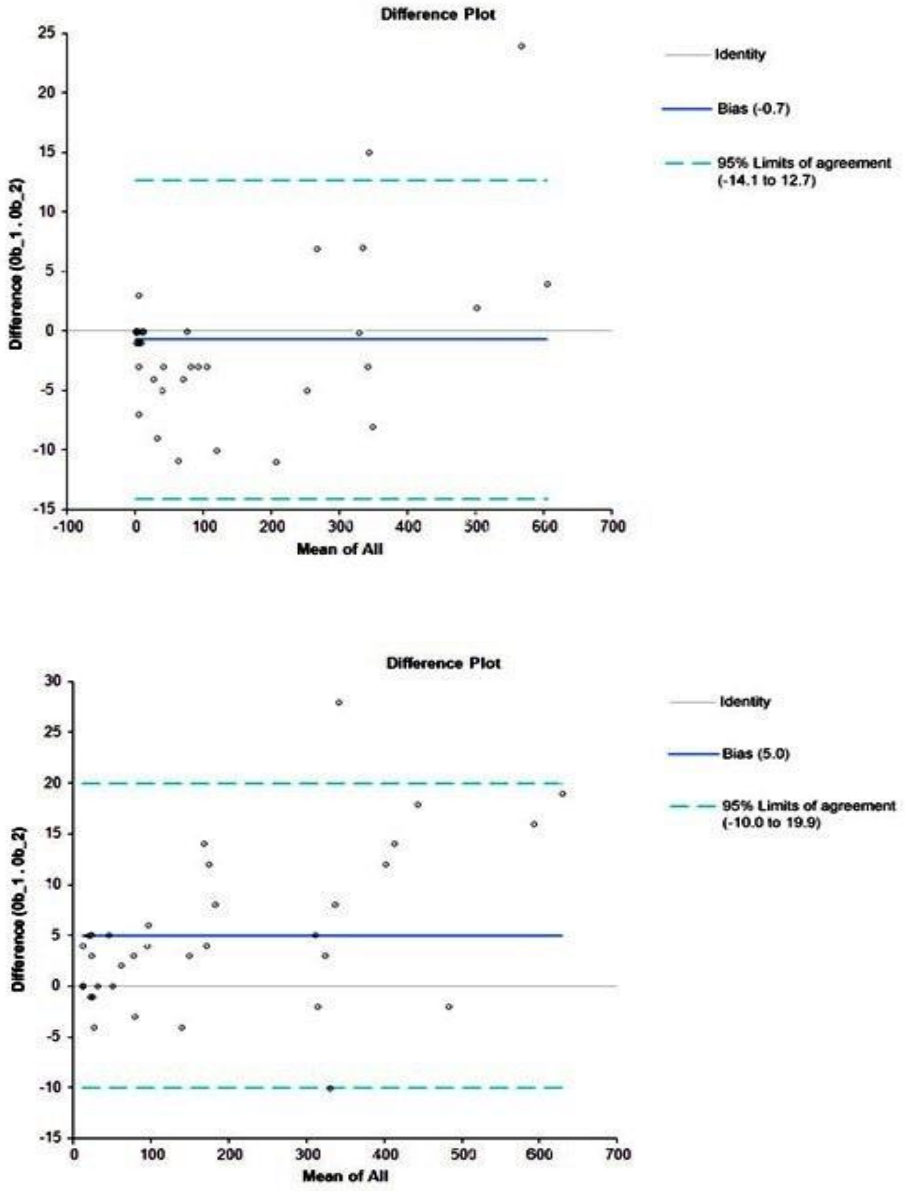

Figure 3.10: Bland-Altman Plots for colony counts of SW-837. Above: SW-837 PN. Below: SW-837 RR. The plots showed that the bias between the two observers was low (-0.7 and 5.0 respectively). More than 95% of values were within the 95% limits of agreement

Similarly, the Bland-Altman plot revealed low bias between the two observers -0.4 and 0.6 for HRA-19 PN and RR cells respectively. Only two of the 36 (5%) values for HRA-19 PN cells and 3 out of 36 (8%) values for HRA-19 RR cells were outside 95% limits of agreement (Figures 3.11).

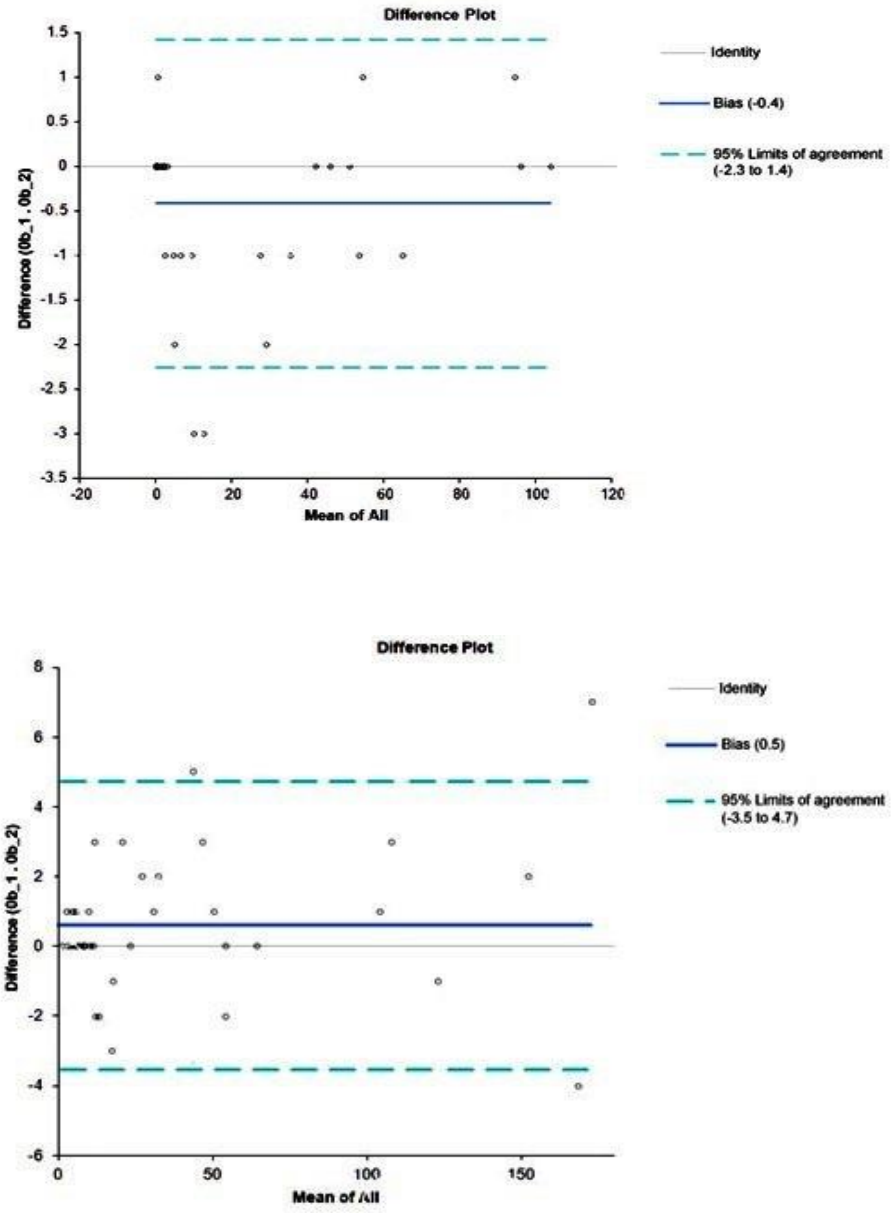

Figure. 3.11: Bland-Altman Plots for colony counts of HRA-19. Above: HRA-19 PN, Below: HRA-19 RR. The plots showed that the bias between the two observers was low (-0.4 and 0.5 respectively).

3.3.6 Comparison of dose response curves: PN Vs RR

3.3.6.1 DRC: SW-837 PN Vs SW-837 RR

To confirm the induction of radioresistance, DRCs of PN and RR cell lines were compared. Table 3.5 shows the modified colony counting assays of SW-837 PN cells versus RR cells. Whereas almost all parental cells were killed at 10 Gy,

formation of a few colonies by RR cells was observed at this dose. The plating efficiency of control cells in RR cells improved to 49.3 compared with 44.6 in PN cells. RR cells demonstrated statistically significant greater survival at radiation doses 4 (p=0.02), 6 (p=0.001), 8 (p=<0.001) and 10 Gy (p=<0.001). At 10 Gy, RR cells demonstrated 31 fold greater survival compared to PN cells (Figure 3.12).

Table. 3.5: Modified colony counting assays: SW-837 PN *Vs* SW-837 RR

Rad. Dose (Gy)	**SW-837 PN**			**SW-837 RR**			**Fold change in SF SW-837 PN *Vs* SW-837 RR**	**P value (*Student's t-test*)**
	Col	**PE**	**SF**	**Col**	**PE**	**SF**		
0	446	44.6	1	493	49.3	1	--	1
2	276	27.6	0.618	324	32.4	0.657	1.09	0.97
4	89	8.9	0.199	162	16.2	0.328	1.71	**0.02**
6	33	3.3	0.073	75	7.5	0.152	2.1	**0.001**
8	4	0.4	0.008	27	2.7	0.054	6.75	**<0.001**
10	1	0.1	0.001	16	1.6	0.034	31	**<0.001**

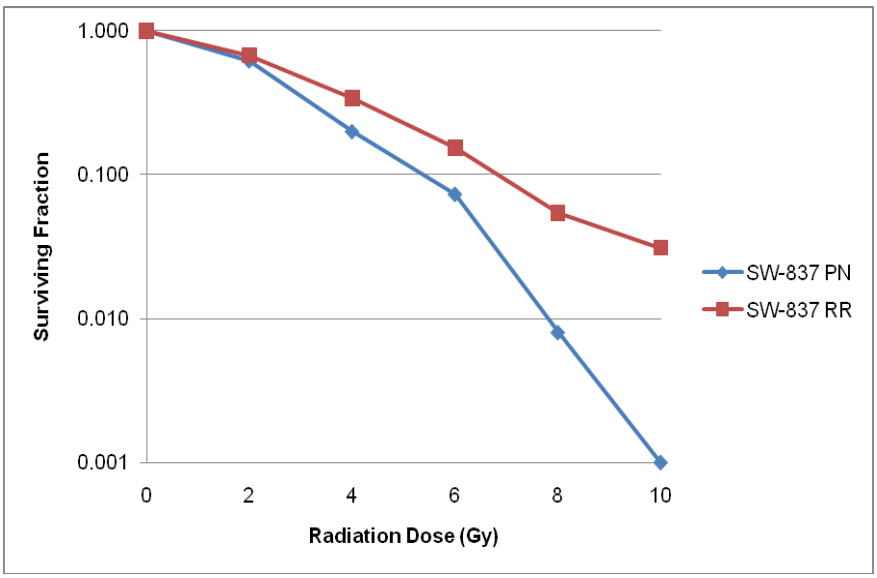

Figure 3.12: Comparative Dose response curve: SW-837 PN *Vs* SW-837 RR. Each data point is a mean of 6 replicates from two independent experiments. The SW-837 RR cells demonstrated better survival as compared with their PN counterparts with a statistically significant survival at 4,6, 8 and 10 Gy doses.

3.3.6.2 DRC: HRA-19 PN Vs HRA-19 RR

HRA-19 cell line was observed to have relatively low plating efficiency compared with SW-837 cell line, however, this improved from 7.8 to 11.0 post induction of radioresistance (Table 3.6). RR cells retained some survival and clonogenic properties at 10 Gy in contrast to PN cells. RR cells demonstrated statistically significant greater survival at radiation doses 4 (p=<0.001), 6 (p=0.003), and 8 Gy (p=<0.002). However, at 10 Gy, significance could not be checked with a statistical test as SF of HRA-19 PN was 0. Nevertheless, RR cells demonstrated highest fold change in SF (59.3 fold). Figure 3.13 compares the DRC of HRA-19 PN versus HRA-18 RR.

