NEUREGULIN-1 (NRG1) IN COLORECTAL CANCER

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Abstract

Previous studies identified a distinct region of interstital deletion at c8p12 locus in colorectal cancer. Deletions at c8p12 were more frequent in early than advanced colorectal tumours. Further studies defined more closely this interstitial deletion, implicating a cluster of genes close to marker D8S259 in early colorectal tumours. Although interstitial deletions normally correspond to tumour suppressor genes, the above findings suggest that this region contains genes with tumour suppressive and oncogenic effects. We investigated NRG1, one of the genes in this region in colorectal tumourigenesis.

To determine the expression profile of NRG1, multiple approaches were utilised. Results from these studies identified deletion (loss of one allele) of *NRG1* in early colorectal tumours. Increased expression of NRG1 protein was identified in advanced tumours compared to early colorectal tumours and adenomas. A significant correlation between NRG1 protein expression and erbB2 protein overexpression was identified. Although the range of NRG1 isoforms expressed was qualitatively similar throughout tumour progression, semi-quantitative real-time RT-PCR, showed overall decreased expression in early tumours compared with normal tissue. In advanced cancers, the results were more heterogeneous, but no consistent pattern emerged between the cases investigated.

Although these studies did not demonstrate any functional relationship, the data strengthens the candidacy of a role for NRG1 in colorectal tumour progression.

To my parents

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor receptor
ErbB	Epidermal growth factor receptor
FISH	Fluorescent *in-situ* hybridisation
GGF	Glial growth factor
HRG1	Heregulin
kDa	Kilo Daltons
LOH	Loss of heterozygosity
mins	minutes
MTA	Multiple tissue array
ndf43	Neudifferentiation factor 43
NRG1	Neuregulin-1
PCR	Polymerase chain reaction
RNA	Riboxynucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
secs	seconds
SMDF	Smooth muscle derived factor
SNP	Single nucleotide polymorphism

Chapter 1

Introduction

1.1 Colorectal cancer

Colorectal cancer is the second most common cause of cancer -related deaths in the western world with an incidence of over 30 000 cases being diagnosed per annum in the U.K. (Conlin et al., 2005; Leslie et al., 2002; Jepson et al., 2005). Despite improvements in medical and surgical treatments the overall 5-year survival is around 40% (Houlston, 2001; Leslie et al., 2002; Association of Coloproctology of Great Britain and Ireland, 2001). The high incidence of colorectal cancer with a modest improvement in overall survival highlights the need for early detection, prevention and improvements in treatment (The Association of Coloproctology of Great Britain and Ireland, 2001).

1.1. 2 The adenoma – carcinoma sequence

The fundamental concept of colorectal cancer involves the stepwise progression of normal colonic mucosa to dysplasia and ultimately to carcinoma through the accumulation of multiple genetic events including chromosomal abnormalities, genetic mutations and epigenetic changes thought to be critical in the development of colorectal cancer (Fearon and Vogelstein, 1990; Hamilton 1992; Massa et al., 1999; Reid et al., 1999; Leslie et al., 2002; Söreide et al., 2006; Leslie et al., 2003; Ilyas et al., 1999; Chung et al., 2000; Conlin, 2005).

Fearon and Vogelstein (1990) proposed that a series of genetic events occurred at key stages in the adenoma-carcinoma sequence, which involved mutational activation (gain of function mutations) of oncogenes such as *ras*, and inactivation (loss of function mutations e.g. loss of both alleles) of tumour suppressor genes,

such as *APC* and *TP53* in the development/progression of colorectal cancer. These functional gain and loss of genes in cancer cells is mirrored by the acquisition of chromosomal aberrations (Reid et al., 1999).

1.1.3 Chromosomal aberrations

Chromosomal aberrations involving the loss or gain of specific regions of the genome have been reported extensively in solid tumours (Albertson et al., 2003; Qin, 2002). Alterations (gains or losses) in DNA copy number, increase with cellular atypia, and seem to correlate with disease progression (Reid et al., 1999). In colon cancer, chromosomal aberrations have been detected in high grade dysplasia and adenomas, but are found increasingly in carcinomas Reid et al., 1996). Similar findings have also been reported in tumours of the breast, brain and cervix uteri where an increase in the prevalence of chromosomal copy number has been reported with progression towards advanced stage disease (Schröck et al., 1994; Schröck et al., 1996; Reid et al., 1995; Reid et al., 1996; Heselmeyer et al., 1996; Reid et al., 1999).

Recurrent chromosomal aberrations are implicated in tumour development and often involve oncogenes and tumour suppressor genes, the expression levels of which are altered by these changes in the genome (Ilyas et al.,1999; Albertson et al., 2003; Qin et al., 2002; Reid et al., 1999). Although a range of chromosome aberrations have been reported in colorectal cancer for the purpose of this study I will focus on chromosome 8.

1.1.4 Chromosome 8 and colorectal cancer

Alterations in the short arm of chromosome 8 have been reported in a wide range of solid tumours including colon cancer (Bardi et al., 2004; Wright et al., 1998). Deletions are the most common aberration in this region, identified through loss of heterozygosity (LOH) studies (Qin, 2002; Chughtai et al., 1999). Although chromosome arm 8p allelic imbalance is frequently observed in colorectal cancer, the segment lost is generally poorly defined and interstitial deletions as well as loss of the whole 8p arm have been observed. Both oncogenes and tumour suppressor genes are thought to reside at these sites of recurrent alterations. LOH analysis has been used to define the precise loci targeted in chromosome arm 8p loss (Nakoa.et al., 2004; Halling et al., 1999).

1.1.5 Loss of heterozygosity analysis of chromosome arm 8p in colorectal cancer

Previous LOH analysis by our group identified two distinct centromeric regions of interstitial deletion, 8p11.2-12 and 8p12 region, in colorectal cancer. Analysis of tumour phenotype revealed isolated 8p12 deletions to be associated with early colorectal disease (4/7 T1, 3/11 adenomas vs 1/63 T2-T4; 2p=0.0001), while retention of this region was seen in advanced colorectal cancer (Chughtai et al., 1999). Fine mapping of the c8p12 deletion region using a panel of P1 artificial chromosome clones (PACs) established the deletion region lay between markers D8S87 (centromeric) and WRN gene (telomeric) as shown in **Figure 1.1**.

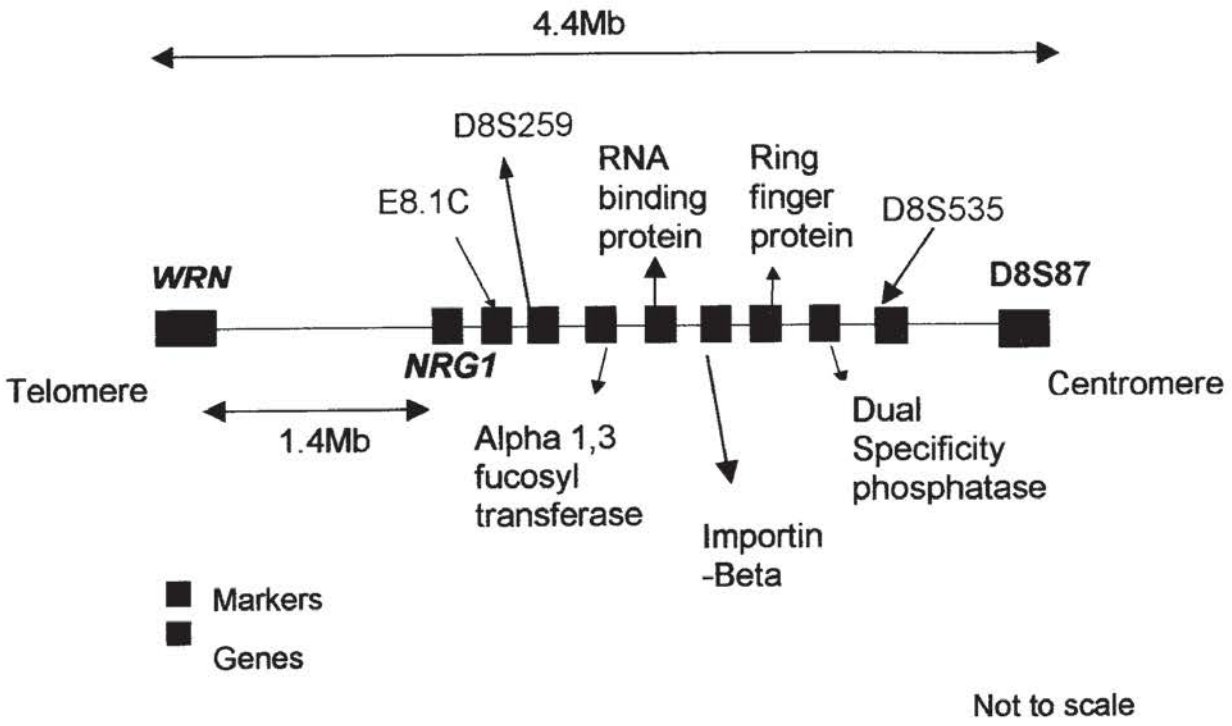


Figure 1.1: 8p12 DELETION REGION: The 8p12 deletion region was established to lie between marker D8S87 centromerically and the *WRN* gene telomerically. Marker D8S535 and E8.1C were found to be deleted and the WRN gene was found to be retained. The *NRG1* gene lies between marker E8.1C and *WRN* gene.