Table 3.6: Modified colony counting assays: HRA-19 PN *Vs* HRA-19 RR

Rad. Dose (Gy)	HRA-19 PN			HRA-19 RR			Fold change in SF: HRA-19 PN *Vs* HRA-19 RR	P value (*Student's t-test*) (a=Incalculable)
	Col	PE	SF	Col	PE	SF		
0	78	7.8	1.000	110	11.0	1.000	1	1
2	38	3.9	0.516	71	7.1	0.612	1.2	0.1
4	8	0.8	0.105	33	3.3	0.294	2.8	**<0.001**
6	2	0.2	0.034	13	1.3	0.124	3.7	**0.003**
8	1	0.1	0.017	8	0.8	0.080	4.8	**0.002**
10	0	0.0	0.000	5	0.5	0.059	59.3	A

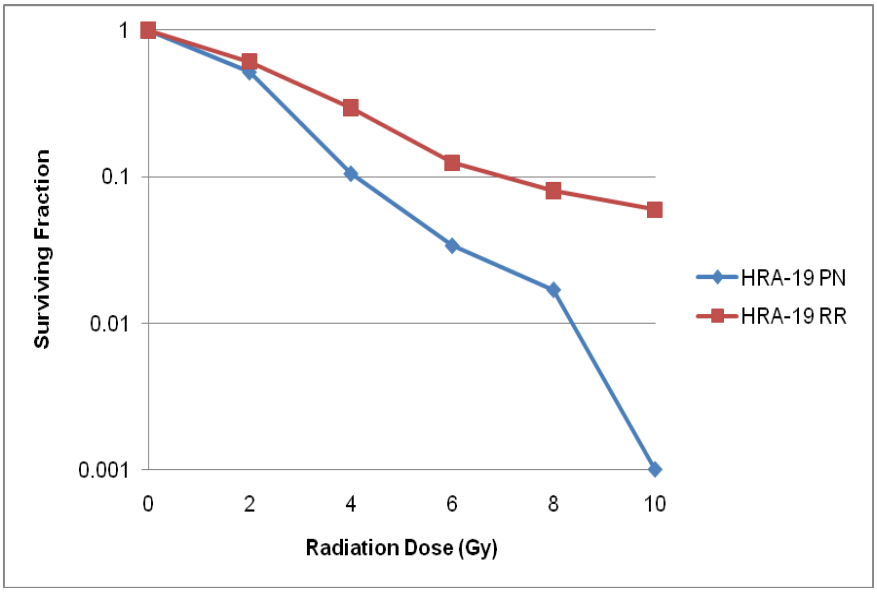

Figure. 3.13: Dose Response Curve: HRA-19 PN Vs HRA-19 RR. Each data point is a mean of 6 replicates from two independent experiments. The HRA-19 RR cells demonstrated better survival as compared with their PN counterparts with a statistically significant survival at 4, 6, and 8 Gy doses.

3.3.6.3 Final dose response curves of radioresistant cell line models

A final plot of dose response of both PN and RR cell lines is given in figure 3.14. The DRCs confirmed that each RR cells displayed better survival at each dose of radiotherapy compared with their PN counterparts. There was statistically significant survival difference between SW-837 PN and RR cells at 4, 6, 8 and 10 Gy and between HRa-19 PN and RR cells at radiation doses 4, 6 and 8 Gy ($p < 0.05$). The DRCs showed that both RR sub-lines retained some clonogenic ability and hence survival at highest dose (10 Gy) of radiotherapy, with up to 31 fold and 59.3 fold better survival in SW-837 RR versus SW-837 PN and HRA-19 RR versus HRA-19 PN, respectively.

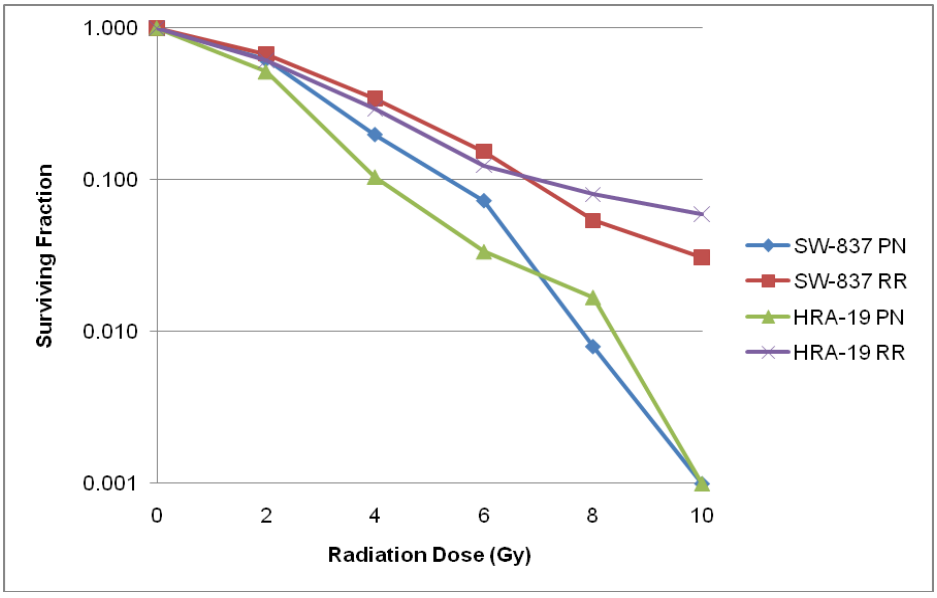

Figure. 3.14: The final Dose Response Curve: Parental (PN) Vs Radioresistant (RR). The comparative radiation response of PN and novel RR cells on the log-linear curve with SF plotted on Y-axis (logarithmic axis) against radiation dose on X-axis (linear). The curves show progressive decline in SF with the incremental radiation dose. However, the RR curves demonstrate better survival compared to the respective PN curves. The statistical analysis confirmed SW-837RR cells to be significantly more radioresistant at 4, 6, 8 and 10 Gy and HRA-19RR at 4, 6 and 8 Gy single doses than their respective parental cells ($p<0.05$).

3.4 Discussion

Interest in the study of cellular behaviour and mechanisms of response to radiotherapy has resulted in the development of cell line models of different cancer types. Such models are usually developed from established cell lines and provide a useful resource for experimental studies to investigate biological pathways involved in the development and treatment of cancer (Chiu, Hsaio et al. 2010). We aimed to establish such an in-vitro model as a means to study molecular determinants of radiotherapy response in rectal cancer.

The cell lines in this experimental model were carefully selected to represent a typical rectal cancer that in most cases would be MMR proficient, and p53 mutant. It was observed that the two cell lines showed different growth characteristics when seeded in 6-well plates. A substantial difference in the PEs of the untreated parental cell lines was observed (SW-837 v HRA-19, 44.6 % v 7.8 %). Such growth characteristics might highlight difference in their molecular factors. The PE of SW-837 was comparable with the published PEs of three colorectal cell lines HT29, HCT8 and HRT18 ranging between 35% to 76% (Fertil, Deschavanne et al. 1980). There is no published data to explain low plating efficiency of HRA-19. However, it was noted that that even lower PEs have been reported for a number of cancer cell lines. Park and colleagues have reported PE of rectal cancer NCI-H630 cell line in the order of 3.5% (Park and Gazdar 1996). Similarly, PE of 8% and 7.7% in MCF-7 and T-47D breast cancer cell lines, respectively, and 1.1% in A427 lung cancer cell line have been described (Ware, Zhou et al. 2007). It is known that PE of continuous cells seldom drops below 10%, but for primary cultures and finite cell lines, it may be as low as 0.5% to 5%, or even zero (Sergey Federoff 1996; Freshney 2005;

Support_ATCC 2011). It is agreed that PE of up to 10% are acceptable (Freshney 2005). Some of the known determinants of plating efficiency include seeding density, optimum culture conditions (temperature, humidity and nutritional elements), and culturing vessels (Hug, Haynes et al. 1984; Von Hoff, Forseth et al. 1986; Support_ATCC 2011).

In our experiments, cells were plated in 6 well plates at a seeding density of 1000 cells per well, whereas it is known that cultures in capillary tubes usually demonstrate up to 5-fold higher plating efficiency (Von Hoff, Forseth et al. 1986). The control, untreated cells (0 Gy dose) were also transported to oncology department, to accompany cells to be irradiated, so that control cells were exposed to same environmental conditions except irradiation. The cells were treated with trypsin for detachment prior to irradiation and then transferred into 6-well plates post irradiation. Others have reported plating 24 hours prior to irradiation because of the fact that plating takes 12-24 hours on an average (Feng, Yi et al. 2010). Plating post treatment in our experiments might have contributed to low PE of treated cells but was not expected to affect PE of untreated cells. However, the time outside incubator for transport to oncology department, and transfer back to incubator might have impacted the PE. The contributory factors likely to be involved include temperature and humidity fluctuations, and unavoidable shaking during transport.

Initially, inherent radiosensitivities of parental cells were determined by performing clonogenic assay. A marked reduction in the surviving fraction was observed when the cells were exposed to the lowest dose of 2 Gy (SW-837 =

0.61, HRA-19 =0.51). However, these observations were comparable with those recorded by Gao et al. in colorectal cancer cell lines that included SW-837 cell line (Gao, Saha et al. 2009), and in pancreatic cancer cell lines (Ogawa, Utsunomiya et al. 2006). It was an interesting observation that the radiosensitivities of both the parental cell lines were markedly different. Therefore, to induce radioresistance in cells, a radiation dose of 8 Gy was used in fractionated irradiation regimen for SW-837 and 4 Gy for HRA-19. These doses represented sub-lethal doses from their respective DRCs. The rationale behind that was to give the highest possible radiation dose at which only a few cells would survive to form colonies. A fractionated irradiation regimen with a sub-lethal dose would therefore induce radioresistant phenotype and has been experimented in several studies (Ogawa, Utsunomiya et al. 2006; Feng, Yi et al. 2010). We followed the most commonly accepted method of confirming radioresistant phenotypes by performing clonogenic assay and comparing the surviving fraction by plotting the DRCs (Ogawa, Utsunomiya et al. 2006; Feng, Yi et al. 2010). However, some investigators have also reported more direct evidence in the form of apoptosis assays (Ogawa, Utsunomiya et al. 2006).

In this study, it was hypothesised that a fractionated irradiation of cells with a sub-lethal radiotherapy dose would leave only a small number of surviving cells after each fraction that might be resistant to radiotherapy. On comparison of the DRCs of respective PN and RR cells, our results indicated significantly better survival for SW-837 RR cells at 4, 6, 8 and 10 Gy and HRA-19 RR at 4, 6 and 8 Gy single doses than their respective parental cells ($p<0.05$). The survival curves demonstrated better survival of up to 31 fold and 59.3 fold in SW-837 RR versus SW-837 PN and HRA-19 RR versus HRA-19 PN, respectively.

There was also a subjective observation that both the RR sublines demonstrated accelerated growth compared with their PN counterparts. In view of demonstrable survival difference, the SW-837 RR and HRA-19 RR were considered radioresistant derivatives of their PN counterparts. It is known that cells are most radiosensitive in G2/M phase, therefore, it could be argued that the observed difference in survival might merely be due to more number of PN cells in G2/M phase compared with RR cells when their radiosensitivities were tested by clonogenic survival assay. Whilst we did not undertake cell cycle assays to exclude such confounding factors, our observations compare favourably with those recorded by others who followed the similar protocols of inducing and checking radioresistance. Smith et al. followed the fractionated irradiation protocol of clinically relevant radiotherapy doses, based on sublethal doses from DRCs to establish RR breast cancer sub-lines. The RR novel derivatives (MCF7RR, MDA-MB-231RR, and T47DRR), along with their parental cell lines, were subjected to modified colony counting assay to prove emergence of radioresistance in much the same way as in our study. The comparison of the DRCs between the PN and novel RR cells demonstrated significant survival difference at 4, 6, 8 and 10 Gy for two cell lines and at 6, 8, and 10 Gy for the third cell line (Smith, Qutob et al. 2009). Our results were closely comparable to above reported by Smith et al. Similar results have been reported in cell line models developed for head & neck cancers (Feng, Yi et al. 2010; Lin, Chang et al. 2010). All of above studies reported differential expression of biomarkers between the parental cell lines and the novel RR derivatives to confirm the differential molecular profile of RR phenotypes.

In summary, we have successfully developed novel radioresistant cell sub-lines by treating parental cell lines with ionising radiation in a fractionated radiotherapy schedule. To our knowledge, this is the first lab model of radioresistant rectal cancer derived from human rectal cancer cell lines. There were demonstrable differences between the parental and the novel radioresistant cell lines as evidenced in better surviving fractions of the RR cells. This in-vitro rectal cancer RR cell line model was used for discovery of biomarkers of radioresistance using comparative proteomics approach of antibody microarray as described in chapter 4.

CHAPTER-4: Proteomic identification of putative biomarkers of radioresistance in radioresistant rectal cancer cell line model using antibody microarray

Aims:

- To use the power of antibody microarray to study the proteomic biomarkers
- To identify the differentially expressed proteins between the parental and radioresistant cell lines
- To identify a panel of common differentially expressed proteins related to radioresistance and to select potential markers for the validation phase of biomarker discovery pipeline

4.1 Introduction

One of the most exciting advancements in medicine to date is the discovery of human genome and the application of that knowledge to the benefit of the patients (Hocquette 2005). However, as discussed in section 1.10.1, not all genetic information is used by the cells. Therefore the true cellular functional environment is determined by the proteins that are the ultimate product of the genome and often regarded as the 'workhorses of biological systems' (Hong, Jiang et al. 2006).

Therefore, in this study, it was hypothesised that proteomics investigations might lead to the discovery of protein biomarkers associated with radioresistance. In chapter 3, establishment of an *in-vitro* radioresistant rectal cancer cell line model was described. The novel radiation treated cells displayed relative radioresistance compared with their parental cells providing us with RR sub-lines to analyse their proteome. The aim of this chapter was to investigate the proteins which were differentially expressed between parental radiosensitive and novel radioresistant cells. For this purpose antibody microarray based comparative proteomics platform was chosen, a high throughput proteomics technique as discussed in section 1.10.1.1.

A number of commercial antibody microarray kits are available with different sets of antibodies to suit the particular research requirements (see section 2.4.2). Panorama cell signalling kit consisting of 224 antibodies (Smith, Watson et al. 2006) and custom made antibody microarray chip (Sreekumar, Nyati et al. 2001) have been used to analyse expression levels of proteins associated with

chemotherapy resistance and radiotherapy resistance, respectively. The work presented in this chapter is based on antibody microarray analysis using Panorama® Antibody Microarray – XPRESS Profiler725 Kit.

4.2 Materials and methods

4.2.1 Cell lines

Proteomic analyses were performed on proteins extracts of SW-837 and HRA-19 rectal cancer lines and their novel radioresistant derivatives SW-837 RR and HRA-19 RR established in this study as described in chapter 3. All proteomic analyses were performed within a short time frame of the final DRCs to ensure that the RR cells did not have time to lose the RR phenotype.

4.2.2 Antibody microarray kit: Panorama XPRESS™ Profiler 725

Comparative AbMa experiments were performed using protein extracts of SW-837 PN and RR, and HRA-19 PN and RR cells using the Panorama – XPRESS^{TM} Profiler 725 kit. Two biological replicates were obtained, one from each cell line model. Further technical replicates would have been ideal, however, that was not feasible due to limitations in funds (cost of a kit = £1435). These experiments were performed in collaboration with Victoria Hodgkinson.

4.2.3 Antibody microarray protocol

The antibody microarray protocol was followed as described in section 2.4.2. Briefly, total protein extracts from the PN and RR cells were quantified using Bradford assay to obtain 1 mg/ml final concentration before and after labelling

with Cy3 and Cy5 dyes. The fluorescent labelling of PN and RR protein samples with Cy3 and Cy5 dyes, respectively, was performed in dark room. In each experiment, an equal amount of labelled PN and RR protein samples (90 µg) with D:P ratio ≥ 2 were co-incubated onto panorama XPRESS profiler 725 microarray slide (hybridisation) for 30 minutes before it was scanned and analysed as described in section 2.4.2. This protocol was previously optimised by my co-investigator (Victoria Hodgkinson) in breast cancer using 90 µg of protein extracts therefore no further optimisation was required (Hodgkinson, ELFadl et al. 2012). The experiments were considered successful when they passed minimum quality control (substance matching of ≥ 90%). Proteins demonstrating a ≥ 1.8-fold change in differential expression between PN and RR cells were considered significant (Hodgkinson, ElFadl et al. 2011).

4.3 Results

4.3.1 Optimisation and quality control

Analysis of AbMa experiment on SW-837 PN vs RR protein extracts revealed that only 83% (637/766) of substances on the array slide were matched – thus failing the quality control criteria. The most likely reason for that appeared to be excessive dye outside substances causing background signal to noise ratio, either due to problems with washing step or due to quality of array slide itself. This was fed back to the supplier of the kit, Sigma Aldrich, UK. Replacement of slide solved the problem and all subsequent experiments passed the quality control criteria of a minimum substance matching of 90%.

4.3.2 Proteomic analysis of SW-837 radioresistant cell subline

The microarray data was normalised by Lowess method. Of the 1536 features spotted on the antibody microarray slide, 1336 were matched. Of the 726 substances, 654 were matched passing the quality control criteria of 90% substances to be matched. GenePix Pro scanner produced two images using laser at 532 and 635 nm wavelengths, before producing the final image of 635/532 ratio, as a layered image. The images scanned at 532 nm and 635 nm detected the corresponding Cy 3 and Cy 5 labelled samples, respectively. The final ratio image acquisition enabled spots to show fluorescent intensities in keeping with differential expression of proteins (Figures 4.1 and 4.2).

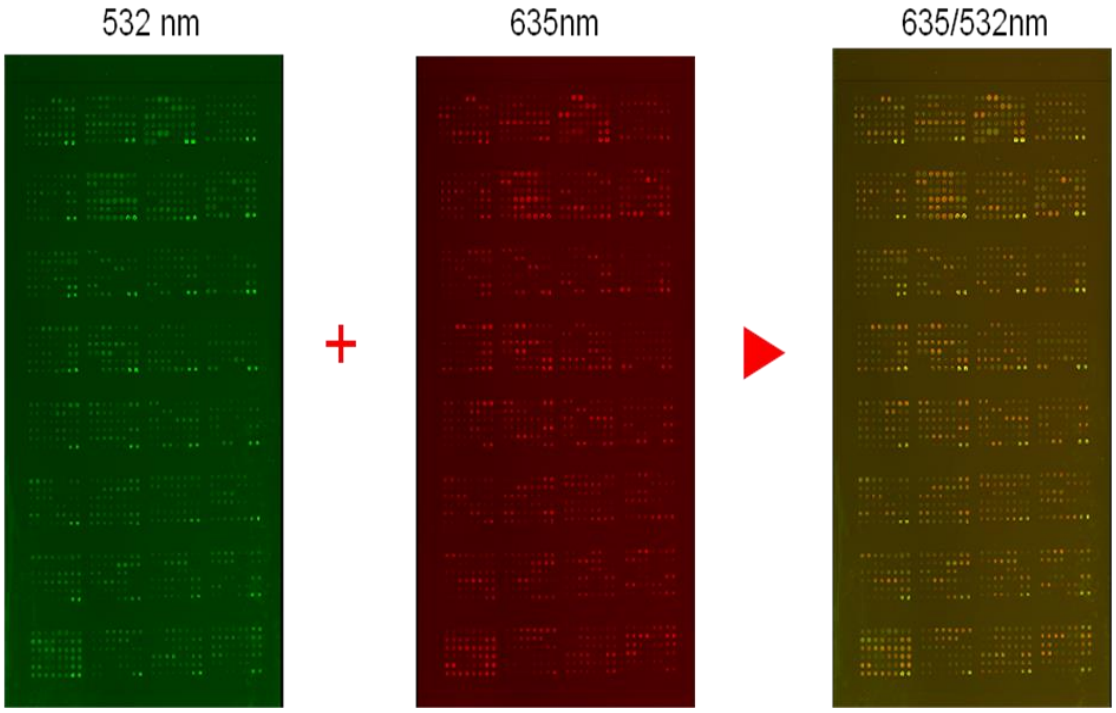

Figure 4.1. Scanned image of antibody microarray slide [SW-837 vs SW-837RR]. Scans were obtained by GenePix Personal 4100A Microarray Scanner (Axon Instruments) and analysed by GenePix Pro software. The colours represent fluorescently labelled proteins post hybridisation. An image taken at 532nm captured the Cy3 labelled SW-837 PN proteins extracts (green), followed by a second image at 635 nm wavelength which captured Cy5 labelled SW-837 RR protein extracts. A final composite image was constructed as a ratio of 635/532 wavelengths as the layering of Cy3 and Cy5 images. In the final image (extreme right) green spots denote down-regulation of the relevant protein in RR sample, red spots denote up-regulation of the relevant protein in

the RR sample and the yellow spots represent the equal amount of protein in both sample. Positive controls anti Cy3 and anti Cy5 also appear yellow and occupied lower right corner of each block in the array.

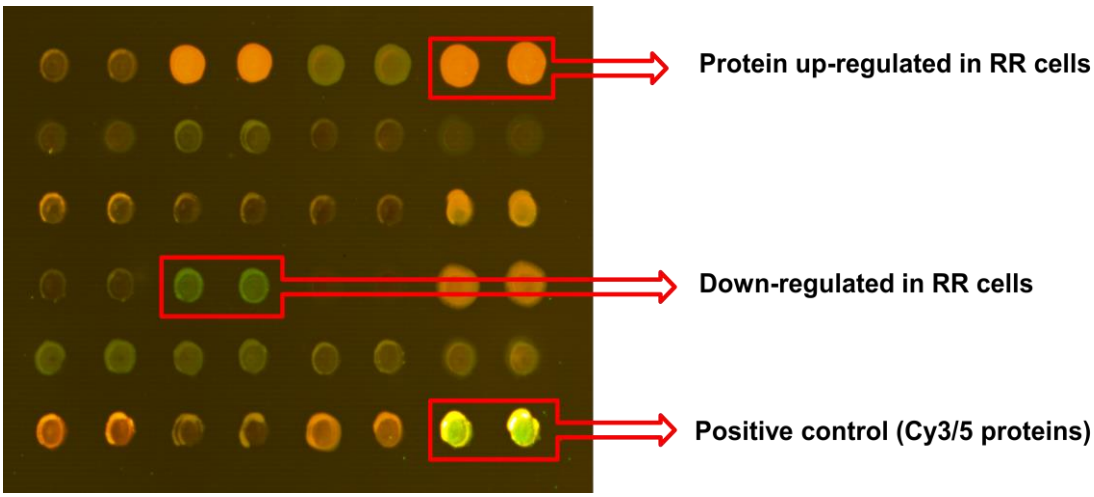

Figure 4.2: Zoomed spots of antibody microarray. The final image of AbMa slide showed different types of spots with varying colour intensities depending upon the relative protein expression. The red spots showed the over-expression of spotted protein in the Cy5 labelled RR sample whereas the green spots showed the down-regulation of the spotted protein in the RR sample. Anti cy3/5 antibody, serving as positive control, occupied right-lower corner position in each grid, appearing more or less uniformly yellow to denote equal amount of the Cy3 and Cy5 dyes uptake. A fourth type of spot, a blank spot, (not present in this image) represented absence of any antibody in that position and served as a negative control (shown in figure 2.5).

The data analysis by Acuity 4.0 software yielded the log ratios of protein expression. These log ratios were converted into the fold changes which revealed 109 DEPs with significant differential expression. Of those, 68 were up-regulated and 41 were down-regulated. Tables 4.1 and 4.2 detail the fold change of DEPs and their known / proposed function. Also, AbMa experiment discovered a few of the previously reported repeatedly identified differentially expressed proteins (RIDEPs) from antibody microarray analysis that have been postulated to be related to stress response (Hodgkinson, ElFadl et al. 2011).

Table 4.1: Differentially Expressed Proteins: SW-837 PN versus SW-837 RR [Up-regulated proteins]. Significantly differentially expressed, **up-regulated proteins** in SW-837 RR cells compared with SW-837 PN cells as discovered by antibody microarray. A fold change of ≥ 1.8 between the differential expression was considered significant. Proteins marked with * belong to RIDEP group. RIDEPs may not necessarily be associated with radioresistance and have been proposed to be expressed as stress response (see discussion)

Up-regulated Proteins	Sigma antibody #	Lowess M Log Ratio	Fold Change	Function
Caspase13	C8854	2.988	7.93	Apoptosis
GRP75	G4170	2.556	5.88	Cell proliferation, stress response
p53DINP1SIP	P4868	2.359	5.13	Apoptosis
JAK1	J3774	2.287	4.88	Interleukin and Interferon signal transduction pathways
Cytohesin 1	C8979	2.183	4.54	Regulation of protein sorting and membrane trafficking
Cyclin D1	C7464	2.071	4.20	Cell cycle regulation
GRP94	G4420	1.961	3.89	Molecular chaperone, signal transduction, protein folding
* Siah2	S7945	1.936	3.82	Ubiquitin-proteasome pathway, apoptosis
* MyD88	M9934	1.890	3.70	Signal transduction
MAP kinase Activated Monophosphoryl	M7802	1.842	3.58	Cell signalling
HSP 27 25	H2289	1.820	3.53	Stress response
hnRNPA2B1	R4653	1.809	3.50	mRNA metabolism and transport
BOB1 OBF1	B7810	1.764	3.39	Octamer-dependent transcriptional activity in B lymphocytes
p38 MAP Kinase NonActivated	M8432	1.735	3.32	Cell signalling
Protein Kinase B α	P1601	1.610	3.05	Cell cycle, apoptosis
Raf1 cRaf	R2404	1.509	2.84	Cell cycle, apoptosis
DcR1	D3566	1.500	2.82	Protection of cells from TRAIL-induced apoptosis.
Heat Shock Protein	H7412	1.497	2.82	Stress response, molecular chaperone for protein