Markers D8S535, D8S259 and E8.1C were found to be deleted and *WRN* gene was retained in the colorectal tumour samples examined. The proportion of colorectal tumours showing deletion positively correlated with advancing tumour stage. This was true when considering deletion at marker D8S87 (2/27 adenomas, 2/9 T1 vs 6/12 T3-T4N0, 6/11 T3-T4N1; chi square for trend = 12.245, p=0.0005). However when considering deletion of the telomeric marker D8S259, this trend was lost (2/27 adenomas, 7/10 T1 vs 6/12 T3-T4N0, 2/11 T3-T4N1; chi square for trend =5.723, p=0.0167) (Adams, 2004). Deletion of maker D8S259 was more frequent in early colorectal cancer compared with advanced colorectal tumours as shown in **Table 1.1** below.

Stage	Polyps	T1-T2		T3 - T4	
		N0M0	N1+	N0M0	N1+
D8S87	2/27 (7%)	2/9 (22%)	0/0	6/12 (50%)	6/11 (54%)
D8S259	3/27 (11%)	7/10 (70%)	0/0	6/12 (50%)	2/11 (18%)

Table 1.1: c8p12 deletions in colorectal tumours

Reference to the Ensemble genomic database (http://www.ensembl.org) (**Build35**) identified six genes within the deletion region; α 1,3 fucosyl transferase, a dual specificity phosphatase 26, an RNA binding protein, a RING finger protein, Importin-beta and Neuregulin-1 (*NRG1*).

1.1.6 Candidate gene

The α 1,3 fucosyl transferase gene is responsible for catalysing the transfer of glycosyl groups from donor to acceptor molecules (NCBI,UniProtKB/TrEMBL). The dual specificity phosphatase 26 (*DUSP*26) is involved in protein tyrosine/serine/threonine phophatase activity, the RNA binding protein is implicated in splicing, the RING finger protein is involved in mediating protein/protein interactions and protein ubiquinitation and Importin-beta functions as a nuclear transport protein (NCBI, UniProtKB/TrEMBL). Neuregulin-1 (*NRG1*), encodes a group of growth factors that belong to the epidermal growth factor (EGF) family of proteins. The protein products of *NRG1* exert a variety of biological effects including cell proliferation, angiogensis, oncogenesis, cellular differentiation and apoptosis (Le et al., 2002). Cleavage studies of neuregulin-1 transmembrane proteins have reported oncogenic activity within the N-terminus of the protein (Montero et al., 2000) and the cytoplasmic tail is reported to have a pro-apoptotic effect (Weinstein et al., 1998).

Although hypotheses can be constructed for any one of the genes, in view of the dual properties of NRG1 proteins, this gene was a clear candidate for further study in colorectal cancer.

NRG1 isoforms and facilitate their relative expression during development and adult life (Steinthorsdottir et al., 2004).

The neuregulin-1 proteins play a fundamental role in normal physiological development of the cardiac and nervous system. Pan-*NRG1* knock out mice have been shown to have severe cardiac and nervous system developmental problems resulting in death during embryogenesis (Meyer et al., 1997)

1.2.1 Structure of NRG1 isoforms

Alternative splicing of the *NRG1* gene results in isoforms that differ in their N-terminal sequence, the presence or absence of the transmembrane domain, and the type of EGF like domain i.e. α or β and the C-terminus. The NRG1 isoforms are classified in to type I isoforms which contain an Ig-like domain at their N-terminus, type II isoforms which contain a signal peptide and kringle-like sequence at their N-terminus plus Ig domain and finally type III isoforms have a hydrophobic domain with cysteine-rich sequence at their N-terminus and share only the common EGF domain with other isoforms. (Falls, 2003; Meyer et al., 1997; Lemke, 1996; Wen et al., 1994; Wang et al., 2001). .

A hydrophobic stretch of amino-acids at the c-terminus of the EGF domain serve as the transmembrane domain and are utilised in membrane insertion (Wang et al 2001; Falls, 2003; Lemke, 1996; Meyer et al., 1997). The type III NRG1 have a hydrophobic region within the cysteine-rich domain (CRD) which serves as a second transmembrane domain in the N-terminal fragment (Falls, 2003; Wang et

al., 2001). A linker region also known as the "stalk" lies in between the bioactive EGF domain and transmembrane domain as shown in **Figure 1.2**. (Falls, 2003; Lemke, 1996; Montero et al., 2000).

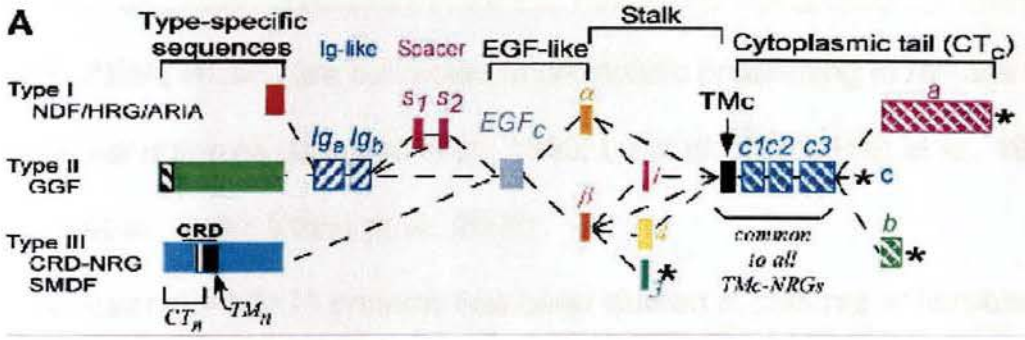


Figure 1.2: Structure of NRG1 isoforms

CRD= cysteine – rich domain; EGF= epidermal growth factor like domain; Ig= immunoglobulin-like domain; CTc= cytoplasmic domain C-terminal of the EGF domain; $^{S}1$ $^{S}2$= spacer domains; TMc= transmembrane domain C-terminal of the EGF domain; CTn and TMn = cytoplasmic and transmembrane domain N-terminal of the EGF domain; * = stop codon. Hydrophobic regions that serve as transmembrane domains are indicated by black boxes. The hydrophobic sequence in the Type II N-terminal indicated by a hatched box is thought to function as a internal signal sequence rather than a transmembrane domain.

Falls, 2003

This structural diversity results in isoforms that are synthesised as soluble or transmembrane proteins and differentiates isoforms with respect to their *in vivo* functions. The bioactive EGF domain common to all NRG1 isoforms is essential for signalling activities (Marchionni et al., 1993; Falls, 2003; Wen et al., 1994; Lemke, 1996; Meyer et al., 1997; Wolpowitz et al., 2000; Montero et al., 2000).

1.2.2 Processing of NRG1 proteins

The mechanism and cellular processing of transmembrane neuregulins has been the subject of several investigations. Numerous membrane anchored proteins such as amyloid precursor protein (APP), TGF-α, including NRG1 proteins (such as NDF, ARIA, HRG1) are subjected to proteolytic processing to release their extracellular domains (Burgess et al., 1995; Lu et al., 1995; Han et al., 1999; Montero et al., 2000; Wang et al., 2001)

The processing of NRG1 proteins has been studied in cultures of fibroblastic cells in which NRG1 isoforms have been expressed by transfection. These studies have indicated that the membrane bound NRG1 isoforms (such as NDF, HRG) are orientated with the amino-terminal ends exposed to the extracellular space with the carboxy terminal in the cytoplasm (Wang et al., 2001; Falls, 2003). The bioactive EGF domain is located extracellularly between the N-terminus and the transmembrane domain as shown in **Figure 1.3**.

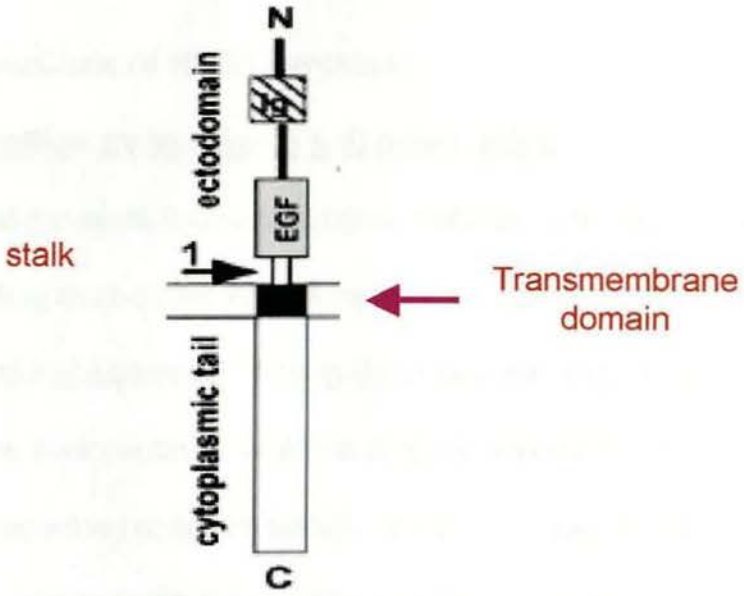


Figure 1.3: Structure of type I neuregulin isoform. The bioactive EGF domain is located at the N- terminus. Cleavage of ectodomain occurs at the stalk region.

(Falls, 2003)

The transmembrane NRG1 proteins are proteolytically cleaved at the extracellular linker region which is considered a critical region for the regulation of balance between soluble and transmembrane forms of membrane anchored polypeptide factors (Montero et al., 2000; Falls, 2003). Type I transmembrane neuregulins are cleaved in the stalk region like the type II NRGs resulting in the release of soluble bioactive EGF domain.