110				folding
ILK	I0783	1.454	2.73	Signal transduction
* 14 3 3	T5942	1.387	2.61	Signal transduction
BAF57	B0436	1.386	2.61	Transcriptional activation of genes, regulation of cell growth
Sin3A	S4445	1.372	2.58	Cellular proliferation, differentiation, apoptosis, oncogenesis
Apaf1	A8469	1.362	2.57	Apoptosis
g Tubulin	T3559	1.331	2.51	Microtubule formation, progression of the cell cycle
Raf1	R5773	1.328	2.51	Cell cycle, apoptosis, cell differentiation and cell migration
Bmf	B1559	1.272	2.41	Apoptosis
Parkin	P6248	1.233	2.35	Ubiquitin-proteasome system
Neurabin I	N4412	1.221	2.33	Cytoskeleton reorganization
DR3	D3563	1.211	2.31	Cell cycle progression, signal transduction, apoptosis
ChK1	C9358	1.183	2.27	Cell cycle arrest, DNA damage response
βTubulin	T5201	1.175	2.25	Microtubule formation, progression of the cell cycle
SynCAM	S4945	1.164	2.24	Formation and differentiation of functional synapses in vitro
Nuf2	N5287	1.157	2.22	Regulation of chromosome segregation
Tumor Necrosis Factor α	T8300	1.132	2.19	Apoptosis, activation of NFkB, regulation of inflammation
Cathepsin D	C0715	1.125	2.18	Lysosomal protein degradation, carcinogenesis, breast cancer
Nitric Oxide Synthase Inducible iNOS	N7782	1.113	2.16	Reactive free radical, neurotransmission, antimicrobial, antitumoural
E2F6	E1532	1.094	2.13	Cell cycle regulation, regulation of tumour suppressor proteins
ROCK2	R8653	1.087	2.12	Regulation of cytokinesis, smooth muscle contraction
Caspase 4	C4481	1.058	2.08	Apoptosis
Acetyl phospho Histone H3 AL9S10	H0788	1.055	2.07	Transcriptional activation of genes

iASPP	A4605	1.055	2.07	Anti-apoptosis
DNASE I	D0188	1.047	2.06	DNA cleavage
MBD 2 ab	M7318	1.044	2.06	Transcription activation
CD40	C5987	1.027	2.03	B cell activation, proliferation, signalling
Ran	R4777	1.011	2.01	Transport, DNA synthesis and cell cycle progression
Tryptophane Hydroxylase	T0678	1.010	2.01	Synthesis of serotonin
Calponin	C2687	1.009	2.01	Smooth muscle contraction and cell adhesion
FOXC2	F1054	1.004	2.00	Development of mesenchymal tissues.
ILK	I1907	0.980	1.97	Cell adhesion, survival, proliferation
MAP 1b	M4528	0.976	1.96	Microtubule formation
Clathrin Light Chain	C1985	0.957	1.94	Formation of coated vesicles
S100	S2644	0.951	1.93	Cell cycle regulation and differentiation
Nedd8	N2786	0.942	1.92	Ubiquitin like protein, cell cycle progression and cytoskeletal regulation
CENPE	C7488	0.935	1.91	Chromosome movement, spindle elongation
Falkor PHD1	F5303	0.928	1.90	Growth suppression
α Tubulin	T6199	0.922	1.89	Microtubule formation
Bim	B7929	0.916	1.88	Apoptosis
Bmf	B1684	0.914	1.88	Apoptosis
FANCD 2	F0305	0.912	1.87	DNA repair
GRK 2	G7670	0.898	1.86	Regulation of G protein-coupled receptors
JNK Activated Diphosphorylated JNK	J4750	0.879	1.83	Cell signalling
Par4 prostate apoptosis Response 4	P5367	0.862	1.81	Transcription activation
hnRNPC1C2	R5028	0.861	1.81	Transcription activation
NcK2	N2911	0.858	1.81	? Cytoskeletal organization
p38 MAPK activated diphosphorylated p	M8177	0.850	1.80	Signal transduction

Table 4.2: Differentially Expressed Proteins: SW-837 PN versus SW-837 RR [Down-regulated proteins]. Significantly differentially expressed, **Down-regulated proteins** in SW-837 RR cells compared with SW-837 PN cells as discovered by antibody microarray. A fold change of ≥ 1.8 between the differential expression was considered significant. Proteins marked with * belong to RIDEP group. RIDEPs may not necessarily be associated with radioresistance and have been proposed to be expressed as stress response (see discussion)

Down-regulated Proteins	Sigma antibody #	Lowess M Log Ratio	Fold Change	Function
Desmosomal protein	D1286	-2.349	-5.09	Cell adhesion
RIP Receptor Interacting protein	R8274	-1.879	-3.67	Activation of NF-kappaB and induction of apoptosis
Serine threonine protein Phosphatase	P7609	-1.873	-3.66	Carbohydrate metabolic process, cell cycle, cell division
Ubiquitin	U0508	-1.840	-3.58	Ubiquitin-proteasome pathway, protein degradation
Tau	T5530	-1.839	-3.57	Microtubule stabilization
Calmodulin	C7055	-1.773	-3.41	Cell cycle, muscle contraction
* Smad 4	S3934	-1.699	-3.24	Cell signalling
MAP Kinase ErK1 ErK2	M5670	-1.572	-2.97	Cell growth, differentiation
Caldesmon	C6542	-1.567	-2.96	Muscle contraction, mitosis
IFI16	I1659	-1.542	-2.91	Modulation of p53 function, cell signalling and growth inhibition
Coilin	C1862	-1.387	-2.61	Mitosis
Growth Factor Independence 1	G6670	-1.359	-2.56	Cell cycle
* IKKa	I6139	-1.321	-2.49	NF-kappa-B activation
TRF1	T1948	-1.319	-2.49	Activation of Caspase activity, cell cycle
Sir2	S5313	-1.277	-2.42	Apoptosis, cell aging, cell differentiation
aActinin	A5044	-1.221	-2.33	Cytoskeleton
Thimet Oligopeptidase 1	T7076	-1.226	-2.33	Cleavage of neuropeptides
Protein Phosphatase	P7607	-1.215	-2.32	Growth, apoptosis, branching morphogenesis

1a				
Rab9	R5404	-1.190	-2.28	Transport, GTPase, recycling membrane receptors
PSF	P2860	-1.179	-2.26	DNA replication
hnRNPU	R6278	-1.174	-2.25	mRNA transport and metabolism, apoptosis
Acetyl Histone H3AcLys9	H0913	-1.126	-2.18	Transfection, transcription, cell cycle progression
Neurofilament 68	N5139	-1.081	-2.11	Neuronal radial growth
mTOR	T2949	-1.082	-2.11	Stress, cell cycle
Bcl-2	B3170	-1.057	-2.08	Anti-apoptosis
aCatenin	C2081	-1.061	-2.08	Aging, cell adhesion, negative regulation of apoptosis
Connexin43	C8093	-1.030	-2.04	Apoptosis, blood vessel morphogenesis, cell-cell signalling
cdc14A	C2238	-1.005	-2.00	Cell cycle
Vitronectin	V7881	-0.995	-1.99	Cellular migration, adhesion, proliferation
AP2 beta	A9856	-0.980	-1.97	Cell proliferation
AP1	A5968	-0.995	-1.97	Cell proliferation
Nitric oxide synthase endothelial NOS	N9532	-0.985	-1.97	Neurotransmission and antimicrobial and antitumoral activities
p300CBP	P2859	-0.953	-1.93	Transcriptional coactivator and pigmentation of melanocytes
DR4	D3813	-0.928	-1.90	Apoptosis, activation of Caspase activity
* Chondroitin sulphate	C8035	-0.926	-1.90	Cell proliferation and recognition, extracellular matrix deposition, and morphogenesis
Transportin 1	T0825	-0.912	-1.88	Protein transport
Anti CY3/5	C0992	-0.908	-1.87	
Cytokeratin 8 1 3	C6909	-0.899	-1.86	Structural integrity of epithelial cells
Rsk1	R5145	-0.887	-1.84	Cell signalling
CaM Kinase Kinase a CaMKKa	C7099	-0.855	-1.80	Cell signalling
hnRNPQ	R5653	-0.851	-1.80	mRNA metabolism

4.3.3 Proteomic analysis of HRA-19 radioresistant cell subline

The AbMa analysis using HRA-19 PN vs RR proteins extracts passed the quality control criteria of 90% substances matching (654 out of 726 substances). A total of 1319 features were matched of the 1536 features spotted on the antibody microarray slide. The scanned images looked very similar to those of SW-837.

The calculations of fold change revealed a number of significantly differentially expressed proteins in HRA-19 RR cells compared with the PN cells. Of the 90 DEPs, 48 were up-regulated and 42 were down regulated. Tables 4.3 and 4.4 enlist the fold change of DEPs and summarise their known / proposed function.

Table 4.3: Differentially Expressed Proteins: HRA-19 PN versus HRA-19 RR [Up-regulated proteins]. Significantly differentially expressed, **up-regulated proteins** in HRA-19 RR cells compared with HRA-19 PN cells discovered by antibody microarray. A fold change of ≥ 1.8 between the differential expression was considered significant. Proteins marked with * belong to RIDEP group. RIDEPs may not necessarily be associated with radioresistance and have been proposed to be expressed as stress response (see discussion).

Up-Regulated proteins	Sigma antibody #	Lowess M Log Ratio	Fold Change	Function
Cytohesin 1	C8979	2.043	4.12	Regulation of protein sorting and membrane trafficking
TRAIL	T9191	1.998	3.99	Apoptosis
BOB1 OBF1	B7810	1.925	3.79	Octamer-dependent transcriptional activity in B lymphocytes
hnRNPA2B1	R4653	1.878	3.67	mRNA metabolism and transport
Cyclin D1	C7464	1.704	3.25	Cell cycle regulation
Cathepsin D	C0715	1.481	2.79	Lysosomal protein degradation, carcinogenesis, breast cancer
JAK1	J3774	1.476	2.78	Interleukin and Interferon signal transduction pathways
MAP kinase Activated Monophosphoryl	M7802	1.374	2.59	Cell signalling
GRP75	G4170	1.286	2.43	Cell proliferation, stress response
Cyclin B1	C8831	1.270	2.41	Cell cycle
Apaf1	A8469	1.264	2.40	Apoptosis
Bim	B7929	1.240	2.36	Apoptosis
MBD 2 ab	M7318	1.206	2.30	Transcription activation
SynCAM	S4945	1.169	2.24	Formation and differentiation of functional synapses in vitro
* Siah2	S7945	1.160	2.23	Ubiquitin-proteqasome pathway, apoptosis
Neurofilament 200	N4142	1.156	2.22	Cytoskleton organization, cell death
Calponin	C2687	1.151	2.22	Smooth muscle contraction and cell adhesion
βTubulin	T5201	1.143	2.20	Microtubule formation and cell cycle progression

Ran	R4777	1.125	2.18	Transport, DNA synthesis and cell cycle progression
g Tubulin	T3559	1.124	2.17	Microtubule formation and cell cycle progression
ILK	I0783	1.112	2.16	Cell adhesion, survival, proliferation
TRAIL	T3067	1.084	2.11	Apoptosis
ILK	I1907	1.065	2.09	Cell adhesion, survival, proliferation
HSP 27 25	H2289	1.049	2.06	Stress response
JNK Activated Diphosphorylated JNK	J4750	1.049	2.06	Cell signalling
Raf1 cRaf	R2404	1.038	2.05	Cell cycle, apoptosis
CENPE	C7488	1.041	2.05	Chromosome movement, spindle elongation
Cyclin D1	C5588	1.038	2.05	Cell cycle
Nedd8	N2786	1.028	2.03	Ubiquitin like protein, cell cycle progression and cytoskeletal regulation
BAF57	B0436	0.981	1.97	Transcriptional activation of genes, regulation of cell growth
a Tubulin	T6199	0.985	1.97	Microtubule formation
S100	S2644	0.972	1.96	Cell cycle regulation and differentiation
Casein Kinase 2b	C3617	0.961	1.94	Signal transduction, transcription, translation, replication
ChK1	C9358	0.946	1.92	Cell cycle arrest, DNA damage response
iASPP	A4605	0.942	1.92	Anti-apoptosis
* Pinin	P0084	0.947	1.92	Cell adhesion
p53DINP1SIP	P4868	0.928	1.90	Apoptosis
Tumor Necrosis Factor α	T8300	0.927	1.90	Apoptosis, activation of NF-kappaB, regulation of inflammation
GRP94	G4420	0.916	1.88	Molecular chaperone, signal transduction, protein folding, protein degradation
NcK2	N2911	0.889	1.85	? Cytoskeletal organization
ROCK2	R8653	0.863	1.81	Regulation of cytokinesis, smooth muscle contraction
Striatin	S0696	0.853	1.80	Negative regulation of cell proliferation, dendrite development

Table 4.4: Differentially Expressed Proteins: HRA-19 PN versus HRA-19 RR [Down-regulated proteins]. Significantly differentially expressed, **Down-regulated proteins** in HRA-19 RR cells compared with HRA-19 PN cells discovered by antibody microarray. A fold change of ≥ 1.8 between the differential expression was considered significant. Proteins marked with * belong to RIDEP group. RIDEPs may not necessarily be associated with radioresistance and have been proposed to be expressed as stress response (see discussion).