Cell –cell interactions of NRG1 proteins

Paracrine signalling by Ig- (type I & II) neuregulins

Paracrine signaling refers to short distance cell-cell communication mediated by diffusible signaling molecules. An example of this cell –cell interaction is seen during cardiac morphogenesis. The Ig (type I&II) neuregulin ligands are expressed in the endocardium, which is directly apposed to the developing myocardium where the receptors (erbB2) for these ligands are expressed. The cleaved ligands interact with the erbB2 receptors via paracrine signalling (Falls, 2003; Lemke, 1996).

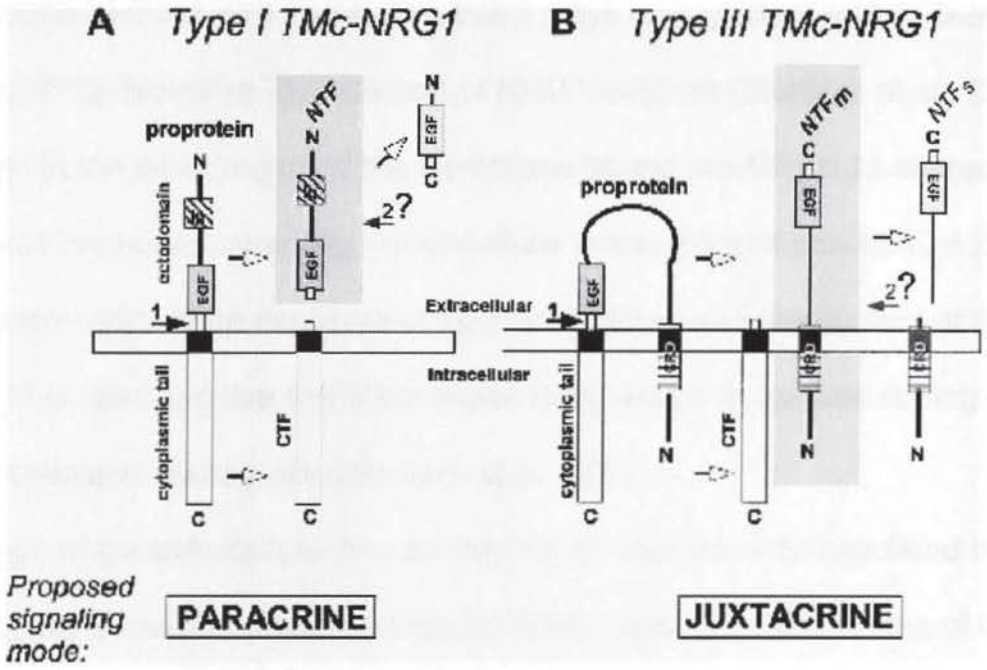
Autocrine Signalling

NRG1 proteins have also been reported to be involved in autocrine signalling (Kim et al., 1999)

Juxtracrine signalling

Studies on processing of N-terminal fragment of type III- β1a and type I –β1a neuregulin-1 isoforms revealed that type III neuregulin –1 proteins serve as juxtracrine signals (Wang et al., 2001; Falls, 2003). The type III-β1a and type I-β1a neuregulins are identical in their EGF like domain, stalk, transmembrane domain but differ in their N-terminal. Wang et al. (2001) compared the processing of type I and type III β1a neuregulin-1 proteins in cell cultures. Western analysis of cell lysates, cell surface biotinylation and immunocytochemical analysis revealed

that the amount of type III- β1a released in to the conditioned medium was less than type I-β1a neuregulin-1 proteins. The type III- β1a was found exposed at the cell surface as a transmembrane N-terminal fragment. Although the sequence of type I-β1a neuregulin-1 proteins is identical except for the N-terminus of the protein, processing of type III- β1a differed from type I-β1a. This transmembrane N-terminal fragment is shown to act as a ligand at the cell surface. Evidence that type III- NRG serve as juxtracrine signals is seen in schwann cells *in vivo* (Morrissey et al.,1995; Salzer et al.,1980). The schwann cells in direct contact with sensory neuron axons have a higher proliferative rate than schwann cells not in contact with axons. This proliferative effect was blocked by antibodies inhibiting neuregulin signalling, providing further evidence of type III -β1a neuregulins interacting in a juxtracrine mode. (Falls, 2003; Morrissey et al., 1995; Salzer et al.,1980). These results suggest that Type III- β1a is involved in cell-cell communication via juxtracrine signalling, whereas Type I-β1a interaction is through a paracrine mode (Falls, 2003; Wang et al., 2001). This is illustrated in **Figure 1.4** below.



led to

an N-

Figure 1.4: **Membrane orientation and processing of neuregulin I- β1a and III- β1a isoforms.** Proteolytic prosessing of type I- β1a isoforms results in the release of a N-terminal fragment containing the bioactive EGF domain. Processing of type III- β1a led to the release of N-terminal fragment containing the bioactive EGF domain, however most of the processing produced an N-terminal fragment that accumulated at the cell surface.

(Falls, 2003)

1.2.3 Linker region of NRG1 proteins

Deletion analysis of the linker region (connects the EGF domain to the transmembrane domain) has shown that it plays an important role in cleavage and release of the bioactive EGF domain of NRG1 isoforms (Montero et al., 2000). Deletion in the linker region of the membrane bound pro-NRGα 2c altered the biological properties, resulting in intracellular entrapment of pro- NRG α 2c and decreased cell surface exposure of the mutant linker and entrapment at the cis-Golgi. This identified that the linker region is implicated in surface sorting of transmembrane neuregulins (Montero et al., 2007).

Cleavage of the extracellular domain has been reported to be regulated by the intracellular domain of membrane bound NRG1 isoforms. Shortening of the cytoplasmic tail or deletion of specific segments with in the cytoplasmic tail can lead the ectodomain not being released (Liu et al., 1998).

The processing of NRG1 proproteins has been shown to be stimulated by activation of protein kinase C, exposure of cells to phorbal esters, serum factors and is sensitive to inhibitors of metalloproteinases (Sunnarborg et al., 2002; Montero et al., 2000; Shirakabe et al., 2001).

1.2.4 Enzymes responsible for cleavage of *NRG1* proteins

ADAMs (a disintegrin and metalloproteases) are a family of membrane anchored glycoproteins (Shirakabe et al., 2001; Sunnarborg et al., 2002; Montero et al.,

2000). Initial experiments using inhibitors of different protease families pointed to metalloproteinases as the enzymes responsible for cleavage of membrane protein ectodomains (Gearing et al., 1994; McGeehan et al., 1994). Use of inhibitors allowed the identification of TNFα converting enzyme (TACE) as the enzyme that participates in the solubilisation of the TNFα from its precursor (Moss et al., 1997). TACE is a type I membrane protein and contains several domains in the extracellular region, including disintegrin and metalloprotease domains characteristic of the ADAM subfamily of metalloproteases. TACE also participates in the cleavage of other transmembrane proteins (Horiuchi et al., 2005). TACE also plays a role in the processing of NRG1 isoforms (Montero et al., 2000). Montero et al. (2000) showed that cleavage of pro-NRG α2c was defective in fibrobalsts derived form TACE-/-animals that express an inactive form of the metalloprotease TACE.

1.3 Epidermal growth factor (EGF) receptors

The neuregulin-1 proteins act as ligands for the epidermal growth factor (EGF) receptors also known as the Her / ErbB receptors (Falls, 2003; Lemke, 1996, Montero et al., 2008, Holmes et al., 1992; Meyer et al., 1997; Wen., 1992; Beerli., 1995). The erb B receptors belong to the receptor tyrosine kinase family of proteins and currently four erbB receptors are known: erbB1, erbB2, erbB3 and erbB4 (Beerli et al., 1995; Slikowski et al., 1994; Chen et al., 1996). All family members have in common an extracellular ligand binding domain, a single membrane spanning region and a cytoplasmic protein tyrosine kinase domain

(Yarden et al., 2001; Riese et al., 1998; Olayioye et al., 2000; Schlessinger et al., 2000). The neuregulin-1 ligands bind the extracellular domain of the erbB receptors leading to the formation of erbB2:erbB3 or erbB2:erbB4 heterodimers and trans-phosphorylation of erbB2 / erbB3 and erbB2 / erbB4 (Falls, 2003; Meyer et al., 1997; Li et al., 2007; Burgess et al., 2003; Montero et al., 2008). The NRG1 proteins are the preferred ligands for erbB3/erbB4 receptors. The phosphorylated tyrosine residues of these receptors serve as docking sites for signalling molecules involved in the regulation of intracellular signalling cascade and serve as mediators of signal transduction (Olayioye et al., 2000; Chen et al., 1996; Pinkas-Kramarski et al., 1996). No soluble growth factor has been identified that binds to erbB2, but it is the preferred dimerisation partner of other erbB family members (Peles et al., 1993). ErbB3 is a binding receptor for NRG1 ligands but lacks intrinsic kinase activity and is not capable of signalling on its own (Funes et al., 2006).

Interaction between receptor and ligand are crucial for cell-cell interaction (signalling) (Yarden et al., 2001; Riese et al., 1998; Olayioye et al., 2000; Schlessinger et al., 2000; Simon et al., 2000; Walker et al., 1998). Recent studies in primary cultures of human airway epithelial cells have demonstrated that activation of the erbB receptors is regulated by segregation of the receptor –ligand complex (Vermeer et al., 2003). In human airway epithelial cells the erbB receptors are located in the basolateral membrane. Heregulin, the ligand (a NRG1 protein) for these receptors is present on the apical surface. Apicobasal cell polarity and tight junctions help to maintain segregation of the receptor from its

ligand. Disruption of tight junctions or of epithelial cell polarity because of injury, will lead to receptor-ligand interaction and cellular proliferation at the site of injury. Conversely, conditions in which epithelial polarity and tight junctions are disrupted can lead to a mitogenic receptor-ligand pair resulting in uncontrolled cell proliferation (Vermeer et al., 2003).

Cleavage by γ-secretase has been reported to result in the release of the soluble erbB4 fragment intracellular domain and facilitation of translocation of this fragment to the nucleus. The soluble erbB4 fragment contains a tyrosine kinase domain and it is possible that nuclear substrates may become phosphorylated by this soluble ErbB4 fragment, further extending the receptors mechanism of action (Ni et al., 2001).

1.3.1 Regulation of ErbB receptor signalling

Following initial activation events, ligand- induced internalisation and degradation of receptor-ligand complexes serves as an important mechanism for attenuating receptor mediated signalling alongside non degradative processes such as dephosphorylation by phosphatases (Wells et al., 1990; Sorkin et al., 1993). Degradation of the erbB receptors is an important mechanism for signal attenuation (Warren et al., 2006). Whereas the EGF domain of NRG1 isoforms is required for the formation of active receptor heterodimers, the N-terminal Ig like domain is shown to be important in accelerating the removal of the activated erbB2 and erbB3, primarily through enhanced degradation of ErbB3 receptor and

in the case of erbB2, dephosphorylation with out receptor degradation appears to be more pronounced. Studies carried out by Warren et al. (2006) have shown that the physical presence of the N-terminal Ig domain was required for these effects. The replacement of the Ig domain with another protein of similar size still resulted in receptor degradation thus highlighting that the effect of the N-terminal Ig domain was sequence –independent.

Some of the negative regulators are actually secreted forms of the cell surface receptors themselves. The secreted receptor isoforms arise through regulated alternate mRNA processing and / or splicing events (Maihle et al., 2002). Lee et al. (2001) identified a secreted receptor isoform arising from the c-erbB3 gene. This study demonstrated that the secreted receptor isoform erbB3 is a naturally occurring inhibitor of heregulin. It blocks heregulin binding to cell surface receptors with high affinity, thereby inhibiting heregulin –induced stimulation of erbB2, 3 and 4 which has shown to be sufficient to effectively block heregulin stimulated cell growth. This novel erbB3 receptor isoform therefore has the potential to be a potent modulator of heregulin regulated cell proliferation and differentiation in normal human tissues.

1.3.2 Overexpression of erbB2 receptor and malignancy

The erbB receptors are expressed in a variety of tissues of epithelial, mesenchymal, and neuronal origin where they play fundamental roles in development, proliferation and differentiation (Yarden et al., 2001; Riese et al.,

1998; Olayioye et al., 2000; Schlessinger et al., 2000; Simon et al., 2000; Walker et al., 1998). Moreover deregulated expression of erbB receptors in particular erbB1 (Rusch et al., 1997; Bartlett et al., 1996; Messa et al., 1998; Grandis et al., 1996) and erbB2 has been implicated in the development and malignancy of numerous types of human cancers (Colomer et al., 2001; Ross et al.,1998). Overexpression of erbB2 has been observed in a significant proportion of breast and ovarian cancers, where it is associated with a poor prognosis (Slamon et al., 1989; Ross et al., 1998; Olayioye et al., 2000; Kraus et al., 1987).

Amplification of erbB2 also known as the Her-2 gene is the major mechanism of overexpression of erbB2 protein. In breast cancer, erbB2 amplification is found to predict decreased survival more consistently than erbB2 protein over expression (Kaptain et al., 2001; Slamon et al., 1987).

Lee.J.C et al. (2002) investigated the expression of erbB proteins in colorectal tumours. Overexpression of erbB3 was identified in early colon cancer, and the percentage coexpression of erbB3 and erbb2 was significantly higher in early stage tumours. The prevalence of erbB3 was reduced in late stage cancers. It was thought that heterodimerisation between erbb2 and erbB4 may play a role in late stages of carcinogenesis.

Studies of erbB2 overexpression in colorectal cancer are inconsistent, with the reported prevalence of erbB-2 overexpression in colon cancer ranging from 0 – 83% (Ross et al., 2001; Caruso et al., 1996). In studies containing smaller

cohorts the rate of gene amplification was in the range of 0-30% (Tal et al., 1998; D'Emilia et al., 1989; Osako et al., 1998). Variations in gene amplification data and erbB2 protein overexpression are thought to be due to studies being underpowered and technical variables in the performance of immunohistochemistry (Nathanson et al., 2003).

1.3.3 Clinical implication of erbB2 receptor overexpression

Monoclonal antibodies to erbB2 receptor (trastuzumab/herceptin) have been approved by the FDA (Federal Drug Administration) for treatment of tumours that express high levels of erbB2 and are in use in patients with breast cancer (Xu et al., 2005). Recent studies have isolated a natural inhibitor of EGFRs called EGFR related protein (ERRP) which is present in most normal human epithelial cells (Yu et al., 2001). Recombinant ERRP has been shown to inhibit the growth of colon cancer cell lines (Yu et al., 2001; Marciniak et al., 2003). In a study by Xu et al. (2005), recombinant ERRP was found to induce apoptosis in colon cancer cell lines in a dose dependant manner. In this study, Herceptin was found to attenuate heregulin (a NRG1 protein) induced activation of cancer cells expressing high levels of erbB-2 but did not induce apoptosis. Xu et al., (2005) concluded that as solid tumours express different EGFRs, identification of ERRP a pan-erbB inhibitor may provide therapeutic benefit to a broader patient population.

1.4 Neuregulin-1 Signalling

Together with fundamental developmental roles (Garratt et al., 2006; Esper et al., 2006; Falls, 2003), the NRG1 proteins have been shown to be involved in a number of cancer types including breast (Tsai et al.,2003; Guerra-Vladusic et al., 1999), ovarian (Gilmour et al., 2002), endometrial (Srinivasan et al., 1999), colon (Venkateswarlu et al., 2002), lung (Gollamudi et al., 2004; Al Moustafa et al., 1999), thyroid (Fluge., 2000), glioma (Flores et al., 2000; van der Horst et al., 2005), medulloblastoma (Gilbertson et al., 1998) and head and neck cancers (O'charoenrat et al., 2000; Breuleux, 2007).

A range of pathways have been reported to be activated by neuregulin-1 proteins including the phosphatidylinositol-3 kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) pathways (Cobb et al., 1995; Venkateswarlu et al., 2002) These pathways regulate cell cycle components at the level of transcription, mRNA stability, translation, post-translation modifications and protein stability (Lee et al., 2000; Wang et al., 1999; Neve et al; 2002).

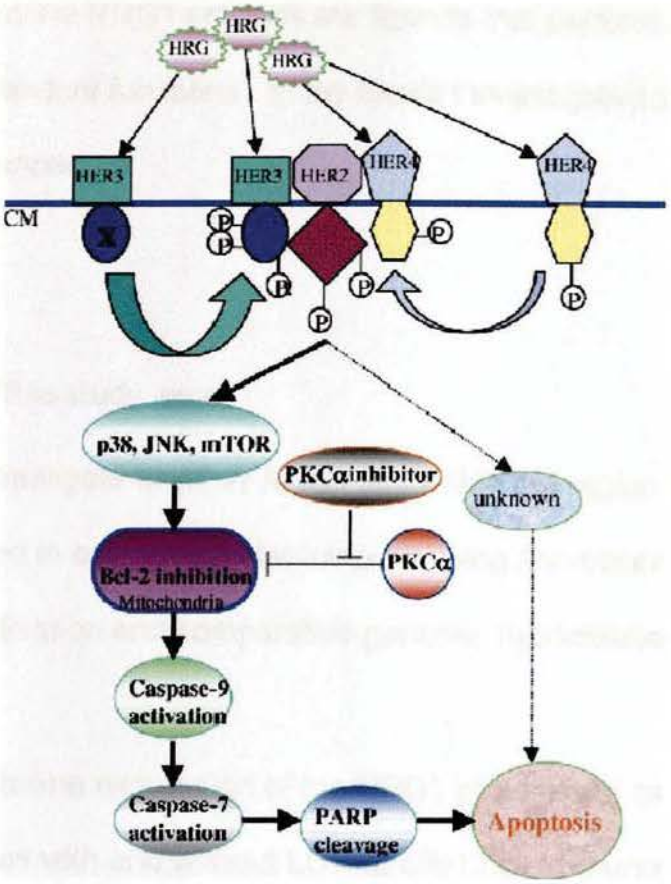
Studies of the NRG1 protein heregulin, have demonstrated that autocrine heregulin activity involving activation of erbB2 contributes to the growth of different types of cancer cells (Slamon et al., 1987; Klapper et al., 2000). A study carried out by Venkateswarlu et al. (2002), examined heregulin and activated erbB2 expression levels in a colon carcinoma (GEO) cell line in different phases of

growth in tissue culture. This study revealed constitutive activation of erbB2 in the absence of exogenous growth factors or serum supplementation in GEO cells in the quiescent state. Blockade of erbB2 receptors with a heregulin neutralising antibody inhibited activation of erbB2 receptor, confirming that endogenous heregulin expression was responsible for activating erbB2 in quiescent cells. These growth arrested GEO cells were subsequently examined for constitutive activation of downstream signalling intermediates. Activation of PI3K and MAPK pathways was analysed in growth arrested GEO cell lysates by investigating expression of activated AKT, p70S6 and ERK1/2 by western blotting. Selective inhibitors to PI3K and AKT and ERK1/2 were also utilized. The findings of this study revealed that activation of erbB2 was the result of heregulin mediated interaction with erbB3 and downstream activation of the ERK and PI3K/AKT pathways. Use of inhibitors confirmed that both the MAPK and PI3K pathways were crucial for re-entry of growth arrested GEO cells into the cell cycle.