Down-regulated Proteins	Sigma antibody #	Lowess M Log Ratio	Fold Change	Function
AP2 beta	A9856	-1.640	-3.11	Cell proliferation
Desmosomal protein	D1286	-1.625	-3.08	Cell adhesion
MAP Kinase ErK1 ErK2	M5670	-1.626	-3.08	Cell growth, differentiation
Protein Phosphatase 1a	P7607	-1.516	-2.85	Growth, apoptosis, branching morphogenesis
PSF	P2860	-1.489	-2.80	DNA replication
DR4	D3813	-1.472	-2.77	Apoptosis, activation of Caspase activity
Sir2	S5313	-1.448	-2.72	Apoptosis, cell aging, cell differentiation
Caldesmon	C6542	-1.414	-2.66	Muscle contraction, mitosis
Growth Factor Independence 1	G6670	-1.399	-2.63	Cell cycle
Bcl-2	B3170	-1.351	-2.55	Anti-apoptosis
Calmodulin	C7055	-1.324	-2.50	Cell cycle, muscle contraction
* Smad 4	S3934	-1.319	-2.49	Cell signalling
IFI16	I1659	-1.293	-2.45	Modulation of p53 function, inhibition of cell growth
Anti CY3/5	C0992	-1.269	-2.40	
Ki67	P6834	-1.251	-2.38	Cell cycle, cell proliferation
p300CBP	P2859	-1.241	-2.36	Transcriptional coactivator, melanocytes pigmentation
RALAR	R8529	-1.222	-2.33	GTP binding protein family, cell signalling
hABH3	A8353	-1.222	-2.33	DNA damage repair
Coilin	C1862	-1.219	-2.32	Mitosis
Acetyl Histone H3AcLys9	H0913	-1.212	-2.31	Transfection, transcription, cell cycle progression
cerbB4	E5900	-1.182	-2.26	ErbB/HER family of transmembrane receptor tyrosine kinase, Oncogene

MTA 2	M7569	-1.169	-2.24	Transcription regulation
ASC2	A5355	-1.152	-2.22	Transcription coactivator
Dystrophin	D8168	-1.145	-2.21	Muscle membrane protein, muscle contraction
TRF1	T1948	-1.146	-2.21	Activation of Caspase activity, cell cycle
Serine threonine protein Phosphatase	P8609	-1.135	-2.19	Carbohydrate metabolism, cell cycle, cell division
aCatenin	C2081	-1.100	-2.14	Signal transduction
Tau	T5530	-1.077	-2.10	Microtubule stabilization
nitric oxide synthase endothelial eNOS	N9532	-1.075	-2.10	Neurotransmission and antimicrobial and antitumoral activities
* Chondroitin sulphate	C8035	-1.056	-2.07	Cell proliferation, extracellular matrix deposition
NBS1 Nibrin	N9287	-1.045	-2.06	DNA damage repair
SNX6	S6324	-1.036	-2.05	Intracellular trafficking
* IKKa	I6139	-1.039	-2.05	NF-kappa-B activation
Rsk1	R5145	-1.038	-2.05	Cell signalling
Ubiquitin	U0508	-0.991	-1.98	Ubiquitin-proteasome pathway, protein degradation
Rab9	R5404	-0.986	-1.98	Transport, GTPase, recycling membrane receptors
Vitronectin	V7881	-0.968	-1.95	Cellular migration, adhesion, proliferation
mTOR	T2949	-0.961	-1.94	Stress, cell cycle
Actin	A3853	-0.956	-1.93	Muscle contraction, cytoskeletal organization
HDRP MITR	H9163	-0.951	-1.93	Transcriptional regulation, cell cycle progression
* Zyxin	Z0377	-0.944	-1.92	Signal transduction, cytoskeletal organization
S6 Kinase	S4047	-0.917	-1.88	Cellular proliferation, apoptosis, growth
b tubulin IV	T7941	-0.891	-1.85	Cytoskeletal organization
Cdk6	C8343	-0.885	-1.84	Regulation of cell cycle progression
Tyrosine Hydroxylase	T2928	-0.879	-1.83	Catecholamine synthesis
BAP 1	B9303	-0.879	-1.83	Tumour suppressor, related to BRCA1 growth control pathway
LIS1	L7391	-0.865	-1.82	Neuronal development
Vinculin	V4505	-0.860	-1.81	Cytoskeletal organization

4.3.4 DEPs common to both RR rectal cancer cell sublines

A comparison of DEP profile of two cell line models revealed a list of 62 common proteins potentially associated with radioresistance in the novel RR cell lines (Table 4.5). Anti-apoptotic protein Bcl-2 was the only DEP common to both cell line models that has been previously demonstrated to be possibly associated with radioresistance (section 1.9.2). The common DEP profile also contained several other interesting proteins that might be related to radioresistance, as discussed later in this chapter.

Table 4.5: DEPs common to both radioresistant rectal cancer cell sublines. A list of 62 significantly differentially expressed proteins common to the two RR cell lines with fold changes in differential expressions in SW-837 PN vs SW-837 RR and HRA-19 PN vs HRA-19 RR. Proteins marked with * belong to RIDEP group. RIDEPs may not necessarily be associated with radioresistance and have been proposed to be expressed as stress response (see discussion). The DEPs have been listed in the order of highest differential expression in SW-837 RR cell line model.

UP-regulated proteins	SW-837 RR Fold Change	HRA-19 RR Fold Change	Down-regulated Proteins	SW-837 RR Fold change	HRA-19 RR Fold Change
GRP75	5.88	2.43	Desmosomal protein	-5.09	-3.08
p53DINP1SIP	5.13	1.90	Serine threonine protein Phosphatase	-3.66	-2.19
JAK1	4.88	2.78			
Cytohesin 1	4.54	4.12	Ubiquitin	-3.58	-1.98
Cyclin D1	4.2	3.25	Tau	-3.57	-2.10
GRP94	3.89	1.88	Calmodulin	-3.41	-2.50
Siah2	3.82	2.23	Smad 4	-3.24	-2.49
MAP kinase Activated Monophosphoryl	3.58	2.59	IFI16	-3.24	-2.45
HSP 27 25	3.53	2.06	MAP Kinase ErK1 ErK2	-2.97	-3.08
hnRNPA2B1	3.5	3.67	Caldesmon	-2.96	-2.66
BOB1 OBF1	3.39	3.79	Coilin	-2.61	-2.32
Raf1 cRaf	2.84	2.05	Growth Factor Independence 1	-2.56	-2.63
ILK	2.73	2.16			
BAF57	2.61	1.97	TRF1	-2.49	-2.21

Apaf1	2.57	2.40	IKKa	-2.49	-2.05
g Tubulin	2.51	2.17	Sir2	-2.42	-2.72
ChK1	2.27	1.92	Protein Phosphatase 1a	-2.32	-2.85
β Tubulin	2.25	2.20	Rab9	-2.28	-1.98
SynCAM	2.24	2.24	PSF	-2.26	-2.80
Tumor Necrosis Factor α	2.19	1.90	Acetyl Histone H3AcLys9	-2.18	-2.31
Cathepsin D	2.18	2.79	mTOR	-2.11	-1.94
ROCK2	2.12	1.81	Bcl2	-2.08	-2.55
iASPP	2.07	1.92	α Catenin	-2.08	-2.14
MBD 2 ab	2.06	2.30	Vitronectin	-1.99	-1.95
Calponin	2.01	2.22	AP2 beta	-1.97	-3.11
Ran	2.01	2.18	Nitric oxide synthase endothelial eNOS	-1.97	-2.10
ILK	1.97	2.09			
S100	1.93	1.96	p300CBP	-1.93	-2.36
Nedd8	1.92	2.03	DR4	-1.9	-2.77
CENPE	1.91	2.05	Chondroitin sulphate	-1.9	-2.07
α Tubulin	1.89	1.97	Rsk1	-1.84	-2.05
Bim	1.88	2.36			
JNK Activated Diphosphorylated JNK	1.83	2.06			
NcK2	1.81	1.85			

4.4 Discussion

4.4.1 The Antibody Microarray

In this chapter, it was aimed to exploit the power of antibody microarray to investigate proteomic markers of radioresistance in rectal cancer using an in-vitro cell line model. As described in section 1.10.1.1, this technique is relatively novel and is now increasingly being used for its ability to provide high throughput results in a relatively short period of time (Angenendt 2005; Smith, Lind et al. 2006; Hodgkinson, Eagle et al. 2010; Hodgkinson, ElFadl et al. 2011). It is also preferred over conventional proteomics tools for its compatibility with lower sample volume and antibody concentration requirements, higher format versatility, and reproducibility (Section 1.10.3.1) (Sanchez-Carbayo 2006). To our knowledge, this study is the first to use Antibody Microarray to study proteomic biomarkers in rectal cancer. The only two proteomics studies in rectal cancer reported to date, used 2D Gel electrophoresis and 2-D PAGE, tryptic digestion and mass spectrometry, respectively (Allal, Kahne et al. 2004; Yeo, Kim et al. 2012).

The application of Antibody Microarray, its quality control criteria and the interpretation of the significant results is currently evolving. Hodgkinson et al recommended that the experiment should be considered successful when the percentage of array's 'substances matched', provided by the software during the data analysis, reaches a threshold of 90% (Hodgkinson, ElFadl et al. 2011). This ensures that array slides of high quality with good dye uptake and a low signal to noise ratio are carried forward for data interpretation. Therefore, in the current study above technical quality control criterion was applied (Hodgkinson,

ElFadl et al. 2011). Our analyses after initial optimisation showed that all the dataset achieved ≥ 90% 'substance matching'. This ensured the overall quality of the data and validity of the results. In this study, the significance level for differential expression of proteins was set at 1.8 fold change as previously recommended in a large series of AbMa analyses (Hodgkinson, ElFadl et al. 2011). We selected Panorama Xpress Profiler 725 kit for these experiments because it has recently shown promising results in discovery of differentially expressed proteins associated with chemoresistance in breast cancer (Hodgkinson, ELFadl et al. 2012). Further, it provides comprehensive (although not complete) proteome coverage from the pathways with possible role in predicting or determining response to radiotherapy (Section 2.4.2).

4.4.2 Differentially expressed proteins

Our results revealed a large number of DEPs in two cell line models separately. Of those, a total of 62 proteins displayed significant differential expression across both novel RR cell sub-lines. A wide range of cellular and biological functions are linked to these proteins as depicted in Figure 4.3. Such a functional pattern represented the selection of antibodies on the array from different cellular pathways. A majority (21%) of proteins belonged to the apoptotic pathway. Additionally, a number of proteins regulating cell growth and the cell cycle appeared to be differentially expressed suggesting such processes may be associated with response and behaviour of cells following radiotherapy.

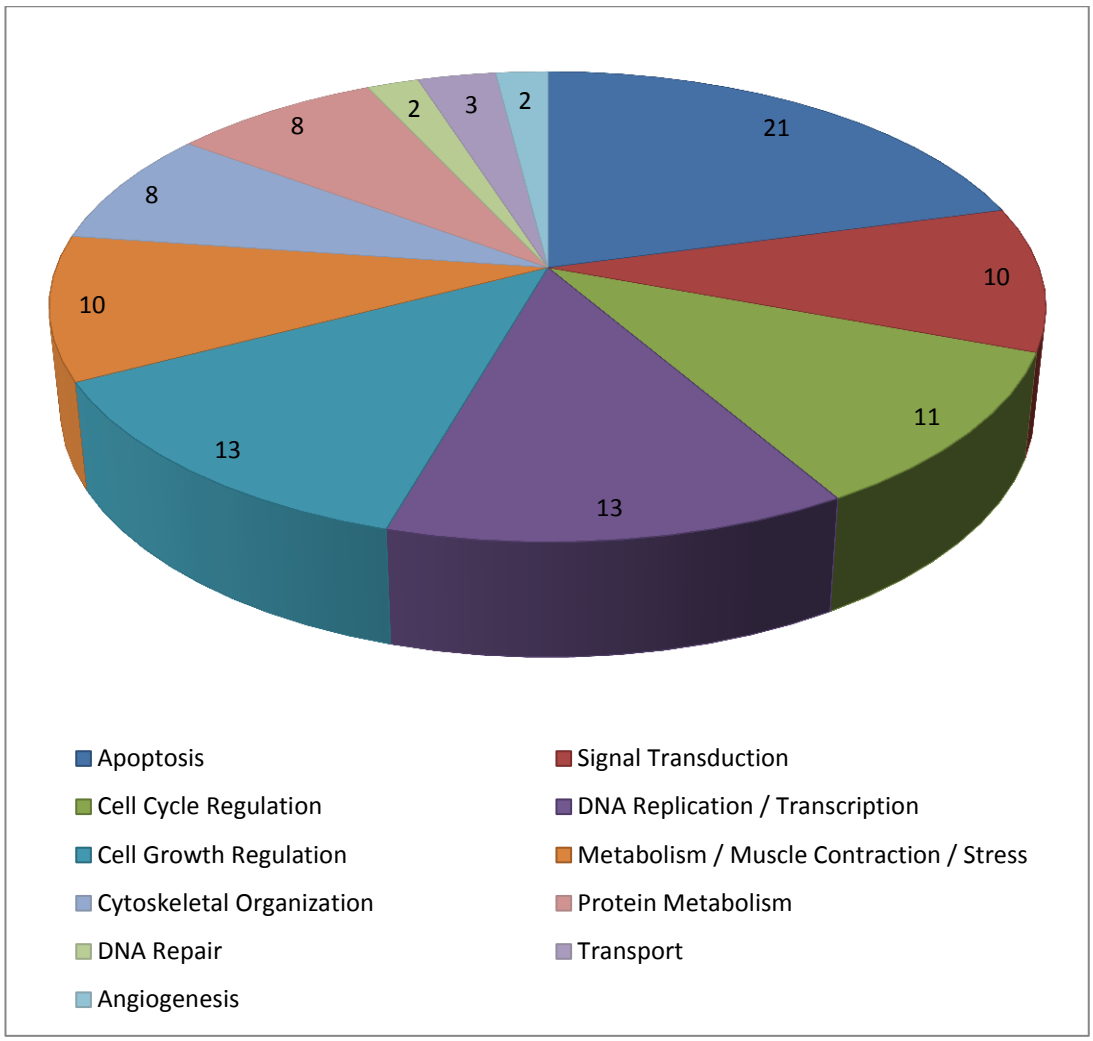

Figure 4.3: Biological functions of DEPs identified by Antibody Microarray. Apoptotic proteins dominated the list with 21% of proteins linked with this pathway. Proteins associated with cell growth regulation, DNA replication / transcription, and the cell cycle regulation represent the next most common groups of proteins associated with radioresistance.

Apoptosis is the major mechanism by which the actions of anti-neoplastic treatments are exerted. Amongst a number of DEPs linked with apoptosis, the members of intrinsic apoptosis - Bcl-2 and APAF1 - were found to be differentially expressed. Bcl-2 has anti-apoptotic function and has been suggested to be linked with radio-resistance, however, evidence to date is not enough to prove this (section 1.9.4). In addition, central mediators of extrinsic apoptosis - TNF α and death receptor DR4 – were also significantly differentially

expressed. The fold change in differential expression of these DEPs in SW-837 RR and HRA-19 RR cells were as follows: Bcl-2 (-2.08 and -2.55), APAF1 (2.57 and 2.40), DR4 (-1.90 and -2.77), and TNF α (2.19 and 1.90). All these proteins are key regulators of apoptosis (Section 1.8.4). These data suggest central role of apoptosis in radiation induced cell injury. Moreover, the role of different apoptotic pathways in determining response to ionising radiation needs to be elucidated. This becomes increasingly relevant because the available evidence suggests that radioresistance is mediated through intrinsic apoptosis (Huerta, Gao et al. 2009). Our data suggest that the extrinsic pathway of apoptosis might play a role in determining the fate of a cell following exposure to ionising radiation via death receptor induced cell death. However, further validation of these results by confirmatory studies is required.

The protein metabolism was observed to be amongst the biological processes linked with radioresistance. Ubiquitin protein, an important member of the ubiquitin-proteasome system (UPS) of protein degradation, was down-regulated in RR cells. Ubiquitin conjugation to target substrates is required for the degradation of proteins via this route. This discovery is consistent with the previous observations about the role of UPS in conferring radioresistance to tumour cells (Russo, Tepper et al. 2001; Smith, Qutob et al. 2009; Elfadl, Hodgkinson et al. 2011). In rectal cancer, one previous study has investigated the role of UPS in radioresistance. High SKP2 expression (a Ubiquitin E3 ligase) was observed to be associated with reduced tumour regression, advanced nodal disease, and reduced recurrence free survival. Bortezomib (Velcade, formerly known as PS-341) is a novel dipeptide boronic acid that reversibly inhibits the chymotryptic activity of the 26S proteasome, the central

member of the UPS. Preclinical studies have shown that Bortezomib decreases proliferation, induces G2-M cell cycle arrest and apoptosis and enhances the activity of chemotherapy or radiotherapy (Richardson, Mitsiades et al. 2005; Petroski 2008; Loeffler-Ragg, Mueller et al. 2009). The association of the UPS pathway with radioresistance, coupled with detection of Ubiquitin protein in current study, warrant further investigations of this protein / pathway in rectal cancer. Cyclin D1, the cell cycle regulatory protein, was found to be up-regulated in both RR cell lines. This protein has previously been investigated in two studies of rectal cancers that failed to find a link between its expression and radioresistance (Moore, Shia et al. 2004; Moral, Fdez-Acenero et al. 2009). However, its consistent differential expression in AbMa analysis means further confirmatory studies are indicated.

In addition to DEPs common to both RR cell sublines, this study found differential expression of a few important proteins that demonstrated differential expression in only one of the two RR cells. Amongst proteins associated with apoptosis, downstream Caspases of intrinsic mitochondrial pathway including Caspase 13 (fold change 7.93) and Caspase 4 (fold change 2.08) were up-regulated in SW-837 RR. Of note, TRAIL, the mediator of death receptor pathway of apoptosis was found to be up-regulated in HRA-19 RR cells. Two different TRAIL antibodies on the array were found to be differentially expressed (#T9191, fold change 3.99 and #T3067, fold change 2.11). TRAIL is a member of the TNF superfamily that promotes apoptosis by binding to the transmembrane receptors DR4 and DR5. TRAIL pathway has been widely linked with enhanced apoptosis and offers one of the most promising molecular targeted strategies in the form of death receptor agonistic antibodies used as

radio-sensitiser or as mono or combination chemotherapy (MacFarlane 2003; Maduro, de Vries et al. 2008; Trarbach, Moehler et al. 2010). Further studies specifically looking at role of TRAIL in radiotherapy response in rectal cancer are certainly recommended.

Similarly, 14 3 3 theta/tau isoform was up-regulated in SW-837 RR cells with a fold change of 2.61. The 14 3 3 protein family is ubiquitously expressed and exists in various isoforms which have been reported to be associated with chemoresistance and radioresistance in multiple cancers (Neal and Yu 2010; Matta, Siu et al. 2012). The 14 3 3 protein family is associated with cellular processes including cell cycle regulation, signal transduction, and apoptosis. Our research group has demonstrated a differential expression of 14 3 3 theta/tau in chemo-resistant breast cancer using antibody microarray and 2-DE MALDI TOF/TOF (Hodgkinson, Agarwal et al. 2012; Hodgkinson, ELFadl et al. 2012). Our results showed that the cellular proliferation protein Ki67 was over-expressed in HRA-19 RR cells (fold change 2.38) but not in SW-837 RR cells (fold change 1.19). It serves as a marker of cellular proliferation and activity, and can be detected in all active stages of the cell cycle. A reduced Ki67 expression is reported to be weakly associated with enhanced tumour regression in response to CRT in rectal cancer only in some studies (Jakob, Liersch et al. 2008; Kuremsky, Tepper et al. 2009). In our study, the observation that above proteins demonstrated differential expression in only one RR subline meant that these proteins would not be considered for confirmation / validation phases of biomarkers discovery.

4.4.3 Repeatedly Identified Differentially Expressed Proteins (RIDEPs)

It is essential that a putative biomarker be carefully selected to undergo further confirmation and validation experiments. Each differentially expressed protein may not necessarily be associated with the cellular behaviour being studied. Recently, a list of 13 proteins has been proposed as the repeatedly identified differentially expressed proteins (RIDEPs) discovered by antibody microarray of chemotherapy and radiotherapy resistant cells / tissues (Table 4.6). It has been proposed that the differential expression of this group of proteins might merely be associated with the cellular stress response as opposed to true molecular alteration as a treatment effect. The proposed RIDEPs panel is based on antibody microarray data from 13 independent experiments on human breast cancer, oral cancer, lung cancer, mesothelioma, and CLL (Hodgkinson, ElFadl et al. 2011).

Our results revealed a number of RIDEPs in rectal cancer cell lines. Of the 13 RIDEPs reported by Hodgkinson and colleagues, eight were found to be significantly differentially expressed in at least one RR cell line model in our study (Table 4.6). These RIDEPs included: Smad4, Chondroitin Sulfate, IKKa, Zyxin, MyD88, 14 3 3, Siah2, and Pinin. Three of these (Chondroitin Sulfate, IKKa, and Siah2) were differentially expressed in both RR cell lines. Whereas frequently observed stress markers have been reported in relation with colorectal cancer (Jimenez, Knol et al. 2010), current study is the first to significantly add to the RIDEP body of evidence in rectal cancer in association with the study of biomarkers of radioresistance. The discovery of such proteins should be treated with caution and it is imperative that the selection of these

proteins for the verification stage of the biomarker discovery pipeline is carefully considered.

Table 4.6: Discovery of RIDEPs in current study. RIDEPs related to radioresistance discovered in current study compared with those identified by Hodgkinson et al. related to different anti-cancer treatments including RT. Of the 13 RIDEPs, eight were discovered in current study using the same proteomics technique with a commercial antibody microarray kit (Sigma XP725). It has been proposed that differential expression of this group of proteins may not be related to the true intracellular events linked with the research question (radioresistance / chemoresistance) and may merely be an indication of cellular stress response. It is recommended that the selection of these proteins for the verification stage of the biomarker discovery pipeline is meticulously planned.

RIDEPs identified by Hodgkinson et al.		**RIDEPs identified in current study**	
RIDEPs	**Cancer type**	**SW-837 RR cell line**	**HRA-19 RR cell line**
Zyxin	CLL, Lung, Oral, Mesothelioma, Breast	--	Down-regulated
BID	CLL, Lung, Oral, Mesothelioma, Breast	--	--
MyD88	CLL, Breast	Up-regulated	--
IKKa	Lung, Mesothelioma, Breast, Oral	Down-regulated	Down-regulated
BclXL	Lung, Mesothelioma, Breast	--	--
Chondroitin sulphate	Lung, Mesothelioma, Breast, Oral	Down-regulated	Down-regulated
14 3 3	Lung, Mesothelioma, Breast	Up-regulated	--
Centrin	CLL, Lung, Mesothelioma, Breast	--	--
SLIPR MAGI3	CLL, Lung, Mesothelioma, Oral	--	--
Pinin	Lung, Mesothelioma, Breast	--	Up-regulated
Protein Kinase C	CLL, Breast, Oral	--	--
Smad4	CLL, Lung, Mesothelioma, Breast	Down-regulated	--
Siah2	CLL, Breast, Oral	Up-regulated	Up-regulated

4.4.4 Conclusion and selection of DEPs for clinical validation

A number of proteins from antibody microarray analysis were identified to be significantly differentially expressed. As discussed above, antibody microarray is a screening proteomics tool and the results need confirmation with complementary techniques or validation by immunohistochemistry. As summarised in table 4.5 and figure 4.3, the common DEP profile identified several candidates that were suitable for IHC study. However, the time and funding constraints meant only two proteins could be selected in the first instance for this thesis. In doing so, we adopted a carefully considered approach in selecting the proteins for IHC analysis:

1. Proteins with direct relevance to mechanism of action of ionising radiation.

2. Proteins with known association with radiotherapy response

3. Avoidance of known stress proteins and RIDEP group of proteins

On the basis of above criteria, we selected the apoptosis related proteins Bcl-2 and DR4 to be the first proteins to be taken to validation stage of biomarker discovery pipeline. Although not the most differentially expressed proteins, the wealth of evidence about their potential role in determining response to RT would justify further testing by IHC (sections 1.8.4 and 1.9.4). This decision was also influenced by the fact that good antibody reagents exist for IHC for these proteins making them a logical first choice. As discussed in section 1.8.4, apoptosis is a major mechanism by which ionising radiations exert their action. Evidence suggests that a cytotoxic stimulus to cell leads to induction of cell death via DNA damage or through apoptosis (section 1.8). Bcl-2 and DR4 proteins remain at the heart of apoptotic process and are linked with intrinsic

and extrinsic pathways, respectively. The role of Bcl-2 in radioresistance has been investigated in 17 published studies as summarised in section 1.9.4. The fact that current evidence is equivocal about the role of Bcl-2, our study would add invaluable data that might help a pooled analysis in future. Similarly, DR4 protein assumes a key role in initiation of extrinsic apoptotic pathway (section 1.8.4). Whereas the role of DR4 protein in radiotherapy response in rectal cancer has not been studied in the past, our research group has recently reported DR4 to be related to radioresistance in breast cancer cell line model and tissues (ELFadl, Hodgkinson et al. 2011). Coupled with findings by ELFadl et al, differential expression of DR4 in RR cells in current study highlighted a possible role of death receptors pathway of apoptosis in determining the outcome of radiotherapy in cancers. Therefore, we selected these two key apoptosis related proteins, out of several candidate proteins, to be the first to undergo validation experiments.

CHAPTER-5: Validation of putative biomarkers of radioresistance using immunohistochemistry

Aims:

- To select a suitable series of rectal cancer patients treated with neoadjuvant long-course chemoradiotherapy
- To categorize the selected patients into ‘good-response’ and ‘poor-response’ groups on the basis of histological tumour regression
- To undertake immunohistochemical analyses of selected DEPs from antibody microarray data to validate the differential expression of biomarkers

5.1 Introduction

Radiotherapy is a well-established treatment modality in solid cancers and has improved the treatment outcomes in rectal cancer. Despite clear oncological benefits, up to 11% of patients are affected by local or distant recurrence of disease (SwedishRectalCancerTrial 1997; Sebag-Montefiore, Stephens et al. 2009). As described in section 1.12, we currently lack the ability to predict whether radiotherapy will be effective in achieving the therapeutic target in individual cases. Efforts have been made to develop such ability by studying the molecular markers for their potential to be used as predictive biomarkers. In chapter 4, we have generated antibody microarray data that revealed several proteins potentially related to the response of rectal cancer to radiotherapy. Our data were based on a cell line model and identified DEPs carry potential for clinical application. However, before such a consideration may be given, these results need further validation in rectal cancer tissues as discussed in section 4.4.4.

Clinical validation of differential expression of biomarkers by IHC requires definition of tumour response to radiotherapy. In this study, the response was assessed by tumour regression grading. TRG has previously been used as a surrogate of tumour response to CRT for biomarker studies as described in section 1.7.2.8. The data from Antibody microarray revealed a number of DEPs with possible association with radioresistance. Of those, apoptosis related proteins DR4 and Bcl-2 were chosen for initial IHC validation as discussed in section 4.4.4. Both DEPs showed consistent differential expression across the two RR sublines and play central role in initiation of and regulation of apoptosis, respectively. This chapter is aimed at IHC analyses of selected DEPs from

antibody microarray data to validate their differential expression. Ethical approval for this pilot IHC study was obtained from the local research ethics committee (Reference 09/00/181 Hull and East Riding Research Ethics Committee).