A study carried out by Hellyer et al. (2001) concluded that the erbB2/erbB3 heregulin co-receptor couples to PI3 kinase in a heregulin-dependant manner. The recruitment and activation of PI3-kinase by the erbB2/erbB3 co-receptor occurs via its interaction with phosphorylated motifs at the c-terminus of erbB3. Mutations within these motifs prevented erbB3 binding the p85 regulatory or p110 catalytic subunit of PI3K. In fibroblasts, downstream signalling to the effector kinase AKT was dependant upon erbB3 association with PI3-kinase. Recruitment of PI3-kinase to erbB3 was required for the heregulin-dependant translocation of AKT to the plasma membrane for its subsequent activation.

Heregulin also activates matrix metalloproteases (MMP-9), which play an important role in tumour invasion and angiogenesis (Yao et al., 2001; Breuleux, 2007). Heregulin has also been shown to promote motility and invasiveness of cancer cells through regulation of autocrine motility factor expression (Talukder et al., 2000) and enhances formation of lamellipodia, filopodia, and stress fibres which are accompanied by increase in cell migration (Adam et al., 1998) through PI3 kinase signalling. PI3-kinase is involved in the proliferation, survival, adhesion and motility of tumour cells (Way et al., 2005; Breuleux., 2007).

As well as the growth promoting effects, the neuregulin-1 proteins have also been shown to have a growth inhibitory effect (Xu et al., 1997; Daly et al., 1997; Le et al., 2000; Grimm et al., 1998; Weinstein et al., 1998; Le et al., 2001). These effects include induction of apoptosis, differentiation and cell cycle arrest (Le et al., 2000, Le at al., 2002). Heregulin mediated apoptosis occurs through the caspase-9 mediated mitochondrial pathway and down regulation of Bcl-2 (a negative regulator of apoptosis) (Weinstein et al., 1998; Le et al., 2001). Several signalling molecules and pathways have been identified that relate to heregulin induced apoptosis which include, JNK pathway and mammalian target of rapamycin (mTOR) kinase and -p70S6k pathways (Le et al., 2002). The p70S6K are mitogen induced serine/threonine kinases that are involved in cell growth and survival (Avruch et al., 2001). The way in which different pathways interact to induce apoptosis is shown below in **Figure 1.5**.



Le et al., 2002

Figure 1.5: **Pathways involved in heregulin induced apoptosis:** Binding of the neuregulin-1 ligands results in receptor hetrodimerisation and tyrosine phosphorylation of Her-2 and Her-3. This creates docking sites for adaptor proteins and activates the downstream signalling pathways. Activation of caspase-7 results in cleavage of PARP and apoptosis (HRG=heregulin, HER=human epidermal growth factor receptor)

In conclusion the NRG1 proteins are ligands that perform a wide range of context dependant functions. In my thesis I investigated the role of *NRG1* in colorectal cancer.

1.5 Aims

The aims of this study were:

- To investigate whether *NRG1* lies within the region of c8p12 frequently deleted in early colorectal tumours using *fluorescent in-situ* hybridisation and comparative genomic hybridisation.

- To examine expression of the NRG1 protein and its receptor (ErbB2) in tumours with and without LOH at c8p12 by immunohistochemistry.

- To further characterise NRG1 protein expression using Western Blotting.

- To investigate differences in protein cleavage by sequencing of the transmembrane domain in matched normal/tumour colorectal tumours.

- To identify which alternative spliced NRG1 isoforms are expressed in matched normal/tumour colorectal samples.

- To investigate mRNA expression levels of NRG1 isoforms in early and advanced colorectal tumours using quantitative real-time RT PCR.

Chapter 2

Materials and Methods

2.1 LIST OF REAGENTS & SUPPLIERS

Bioline, 16 Edge Business Centre, Humber Road London, NW2 6EW, U.K. 10xNH4 buffer, Biotaq DNA polymerase, RQ1 Rnase-Free Dnase **Amersham Pharmacia Biotech, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, U.K.** Deoxynucleoside triphosphate, ECL chemiluminescence detection, Ready-to –go-you Prime First strand beads **Applied Biosystems, Lingley House, 120 Birchwood Boulevard, Warrington, U.K.** Real-time PCR master-mix, SYBR Green, SYBR green dye solution. BDH Ltd, Church Farm Business Park, Corston, Bath BA2 9AP,U.K. Bio-Rad detergent compatible assay, methanol, sodium citrate, sodium dodecyl sulphate (SDS), acetic acid. **Boehringer Mannheim, Bell Lane, Lewes, East Sussex, BN7 1LG, U.K.** Anti-avidin Cy-3, anti-dig FITC, 6-diamidine-2-phenylindole (DAPI), dig blocking reagent, digioxigenin-11-UTP. **Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, U.K.** Ethylenediaminetetraacetic acid (EDTA), formaldehyde, glycine, sodium chloride. **Invitrogen Ltd, 3Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF, U.K.** Human Cot-1DNA **New England Biolabs, 240 County Road, Ipswich, MA, U.K.** 100 basepair DNA ladder. **Promega Corporation, Delta House, Chilworth Science Park, Southampton, SO16 7NS, U.K.** 10xDnase buffer, Dnase I, random hexamers. **GeneFlow, Fradley Business Centre, Wood End Lane, Fradley, Staffordshire, WS13 8NF, U.K.** Bisacrylamide bisulphate, protomarkers. **Roche biochemicals, Hexagon Place, 6 Falcon Way, Welwyn Garden City, Hertfordshire, AL7 1TW, U.K**. Biotin-16-dUTP, Biotin-nick-mix. **Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, U.K.** Ammonium persulphate, 3-[N-morpholinol] propanesulfonic acid, butanol, β-mercaptoethanol, bovine serum albumin, bromophenol blue, chloramphenicol, diaminobenzidine tetrahydrochloride, ethanol, ethidium bromide, formamide, glucose, glycerol, guanidine hydrochloride, haematoxylin, hydrochloric acid, hydrogen peroxide, isopropanolol, L-B both, marvel, normal goat serum, phenol, phosphate buffered saline (PBS), potassium acetate, proteinase K, sodium

acetate, sodium hydroxide, strepavidin-peroxidase conjugate, tetramethylethylenediamine (Temed), Tris-HCL, Trizol lysis buffer, Tween 20, urea, agarose. **Vector Laboratories, Ltd, 3 Accent Park, Bakewell Road Orton Southgate, Peterborough, PE2 6XS, U.K.** Vector shield mounting solution. **Alta Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TH, U.K.** Oligonucleotides. **Qiagen House, Fleming Way, Crawley West, Sussex, RH10 9NQ, U.K.** Qiagen PCR purification kit. **Zymed Laboratories Inc, 561 Eccles Avenue, South San Francisco, CA, USA.** Mouse anti-cytokeratin 8 antibody, anti-mouse IgG conjugated with horseradish peroxidase. **Santa Cruz Insight Biotechnology Ltd, PO Box 520, Wembley, Middlesex, HA9 7YN, U.K.** HRG (C-20) rabbit polyclonal antibody, heregulin blocking peptide. DakoCytomation, Denmark House, Angel Drove, Ely, Cambridgeshire, CB7 4ET, U.K. HER2 (human epidermal growth factor receptor) rabbit anti-human antibody, goat anti-rabbit immunoglobulin conjugated with HRP.

Cell Lines used in this study

All cell lines were purchase from American Tissue Type Culture (ATCC). H29 (Colon carcinoma), HEK293 (Human embryonic kidney), Colo320 (Sigmoid colon carcinoma), Panc1 (Pancreatic ductal carcinoma), BxPC3 (Pancreatic carcinoma), HS578T (Breast normal tissue), HS174T (Colon adenocarcinoma), SW480 (Colon adenocarcinoma), Caco-2 (Polarized cell line), HCT116 (Colorectal carcinoma), MCF-7 (Breast carcinoma).

2.2 PRIMER SEQUENCES

Table 2.1: Primer sequence of the transmembrane domain.

TM F'	*GGC AAG TGG AAG TGA CCT GT*
TM R'	*AGG CTG TGG AGC TCA GAT TC*

Table 2.2a: RT-PCR primer sequences used for NRG1 isoform analysis

Primer	Primer Sequence 5'-3'	Annealing temperature	Product size
All	CAG TAT CCA CAG AAG GAG CAA AT CTC GAG GGG TTT GAA AGG T	56	150bp
Gamma	GAG TAT CCA CAG AAG GAG CAA AT AGG AGT GAT GAC GAA TGG TG	56	333bp
Beta1	GTG CCC AAA TGA GTT TAC TGG TAG GCC ACC ACA CACATG AT	56	174bp
GGF2	GAG GCT CAA GGA GGACAG C AAC TGG TTT CAC ACC GAA GG	56	236bp
Beta 3	TAC ATC CAC CAC TGG GACAA CCA ACT AGTCGC GAA CAG AG	56	379bp
SMDF	CCG ACA CCG AAG AAT CGT AT TCT CCT TCT CCG CAC ATT TT	56	238bp
Alpha	CTG AGA ATG TGC CCA TGA AA GAA TGA TGG GCT GTG GAA GT	57	367bp
ndf 43	GAG GCC CTC GTG AAT GTA AC TTG CTG ATA GCT GGA TCT GC	56	156bp

Table 2.2b: B-actin primer sequence

Primer	Primer sequence 5'-3'	Annealing Temperature °C	Product size
B-actin	ACA TCC GCA AAG ACC TGT AC ATA CTC CTG CTT GCT AAT CC	55	223bp

Table 2.3: Real-Time Primer Sequences used for NRG1 isoform analysis

Primer	Primer Sequence 5'-3'	Product size
Gamma	GGT TTT CTT GGT TGG TTA TTC AGA A TCT TAC AGC TTA ATT CAT ACA TTG GAT GT	103bp
Beta-1	ACG TAA TGG CCA GCT TCT ACA AG CAC ATG ATG CCG ACC ACA AG	124bp
Beta -3	TCC ATC TTC TTT ATA CAA TGA CCA CAT AAT TAA CTT ACC ATC ATT TCA AGT GCA	87bp
ndf -43	AGG AGG CCC TCG TGA ATG TAA CAT TTG GAT CTG TGT GCC TTG T	135bp
KRT8	GAT CGC CAC CTA CAG GAA GCT ACT CAT GTT CTG CAT CCC AGA CT	100bp

2.3 Extraction of RNA, DNA and protein

Isolation of RNA, DNA and protein from colorectal tissue samples and cell lines was carried out using TRI reagent as per manufacturers instructions.

Colorectal tissue samples were homogenised in 1ml of cold TRI reagent buffer and the samples were transferred to liquid nitrogen for subsequent triple extraction. Cells in 25cm^2 flasks were lysed using 0.5ml of TRI reagent buffer. Cells were left to lyse for 5 mins and were then transferred to a sterile tube and stored at -80°C.

2.3.1 Isolation of RNA

Colorectal tissue and cell line homogenates in TRI reagent buffer were thawed at room temperature (RT). 0.2ml of chloroform was added to each sample, mixed vigorously and stored at RT for 15 mins. All of the samples were centrifuged at 12,000g for 15 mins at 4°C separating the solution into organic phase, interphase and aqueous phase.

To precipitate RNA, the aqueous phase was transferred in to separate fresh tubes and 0.5ml of isopropanol was added to each tube. Samples were stored at RT for 10 mins and then centrifuged at 12,000g for 8 mins at 4°. The supernatant was removed and RNA pellet was washed with 75% ethanol, then centrifuged at 7500g for 5 mins at 4°C. The ethanol was removed and the RNA pellet was air-dried and resuspended in 30µl of distilled water. RNA was stored at -80°C for subsequent use.

2.3.2 Extraction of DNA

DNA was recovered from the interphase and organic phase using ethanol. 0.5ml of ethanol was added to each tube and samples mixed by inversion and incubated at RT for 3 mins then centrifuged at 2000g for 5 mins at 4°C. The phenol-ethanol supernatant was transferred to a fresh tube, and stored at 4°C. The DNA pellet was washed twice in 1ml of 0.1M sodium citrate in 10% ethanol (10ml of 1M sodium citrate, 10ml of ethanol, 80ml of H_20). During each wash the samples were stored at RT for 30 mins, with periodic mixing and then centrifuged at 2000g for 5 mins at 4°C. The DNA pellet was washed in 75% ethanol for 10-20 mins at RT, and centrifuged at 2000g for 5 mins at 4°C. The ethanol wash was removed and the DNA pellet was air dried at RT. Finally, the DNA pellet was resuspended in 8mM NaOH.

2.3.3 Protein extraction

Protein was precipitated from the phenol-ethanol supernatant with isopropanol. 800µl of isopropanol was added to each tube and samples were incubated at RT for 10mins then centrifuged at 4^0C at 12000g for 10mins. The supernatant was discarded and protein pellet was taken through a series of 3 washes in 1ml of 0.3M guanidine hydrochloride in 95% ethanol for 20 mins at RT and centrifuged at 7500g for 5 mins at 4^0C. The protein pellet was resuspended in 2ml of 100% ethanol, incubated at RT for 20 mins and centrifuged at 7500g for 5 mins at 4°C. The ethanol wash was removed and the pellet was air-dried and dissolved in 100ul of 6M urea. Samples were stored at RT overnight to allow protein solubilisation.

2.3.4 Extraction of DNA using proteinase K

DNA from matched normal/tumour colorectal samples was extracted using proteinase K and used in comparative genomic hybridisation and sequencing reactions. 0.5ml of DNA digestion buffer (50mM Tris-HCL pH8.0; 100mM EDTA pH8.0; 100mM NaCl ; 1% SDS) with 0.5mg/ml proteinase K, was added to a small section of frozen tissue and samples were incubated overnight at 37°C. The samples were rotated overnight to allow mechanical agitation to aid in disruption of the tissues. 0.7 ml of phenol was added to the samples which were then centrifuged at 12000g for 5 mins. The upper phase (0.5ml) was recovered and 0.7 ml of chloroform was added. The samples were mixed and centrifuged for a further 5 mins and the aqueous phase removed. 1ml of 100% ethanol was added and the samples were mixed by inversion. Samples were microfuged for 5 mins at 13 000g and the supernatant was carefully removed and discarded. 0.5ml – 1ml of 70% ethanol was added to the samples and microfuged for 5 mins at 13 000g after which the supernatant was carefully removed and discarded. The samples were air dried and DNA was resuspended in 100 – 200 µl of TE buffer.

2.4 Isolation of DNA from bacterial artificial chromosome clones

Clones were streaked on to agar containing 25µg/ml of chloramphenicol and grown overnight at 37° C. 5ml of autoclaved LB broth containing 25µg/ml of chloramphenicol was inoculated with a single colony and incubated overnight at 37°C in a shaker to provide constant agitation. The overnight culture was centrifuged at 1,500g for 10 mins following which the LB broth was discarded and

the pellet was resuspended in 200µl of lysis buffer (10mM Tris pH 8.0, 25mM EDTA pH 8.0, 50mM Glucose) and incubated on ice for 5mins. 400µl of a freshly prepared solution of 0.2M NaOH, 1% sodium dodecyl sulphate, was added. The contents were mixed gently by inversion and incubated on ice for a further 5 mins. 300µl of prechilled 3M potassium acetate was added and the contents mixed gently by inversion and placed at -80°C for 30 mins. The suspension was allowed to thaw following which it was centrifuged at 13,000rpm for 15 mins to collect cell debris and precipitated salts. The resulting supernatant was transferred to a fresh tube and 0.6 volumes of isopropanol was added and contents mixed by inversion and left to precipitate at RT for 10 mins. The contents were centrifuged at 13,000 rpm for 15 mins to pellet the DNA. The supernatant was discarded and the DNA pellet washed was with 1ml of 70% ethanol and centrifuged at 13,000 rpm for 2 mins. Following centrifugation the ethanol was removed and the DNA pellet was dried and resuspended in 40µl of TE pH 8.0 (50mM Tris, 50mM EDTA) overnight. Finally 1µl of 10µg/ml of RNAse A was added and samples were incubated at 37°C for 1 hour. The DNA was stored at -20°C.

2.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to resolve DNA, reverse-transcriptase and real-time PCR products. The agarose gels were made to a concentration of 1-3%, depending on the size of fragments to be resolved in 100ml of 1x TAE buffer pH 7.8 (Final working buffer concentration (1X): 40mM Tris acetate, 1 mM EDTA) Tris base, EDTA, NaOH) and 0.01mg/ml ethidium bromide. The type of comb

used to cast the wells depended on the number and volume of samples. The gel was allowed to set for 1hour after which it was immersed in a gel tank containing enough running buffer (the same used for casting the gel) to cover the gel. Prior to loading the wells, 1ul of loading dye (6X Loading dye 15% w/v Ficoll 400, 0.025% w/v Bromophenol Blue, 0.025% w/v Xylene Cyanol) was added to 5µl of each sample. 0.5µg of molecular weight marker 100bp DNA ladder was also run alongside the samples. The gel was run at 100V for approximately 40 mins and photographed on a UV transilluminator.

2.6 Quantification of RNA, DNA and protein

2.6.1 Quality and quantity of extracted RNA

Formaldehyde gel electrophoresis and spectophotometry were used to determine the quality and quantity of extracted total RNA respectively.

RNA quality

This was determined by Northern gel electrophoresis. A 50 ml formaldehyde gel (0.75g agarose, 37 ml H_2O and 5 ml of 10x MOPS buffer (0.2 M MOPS (3-[N_morpholinol] propanesulfonic acid, 0.05M sodium acetate, 0.01 M EDTA, made up to pH 7.0 with 1M NaOH) was made. The gel was melted and allowed to cool to 50 °C prior to adding 8ml of 37% formaldehyde, The gel was placed in a tank with 1 X MOPS but was not fully submerged.

5 µl of RNA (1 µg) was added to 15 µl of loading buffer (0.72 ml Formamide, 0.16 ml 10 X MOPS buffer, 0.26 ml Formaldehyde (37%), 0.08 ml Bromophenol blue

(saturated solution), 0.18 ml H_2O, 0.10 ml 80% glycerol) and heated to 60° C for 5 mins and placed on ice. The gel was loaded and run at 80 to 100 volts. The gel was washed in 10% glycine for 20 mins after which 100 µl of 10 mg/ml ethidium bromide was added per 250 ml of glycine and the gel was incubated for a further 10 mins. The gel was destained in H_2O for 2 X 20 mins and 10X SSC (1.5M NaCl, 0.15 M Na_3 Citrate, adjusted to pH 7.0 with 1 M HCL) for a further 20 mins, and photographed.

Quantitation of RNA

A spectophotometer was used to quantify the amount of RNA present in the samples. The absorbance of RNA was measured at wavelengths of 260 and 280nm using a quartz cuvette. The absorbance reading at 260nm allows for the calculation of the concentration of nucleic acids in the sample. An absorbance of one unit at 260 nm corresponds to 40 µg of RNA per ml (*A*260 =1= 40 µg/ml). The ratio of A260/A280 provides an assessment of the purity of the nucleic acid.