5.2 Materials and methods

IHC analyses were undertaken on FFPE rectal cancer specimens from eligible patients as described below.

5.2.1 Case selection for Immunohistochemistry

The National Bowel Cancer Audit Programme (NBOCAP) database was searched to identify rectal cancer patients treated with neoadjuvant LCCRT at Castle Hill Hospital from July 2006 to August 2011. All patients received neoadjuvant LCCRT consisting of five fractions a week of 1.8 Gy radiation aiming for a total dose of 45 Gy and continuous oral Capecitabine at dose of 825 mg/m^2 twice daily. The total duration of uninterrupted treatment was 5 weeks (35 days) with 25 fractions of radiotherapy. The patients subsequently underwent oncological resection 5 - 6 weeks post completion of LCCRT. This interval ensures adequate tumour regression and return of bowel wall and surrounding tissues to near normal state to allow optimum healing and avoidance of anastomotic dehiscence.

5.2.1.1 Inclusion and exclusion criteria

Following inclusion criteria were defined for the patients to be included in this study:

1. Patients who received preoperative long-course chemoradiotherapy (Consisting of 45 Gy RT + oral capecitabine)

2. Invasive adenocarcinoma on pre-treatment biopsy

3. Surgical resection post LCCRT

4. Pathological confirmation of rectal adenocarcinoma on resection specimen

5. Documented tumour regression grade (Mandard's TRG 1 to 5 or 3-point TRG 0 to 2), assessed from histological examination of resection specimen

6. Availability of pre-treatment tumour biopsy specimen for IHC

The patients meeting the following criteria were excluded from the study.

1. TRG grade-1 of 3-point grading system (for explanation see section 2.5.2)

2. Failing to fulfil any of the above inclusion criteria

5.2.1.2 Study groups

The immunohistochemical expression of putative biomarkers was compared between the two groups of tumours; Good-Response versus Poor-Response groups (response categorisation discussed in detail in chapter 2, section 2.5.2). Those patients with significant tumour regression post LCCRT were categorised into Good-response group and those with poor or absent tumour regression were considered Poor-response group. The two study groups are outlined in table 2.6.

5.2.2 Immunohistochemistry protocol and antibody staining criteria

The experiments were performed in close collaboration with Lucy Scaife and Dr Lynn Cawkwell. The IHC was performed as per protocol described in section 2.5.4. Briefly, from each FFPE specimen block, 4µm sections were cut onto superfrost microscope slide, antigen sites retrieved, and antibody incubation was undertaken as described in section 2.5.4. Both the selected antibodies were optimised on colorectal specimens consisting of normal and cancer tissues. A slide consisting of the same section was also included in all IHC experiments which served as positive and negative control. Negative controls were treated identically, with primary antibodies omitted. Scoring for antigen staining was performed as described in section 5.2.2.1. The primary antibodies used for immunostaining with dilution, incubation time and the scoring criteria are outlined in table 5.1.

Scoring was performed by an independent observer, experienced in IHC staining scoring and was blinded to the study groups at the time of scoring (Lynn Cawkwell). As explained in section 2.5.4.9, the scoring was limited to only one observer's scores due to lack of training opportunities and trained researchers for IHC scoring. However, efforts were made to minimise the chance of bias by blinding the observer to study groups.

Table 5.1: The primary antibodies used for IHC analysis. The dilution concentration and the antibody staining scoring are given.

Antibody name	Catalog #	Dilution& Incubation time	Scoring criteria
DR4	Ab13890 (Abcam)	1:50 2 hours	**POSITIVE:** Strong cytoplasmic staining (majority of area) **NEGATIVE:** No / very weak cytoplasmic staining (majority of area)
Bcl-2	Ab692 (Abcam)	1:25 2 hours	**POSITIVE:** Medium - strong cytoplasmic staining (≥ 10% of area) **NEGATIVE:** No / very weak cytoplasmic staining (< 10% of area)

5.3 Results

5.3.1 Patients' characteristics

A total of 125 eligible patients were identified. Of those, 33 patients met the inclusion criteria and were included in the study. Of the 92 patients excluded from the study, 47 had 3-point old TRG grade 1 (intermediate grade), 32 had no TRG assessed on post LCCRT resection specimen, and the remaining 13 were excluded due to miscellaneous reasons (Figure 5.1).

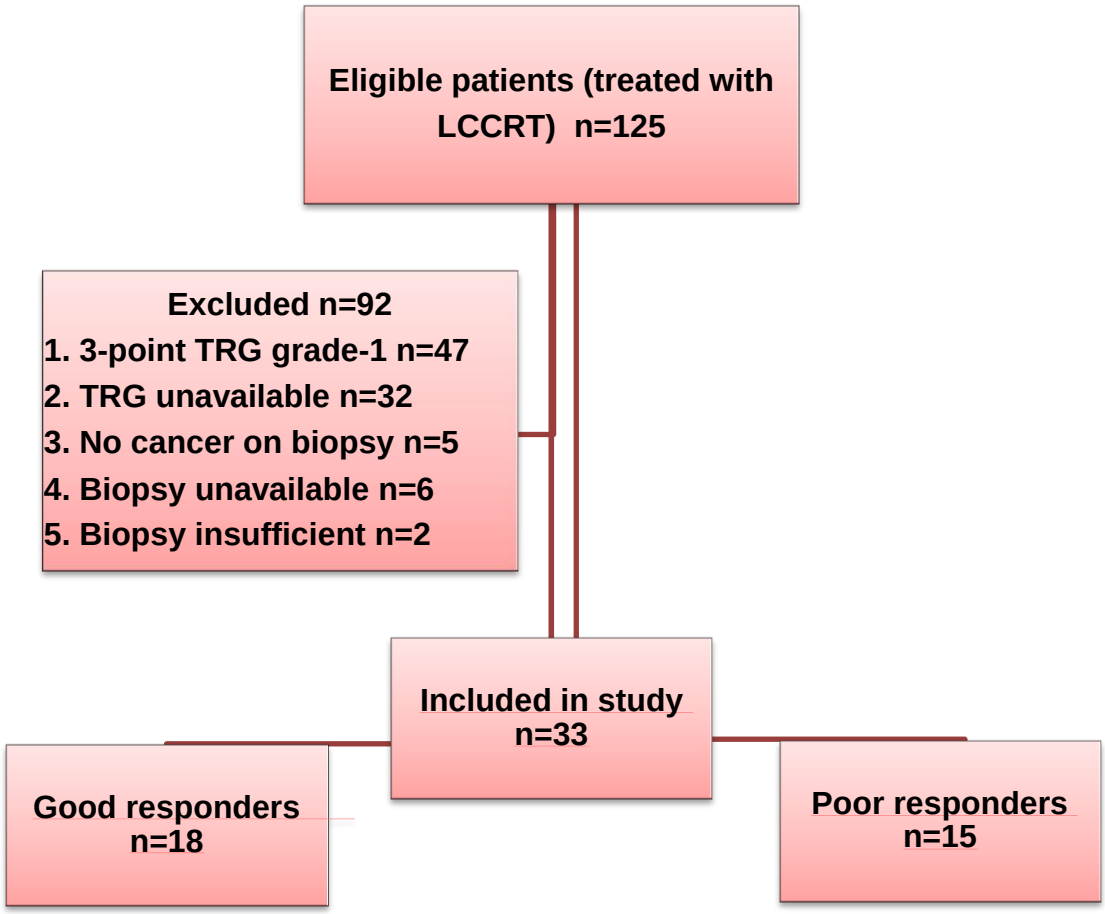

Figure 5.1: Case selection for Immunohistochemistry. Of the 125 patients who underwent LCCRT over the study period, only 33 met the inclusion criteria (described in text) and were included in the study.

The groups were matched for age, gender, preoperative TNM staging, pathological characteristics, and time since CRT to surgical resection of tumour i.e. time to assessment of TRG (Table 5.2).

Table 5.2: Clinico-pathological characteristics of groups: Good responders versus Poor responders. Comparison of both groups revealed no significant differences between the groups in terms of preoperative staging and pathological characteristics. * Chi-square test unless otherwise specified. # Student's t test. a = incalculable

Variables	Good responders (n=18)	Poor responders (n=15)	p-value*
Gender			0.8
Male	15	12	
Female	3	3	
Age (years)	63.6	66.7	0.37#
Time to TRG (days)	98.7	110.3	0.14#
Preoperative T-stage			0.41
T1	0	0	
T2	2	2	
T3	15	10	
T4	1	3	
Preoperative N-stage			0.51
N0	7	5	
N1	9	6	
N2	2	4	
Preoperative M-stage			A
M0	17	15	
M1	0	0	
Tumour differentiation			A
Well-moderate	5	14	
Poor	0	0	
Pathological N-stage			0.14
N0	16	9	
N1	1	4	
N2	1	2	
Apical lymph node			0.26
Involved	0	1	
Not involved	18	14	
EMVI			0.12
Positive	0	2	
Negative	17	13	

5.3.2 DR4 staining

The DR4 antibody was optimised on colorectal tissues at a concentration of 1:50 for an incubation time of 2 hours. Strong cytoplasmic staining was observed for the majority of CRC area, as opposed to weak staining of the

normal colorectal tissue (Figure 5.2). The IHC staining for DR4 protein was observed to be positive in all 33 specimen (18 good and 15 poor responders) of rectal cancer in this study.

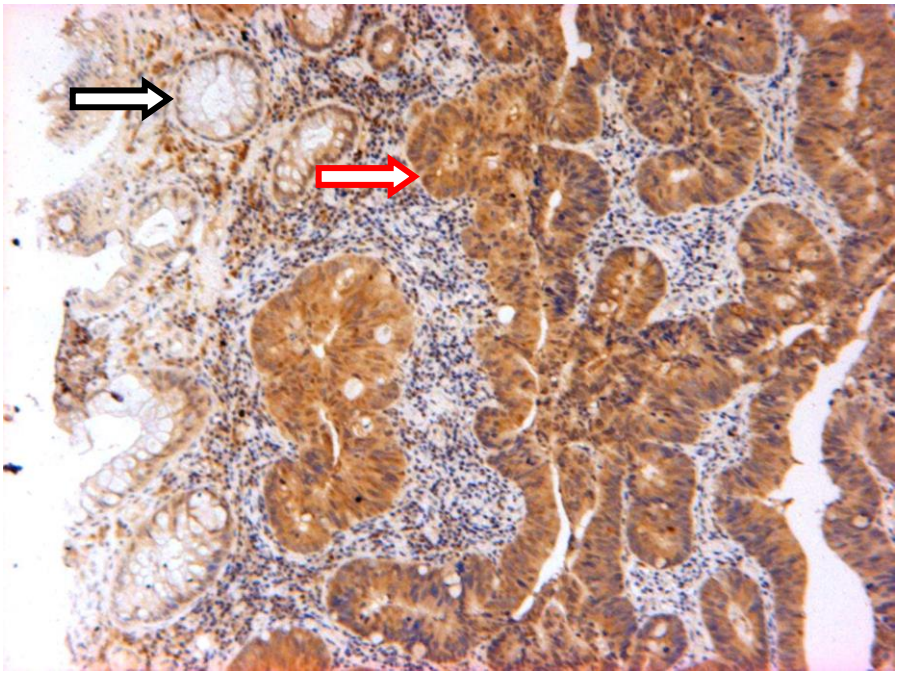

Figure 5.2: DR4 Immunohistochemistry in CRC. The immunostaining of DR4 protein in colorectal adenocarcinoma cells showing strong cytoplasmic staining (red arrow). Note the adjacent normal colorectal cells showing weak staining compared with the cancer cells (black arrow), and minimal staining in the background. Original magnification 63x. Each biopsy specimen was represented by three 4 µm sections on the slides.

5.3.3 Bcl-2 staining

The Bcl-2 antibody was optimised in a similar way at a concentration of 1:25 using a section of colorectal tissue containing both the normal colorectal tissue and the invasive cancer. Figure 5.3 is one optimisation slide showing positively staining cells of normal colorectal tissue with no staining of CRC (some optimisation slides showed positive staining for cancer tissue as well).

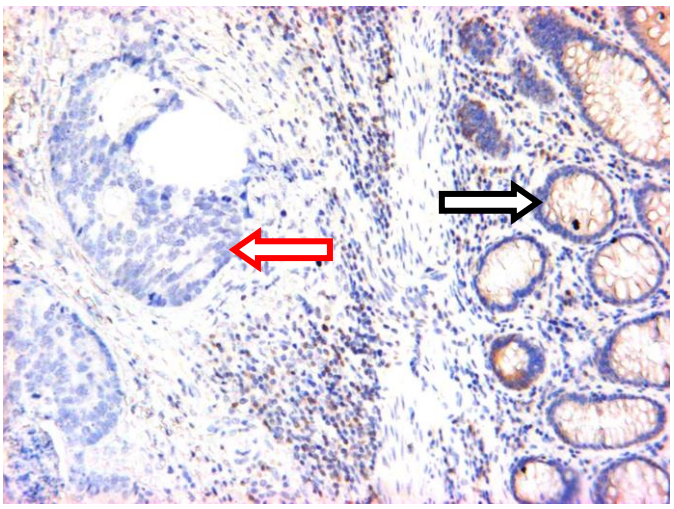

Figure 5.3: IHC staining pattern of Bcl-2 in CRC. Optimisation of Bcl-2 staining of CRC showing negative cytoplasmic staining of CRC (red arrow) compared with positive staining of normal colorectal tissue (black arrow). Original magnification 100x.