To calculate the RNA concentration the following calculation was used:

RNA conc ug/ml = A260 x 40 X dilution factor.

2.6.2 DNA quantification and Quality

DNA quality

The quality of the extracted DNA was assessed by direct analysis of 0.5µg of DNA on a 1% agarose gel.

DNA quantitation

Spectrophotometery was used to quantify the amount of DNA extracted. Spectrophotometer readings were taken at wavelengths 260nm and 280nm. The 260nm reading allowed the concentration of DNA to be calculated and an OD reading of one corresponds to approximately 50 µg/mL for double-stranded DNA. The DNA quality measurement is based on the fact that OD at 260 nm is twice that at 280 nm if the solution contains pure DNA. Clean DNA has an OD-260/OD280 between 1.8 and 2.0. DNA was quantitated using the following calculation:

DNA conc ug/ml: A260 x 50 X dilution factor.

2.6.3 Protein Quantification

Protein concentration was determined using the Bio-Rad detergent compatible assay. Depending on the volume of protein this assay can be adapted to determine high (0.2-1.5mg/ml) and low (5-250µg/ml) concentrations of proteins. Bovine serum albumin (BSA) was used as a control to plot a standard curve for Bio-Rad protein assay. The BSA was dissolved in 6M urea (1mg/ml) and serial dilutions were carried out. In brief, a 10-step two-fold dilution series of BSA was carried out in a 96 well microplate and used as controls.

10µl aliquots of protein samples were distributed into further wells on the plate and 200µl of Bio-Rad DC mix (Reagent A and B) at a dilution of 1:5 respectively

was added to each well. A blue colour change indicated the presence of protein in the samples.

The absorbance was measured at 595nm using a microplate reader and a standard curve of absorbance vs. BSA concentration (mg/ml) was plotted to determine protein concentrations of the colorectal samples.

2.7 cDNA synthesis

First strand cDNA synthesis was carried out using 1ug of RNA following DNase digestion. 1µl of 10 x DNase buffer and 1µl RQ1 Rnase-Free DNase (1 unit) was added to 1µg of RNA in a fresh tube the solution was mixed and incubated at RT for 15 mins. The reaction was terminated by adding 1µl of Stop solution (25mM EDTA pH 8.0) and incubating at 65° C for 15 mins.

22µl of DEPC – treated H_20 was added to DNase -treated RNA and incubated at 65°C for 10 mins. The samples were placed in ice for 2 mins following which the contents were transferred to a fresh tube containing ready-to-go-you Prime First strand beads. 1µl of random hexamers was added to the tube and samples were incubated at RT for 1 minute. The samples were vortexed, centrifuged and incubated at 37°C for 1 hour. The cDNA produced was stored at -20°C.

2.8 *Fluorescent in-situ* hybridisation

2.8.1 Probe labelling

Probes were labelled by nick translation. Centromeric probes (D8Z2, C8 centromeric probe) were labelled with digioxigenin-11-UTP and probes to the region of interest (*NRG1*) were labelled with biotin-16-dUTP. Probe DNA (5.5ug, 3ul) was nick translated with 4ul digoxin or biotin-nick mix stabilized reaction buffer (50% glycerol and DNase polymerase 1, DNase 1, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP and 0.08 mM Biotin-16-dUTP/digioxigenin-11-UTP). The reaction was made up to a final volume of 19µl with distilled H_20, mixed, centrifuged briefly and incubated for 90 mins at 15°C. The reaction was terminated by adding 1µl of 0.5 M EDTA (pH 8.0) and incubating at 65°C for 10 mins.

2.8.2 Preparation of touch-prep and lymphocyte metaphase spreads

To prepare touch-prep slides a small piece of tumour tissue was cut from the frozen tumour sample, thawed and blotted on Whatman 3MM paper before touching onto a polylysine coated slides. The slides were immediately fixed in methanol: acetic acid (3:1) for 20 mins, air-dried and stored at -20°C.

Lymphocyte metaphase spreads

Cultured lymphocyte suspension (gift from Genetic Unit, Birmingham Womens Hospital NHS Trust) was placed on to a slide and allowed to air dry prior to

fixation with freshly prepared (3:1) methanol: acetic acid for 20 mins. Slides were air dried and stored at 20°C until used.

2.8.3 Pre-treatmemt of slides

Slides were pre-treated prior to hybridisation by washing in 3:1 methanol and acetic acid for 5 mins, followed by 2xSSC (0.3M sodium chloride, 0.03M sodium citrate) for 10 mins. Slides were incubated in 0.01% pepsin solution (0.01g pepsin in 1M HCL) at 37°C for 10 mins, washed in 4% paraformaldehyde for 5 mins then in distilled H_2O for 5 mins and allowed to air-dry. Slides were denatured in 70% formamide at 80°C for 5 mins, dehydrated in 70%, 85% and 100% cold ethanol series for 2 mins each and allowed to air-dry.

2.8.4 Hybridisation

The hybridisation mix consisted of 14µl of mastermix (5ml of deionised formamide: (200ml formamide, 10g mixed resin beads); 1ml of 20x SSC: (3M sodium chloride (175.2g/l), 0.3M sodium citrate (88.2g/l); 1g of dextran sulphate and 7µl of dH_2O) 5.5µg (3µl) of labelled probe, specific to the region of interest (*NRG1* gene), 1µg (2µl) of labelled centromeric (D8Z2) probe and 1µl of human Cot-1 DNA. The hybridisation mix was denatured at 90°C for 5 mins and then incubated at 37°C for 15 mins. The pre-treated slides were placed on a hot block at 37°C and 30µl of hybridisation mix was added, a coverslip was placed over and sealed with rubber solution. The slides were incubated in a moist chamber at 37°C overnight.

2.8.5 Detection of probes

The coverslips were gently removed from the slides which were taken through a series of post hybridisation washes at 45°C in 50% formamide for 5 mins x 3, twice in 2 x SSC, once in 4 x SSC, 0.05% Tween 20 (200ml of 20x SSC, 800ml dH_2O, 0.5ml Tween 20) and finally in 4 x SSC, 0.05% Tween 20 for 5mins at RT. To detect biotin labelled probes slides were incubated with 100µl of anti-avidin Cy3 in blocking reagent (final working concentration:

0.25µl of 2mg/ml stock of anti-avidin Cy3 in 99.75µl of blocking reagent : 500ml of 4 x SSC, 0.05% Tween 20, 25g non-fat milk powder) under parafilm for 20 mins at 37°C. Slides were then washed 3 times in 4 x SSC, 0.05% Tween 20 for 5 mins each.

To detect the digoxygenin probes slides were incubated with 100µl of anti-digoxygenin conjugated to FITC in blocking solution (final working concentration: dilute 5µl of anti- digoxygenin conjugated to FITC in 200µl of digoxygenin blocking reagent: 4 x SSC, 0.05% Tween 20, 0.5% dig blocking reagent) under parafilm at 37°C for 20 mins. Slides were taken through a further series of washes as described previously and were counterstained in 4, 6-diamidine-2-phenylindole (DAPI; 0.5µg/ml) in vector shield mounting solution prior to microscopy.

2.8.6 Image analysis and quantification

Slides were examined under a fluorescent microscope using Smart Capture software. For each tumour sample, 100 tumour cell nuclei were identified and scored for both Cy3 (*NRG1*) and FITC (8 centromere) signal. Deletion was defined as cells, which had fewer Cy3 signals than FITC signals. Cells, which appeared intact and were not overlapping, were counted. In order to limit false positives, cells with no FITC signal were discarded for deletion scoring.

2.9 Comparative genomic hybridisation (CGH)

This work was carried out in collaboration with Dr P.Edwards (Cambridge MRC centre). DNA from matched normal/tumour samples was sent to the MRC centre in Cambridge for CGH analysis. The method employed for CGH has been described by Huang et al. (2004).

DNA extracted from tumour samples (Proteinase-K) and normal reference DNA were differentially labelled with fluorescent dyes and co-hybridised in equal amounts to normal metaphase spreads. The difference in fluorescence intensity that reflects the ratio of tumour DNA to control DNA hybridised to chromosomal spread were measured using digital imaging and software analysis to determine fluorescence values. The amplification and deletion of specific chromosome regions were detected by the increased and decreased ratios of tumour specific fluorescence to control respectively.

2.10 Immunohistochemistry

Paraffin tissue sections/slides were dewaxed in xylene for 5 mins and rehydrated in ethanol series (100% ethanol x 2, 70% ethanol x1 for 5 mins each). Slides were then taken through a series of three washes in phosphate buffered saline (PBS) for 5 mins each. Slides and cells mounted on coverslips were blocked with hydrogen peroxide in methanol (15ml of 30% hydrogen peroxide in 500ml of methanol) for 15 mins and then further washes with PBS were carried out as described above. Slides and coverslips were blocked with 10% normal goat serum for 30 mins and then incubated with the primary antibody of appropriate concentration in 10% normal goat serum overnight at 4°C. A further 3 washes with PBS were carried out, after which sections and coverslips were incubated with biotinylated secondary antibody of appropriate concentration for one hour. Serial PBS washes were performed and the sections were incubated for 30 mins with streptavidin-peroxidase conjugate before incubation with diaminobenzidine tetrahydrochloride. Finally, further serial PBS washes were carried out after which sections and coverslips were counterstained with haematoxylin, dehydrated in ethanol series (70% ethanol x1, 100% ethanol x 2 for 5 mins each) and fixed in xylene for 5 mins. Slides and coverslips were mounted using non-aqueous permanent mounting medium and a coverslip was placed. The primary and secondary antibody concentrations were titrated until good staining with little or no background was achieved with a primary antibody concentration of 1:200 and a secondary antibody concentration of 1:500. The heregulin blocking peptide was used as a control. The blocking peptide was prepared as per manufacturers instructions. 1µl of primary antibody was added to a five-fold excess of blocking

peptide (1µg /5ul) in a small volume (50µl) of PBS for neutralisation. The antibody /blocking peptide mixture was incubated at RT for 2 hours following which the mixture was diluted in a 150µl of PBS for use.