IHC of rectal cancer specimens showed that 5 of 15 (33%) poor responders demonstrated Bcl-2 expression (positive staining) compared with 7 of 18 (39%) good responders (Figure 5.4). There was no difference in expression levels between the two groups (p=0.74, Chi-square test).

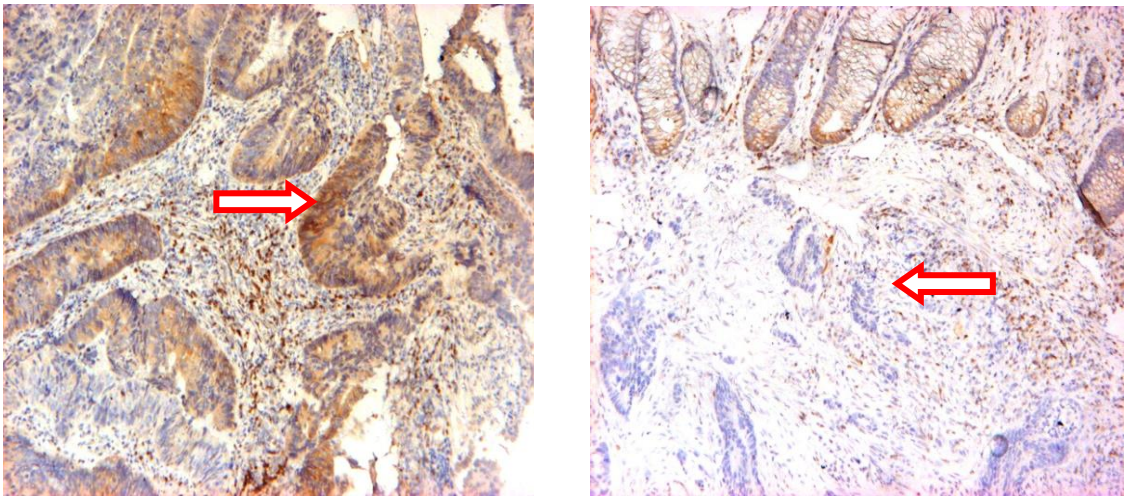

Figure 5.4: IHC staining of Bcl-2 in rectal cancer. Three sections per case were included on the slides. **Left (positive expression):** Positive cytoplasmic staining of ≥ 10% rectal cancer area (arrow). **Right: (negative expression)** Negative cytoplasmic staining of rectal cancer (arrow). Note positive staining of adjacent normal colorectal tissue and lymphocytes.

5.4 Discussion

Antibody microarray based comparative proteomics revealed a number of potential biomarkers of interest and several of those would warrant validation IHC analyses (Section 4.3.4). Our initial consideration was given to validation of two of those, DR4 and Bcl-2, as discussed in section 4.4.4

5.4.1 Immunohistochemistry of DR4 and Bcl-2

The DR4 protein is located in the cytoplasm. In this study, the expression of DR4 was considered positive when the majority of cells in the stained slide demonstrated strong cytoplasmic staining. In addition to qualitative staining pattern, quantitative scoring systems for DR4 expression also exist. Koornstra et al. estimated percentage of positive cells semi-quantitatively by classifying the DR4 expression into four categories based on the total percentage of stained cells in colorectal tissues [scores: 0 <10%, 1 = 10% - 50%, 2 >50%] (Koornstra, Kleibeuker et al. 2003). Similarly, a more extensive 5-tier quantitative scoring system for DR4 staining in colorectal cancer (n=169) has been described that demonstrated that DR4 expression predicted DFS in patients with colon cancer (Strater, Hinz et al. 2002). Our results from this pilot study indicated that DR4 is widely expressed by rectal cancer tissues. All samples from both the responder and non-responder groups demonstrated positive staining with essentially no difference in the expression levels.

The anti-apoptotic protein Bcl-2 is localised in cytoplasm and a positive expression in this study was defined as staining of 10% or more of the tumour cells (Chang, Jung et al. 2005; O'Kane, Pound et al. 2006). Like DR4,

quantitative scoring system for Bcl-2 expression in rectal cancer has been described. Chang and colleagues described quantitative scoring of Bcl-2 expression into three categories based on the total percentage of staining cells [scores: 0 <10%, 1 = 10% - 50%, and 2 >50%] (Chang, Jung et al. 2005). The IHC analysis in our study showed that the Bcl-2 expression was similar between the two groups. Some 33% of poor responders demonstrated Bcl-2 expression compared with 39% of good responders (p=0.74).

Our results failed to confirm the differential expression of DR4 and Bcl2 proteins that was demonstrated in the AbMa analysis. There were several limitations in this study which might explain non-significant results. It should be considered that our data is based on a small pilot study and a type-2 error might have impacted the results owing to small sample size. Moreover, a quantitative scoring system would have yielded more meaningful results as opposed to mere qualitative staining pattern (positive / negative expression). However, we were unable to follow a quantitative scoring system for antibody staining owing to small sample size that would make quantitative scoring very difficult to interpret. Furthermore, this study highlighted the real challenges in obtaining a suitable archival series of clinical samples for IHC studies. Of the identified cases over a 5-years period, only a quarter (33 / 125) of cases were eligible for the study. The main determinants were different tumour regression scoring systems, and unavailable or insufficient biopsy specimens. Such limitations should be considered while planning IHC studies on the archival series of clinical samples. A desired number of suitable samples may be obtained by collaboration with other centres with access to a large number of samples. Future studies should endeavour to recruit more cases to reliably determine the

expression difference of putative biomarkers between good and poor responders.

5.4.2 Conclusion

In summary, whereas we provided the first time evidence that Bcl-2 and DR4 were differentially expressed in a proteomic microarray based study of radioresistant cell line model, our immunohistochemistry analysis of rectal cancer biopsies did not confirm such differential expression. Our results might represent the true cellular behaviour, but in view of the limitations of the study discussed above further testing in a larger series of good-response versus poor-response categories is strongly recommended. Large and preferably prospectively designed studies are required to derive meaningful conclusions. Such studies would benefit from prospective patient selection and assessment of the TRG.

CHAPTER-6: Conclusions and future directions

6.1 Summary of work and future directions

In recent times, there has been a phenomenal development in our understanding of the tumour biology and the focus of research has shifted to molecular targeted strategies for use in fight against cancer (Chung, Levy et al. 2007). The work presented in this thesis generated a number of differentially expressed proteins that may be related to radioresistance thus setting foundation for confirmatory studies. Our initial consideration for validation experiments went to DR4 and Bcl-2. A number of other interesting, novel biomarkers remain to be tested for their relationship with radiation treatment (table 4.5). For example, Ubiquitin, the central protein of Ubiquitin-Proteasome pathway was differentially expressed across both RR cell lines and should be taken to validation phase. The AbMa kit used in this study did not contain the key enzyme complex of UPS – 26S proteasome which has recently been linked with radioresistance. Future studies are strongly recommended to test 26S proteasome using complementary techniques.

Following are the recommendations for future studies:

1. Use of complementary techniques for comprehensive proteome coverage: The practical value of the proteomics approach is to give a global overview of cellular processes. The current study was limited to proteins on one

commercially available array. Different approaches that may be adopted in future for more comprehensive proteome coverage include use of additional or custom made antibody microarrays (sections 1.10.1.1 and 2.4.1) and complementary proteomics approaches (section 1.10.1). Data from global techniques would set the foundation for targeted confirmatory studies using e.g. western blotting or immunohistochemistry. Comprehensive proteome coverage would also allow robust data mining and identification of key proteins and pathways that might have a central role in radiotherapy resistance.

2. Increasing accrual of patients for IHC study:

For IHC validation experiments, we categorised Mandard's TRG-1, 2 into 'good-response' and TRG-2, 3, 4 into 'poor-response' categories. Due to different grading systems, a large number of patients belonged to old TRG-1 necessitating their exclusion from this study. In future, efforts to increase patient accrual in confirmatory studies should be made. Collaboration with regional centres to obtain more samples should be actively sought. A large collection of samples might be sourced from the tissue banks that are central repository of tissue samples collected from clinical trials. The importance of such sources could be appreciated from the fact that tissue bank of NSABP (National Surgical Adjuvant Breast and Bowel Project) stores more than 90,000 breast and colon cancer specimens. Some of the tissue banks specifically promote the scientific research in the areas of prognostic and predictive biomarkers and maintain the valuable tissue materials with clinical follow-up information (http://www.nsabp.pitt.edu/).

3. Quantitative scoring for immunohistochemical staining:

Our IHC pilot study was also limited by qualitative scoring for staining patterns of proteins. It appears that quantitative scoring system provides more meaningful results. Future studies should aim to score IHC staining quantitatively which is possible in case of large sample size.

6.2 Impact of work on surgical oncology and concluding remarks

The treatment of rectal cancer has undergone a paradigm shift with the advancements in surgical and neoadjuvant treatment options over the last two decades. Admittedly, advancements in the radiotherapy regimens have substantially improved oncological outcomes (section 1.7). Unfortunately, up to 11% of rectal cancer patients still remain at risk of local or distant recurrence despite standard treatment. The challenge faced by surgical oncology is *"how to determine what treatment option is best for a given patient"*. It is imperative to offer radiotherapy to those who will benefit, and the predicted radioresistant tumours may proceed straight to surgery, if deemed resectable. To ensure right tumours are treated with correct treatment modality, *"individualisation of treatment"* is necessary. The published data about the biomarkers associated with radioresistance present inconclusive evidence (section 1.9). The work presented in this thesis adds comprehensive data to that body of evidence in that a number of novel biomarkers from screening proteomics were discovered that may be related to radioresistance. Although our initial confirmatory IHC study failed to validate the differential expression of DR4 and Bcl-2 proteins, several other DEPs remain to be tested. The successfully validated molecular targets could be considered in formulating strategies to potentiate the

effectiveness of existing therapies (e.g. RT) and to possibly open newer avenues.

In general, the molecular targeted strategies could be applied at two levels. First, the predictive biomarker may be analysed on initial biopsy specimen at the time of histological diagnosis of the lesion and further treatment decisions may be based on its expression status as appropriate. The biomarkers with significant predictive potential can help identify the radioresistant tumours which may proceed directly to surgical resection without receiving futile radiation treatment. Such an approach would ensure that only the patients likely to derive benefit from RT are actually subjected to this toxic treatment modality.

The second approach employs using the knowledge of the tumour biology in translational research. Molecular targeted agents could be developed that modify the function of the putative molecule in order to counteract radioresistance or confer radiosensitivity. It is envisaged that such combination treatment to radiosensitise tumours would open new possibilities in the management of rectal cancer. The use of radiosensitisers to enhance the effect of RT on malignant tissues will impact the oncological outcomes. The role of molecular targeted agents (e.g. anti-EGFR, anti-VEGF and anti-proteasomal agents) to radiosensitise the tumours is actively being sought (Wadlow and Ryan 2010). In addition, novel radiosensitising agents prior to RT / CRT have been proposed indicating that this avenue holds promise (Chiu, Hsaio et al. 2010; Dewdney and Cunningham 2012). Thus the attempts to potentiate the

effect of RT in cancer tissues should continue until a promising tissue specific radiosensitising agent could be incorporated into routine treatment regimens.

The combination treatment with radiosensitising agents offers several possibilities as it would allow therapy to be tailored on an individual patient basis. In the first instance, such approaches would allow potentiating the therapeutic response of RT in locally advanced cancers to enhance downsizing or down-staging. Thus, the surgical resection following neoadjuvant treatment might become virtually curative. Likewise, low rectal cancers might be more effectively treated to obtain the higher rate of sphincter sparing surgery. However, the most exciting aspect of the radiosensitising strategies is that novel management approaches might be used as curative treatment. Early stage (T1, T2) tumours might become amenable to full cure when treated with CRT used alongside novel radiosensitisers. Admittedly, the early stage tumours could potentially be spared the oncological resection and its associated risks. Therefore, the spectrum of surgical and oncological outcomes influenced by radiosensitising strategies would encompass greater curative resections, higher rates of sphincter preserving procedures, and avoidance of surgical resection in early stage cancers. Such combination approach would substantially improve the oncological outcomes in patients with rectal cancer in the form of prolonged survival and improved quality of life.

In summary, the work presented in this thesis provided first time evidence from antibody microarray proteomics that a number of proteins might be related to radioresistance. Whereas our initial validation experiments did not confirm the

value of the tested proteins, the array data provides several markers for future validation studies. The validated protein biomarkers would provide an ability to predict the response to RT that could facilitate judicious use of this treatment modality. Only the patients predicted to derive therapeutic benefit from RT would be given RT. Those tumours predicted to respond poorly, would be treated with surgery alone or with RT in combination with molecular targeted treatments in the form of radiosensitising agents. Admittedly, molecular targets offer individualised treatment options that would be welcomed by both clinicians and patients alike!

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