Slides were analysed by light microscopy. The intensity of staining and the proportion of positively stained tumour cells were scored. Immunoreaction was determined as: 1+ weak staining, 2+ moderate staining and 3+ strong staining patterns. The proportion of tumour stained was divided in to 4 categories, 0-10%, 10-25%, 25-50%, 50-75% and >75% tumour stained.

Slides were scored by a second independent observer (T.Philippe).

2.11 Immunocytochemical staining: HercepTest™

The HercepTest assay was used to investigate ErbB -2 overexpression. This work was carried out in collaboration with Dr.P.Taniere (Pathology Department, University Hospital Birmingham).

Paraffin sections were dewaxed in xylene for 5mins and rehydrated in ethanol series (100% ethanol x 2, 95% ethanol x2 for 4 mins each).

Slides were placed in distilled water for a minimum of 30 secs and were then immersed in preheated epitope retrieval solution (0.1mol/L citrate buffer with an antimicrobial agent) and incubated in a water bath at 95-99°C for 40 mins.

Slides were removed and allowed to cool at RT for 20mins and were then rinsed in wash buffer (x10Tris/HCL buffer working dilution 1:10)

and blocked with 200ul of peroxidase –blocking reagent (3% hydrogen peroxide containing 15mmol/L sodium azide) for 5 mins. Slides were then rinsed in wash buffer, and incubated with 200ul of primary Anti-HER2 protein antibody (or

negative control reagent- immunoglobulin fraction of normal rabbit serum at an equivalent protein concentration as the antibody to HER2 protein) for 30 mins. Following incubation, slides were rinsed and incubated with 200ul of visualization reagent (dextran polymer conjugated with HRP and affinity-isolated goat anti-rabbit immunoglobulins in Tris/HCL buffer containing stabilising protein and an antimicrobial agent) for 30 mins. Slides were rinsed in wash buffer x2 and incubated in diaminobenzidine tetrahydrochloride (DAB) for 10 mins, rinsed with deionised water and counterstained in a bath of haematoxylin for 2-5 mins. Residual haematoxylin was removed by gently rinsing the slides in a distilled water bath for 2-5 mins. All slides were mounted with non-aqueous, permanent mounting medium prior to viewing.

Slides were analysed using light microscopy and scored using the HercepTest guidelines. Sections of breast carcinoma known to be strongly, moderate and weakly positive for erbB-2 receptors were used as controls. The Hercep Test was reported as being negative if no membrane staining was observed or <10% of tumour cells were stained (score 0) and if faint staining was noted in only part of the membrane of >10% of tumour cells (score 1+). A positive Hercep Test for erbB2 overexpression was reported when >10% of tumour cells had weak to moderate complete membrane staining (score 2+) and if strong complete membrane staining was noted in >10% of tumour cells (score 3+). Slides were scored by a second independent observer (T.Philippe).

Tumour samples were excluded from further analysis if staining artefacts were noted and if tissue sections on slides were incomplete and therefore not interpretable.

2.12 Multiple Tissue array (MTA) construction

A multiple tissue array was used to investigate heregulin expression and erbB2 overexpression in a large cohort of colorectal tumours. The MTA was constructed at the Pathology Department (Queen Elizabeth Hospital, University of Bimingham, NHS Trust) and contained 250 paraffin embedded colorectal tumours. All tumour samples were collected according to local ethical guidelines. For the purpose of this study, permission was obtained to use the tissue array. Each slide contained sections of 20 different tumour samples as shown in **Figure 2.1** below.

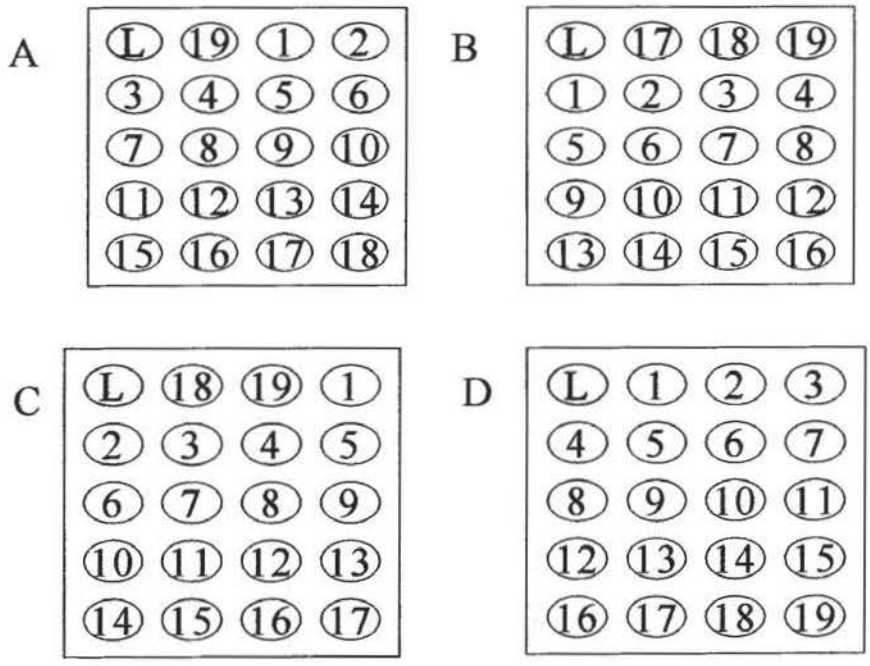


Figure 2.1:Multiple Tissue Array Key. The above figure illustrates the orientation of colorectal tumours samples in each section. Liver tissue was used to orientate the slides.

In order to take into account tissue fixation and variation in reproducibility of immunohistochemical data, four slides containing the same group of 20 tumours (eg 1A, 1B, 1C, 1D) were obtained in which each of the individual tumour sample were embedded at different locations. Liver tissue (a positive control for heregulin expression) was used to help with orientation of the slides. A replicate tissue array was obtained so that the same sections were used to investigate heregulin expression and ErbB2 overexpression.

2.12.1 Clinical and pathological data

Clinical and pathological data were collected in accordance with ethical guidelines. Tumour grade, stage, lymph node involvement and evidence of metastases were collected. The array was constructed with tumour sections from patients who had undergone surgical resection between June 1997 and Feb 2002. Follow up data was collected till June 2005.

2.13 Western Blotting

A separating gel (1.5M Tris pH 8.8, 4.0ml of bisacrylamide bisulphate, 100ul of 10% sodium doceyl sulphate (SDS), 50ul of 10% ammonium persulphate, 3.4ml of distilled H20 and 10ul of 99% Temed) was made and poured between two BIORAD mini gels plates. The top surface of the gel was covered with water saturated butanol and allowed to set at RT for 1 hour. Once the gel had set, the water saturated butanol layer was poured off, and the top surface of the gel was rinsed with distilled H_20. A stacking gel was made (6.1ml of dH_20, 2.5ml

bisacrylamide (37.5:1), 1.5ml of 0.5M Tris pH 6.8, 100ul of 10% SDS, 50ul of 10% ammonium persulphate and 10ul of 99% Temed) and poured on top of the separating gel. A comb was placed between the two glass plates. The size of comb used depended on the number and volume of the samples. The gel was allowed to set for 40 mins.

Cell lysates and colorectal protein samples were prepared by adding 2 x SDS-PAGE sample buffer (1.0 ml of 0.5 M Tris pH 6.8, 0.8ml of Glycerol, 1.6ml of 10% SDS, 0.4ml of B-mercaptoethanol and 0.2ml of bromophenol blue). Prior to loading the samples were boiled for 5 mins to denature the protein. The gel was placed in a tank containing 1x SDS-PAGE buffer (3g Tris, 14.4g Glycine, 1g sodium doceyl sulphate/L). 20µg of protein was loaded in to each well and 5µl of protein marker (ProtoMarkers) was run alongside the protein samples. The gel was run at 100V until the tracking dye had just run off the gel. The stacking gel was removed and the separating gel was used for transfer.

2.13.1 Transfer

Hybond ECL Nitrocellulose membrane, sponge pads and 3MM Whatman paper were soaked in Towbin transfer buffer (3.03g/l of 25mM Tris, 14.4g/l of 192mM glycine and 200ml/l of 20% methanol) for 10 mins to allow equilibration of the membrane. A sandwich consisting of sponge pad, 3MM Whatman, nitrocellulose membrane, separating gel, 3MM Whatman paper and sponge pad was made and placed in an electroblotting cassette. The Hybond ECL nitrocellulose membrane was placed on the anode side of the gel to allow transfer of proteins from gel to nitrocellulose membrane Transfer was carried being running at 110V for 90 mins.

Following transfer the membrane the orientation of the gel was marked on the membrane.

2.13.2 Optimisation of antibodies for Western blotting

A range of primary and secondary antibody dilutions were used to optimise the antibody concentration for Western Blot analysis. The primary and secondary antibody concentrations were titrated until bands with little or no background staining were achieved. Following transfer, the nitrocellulose membrane was incubated in 4% Marvel in 1xTBST (10mM Tris pH 8, 0.15M NaCl and 0.05% Tween 20) for 1 hour to block non-specific binding and reduce background staining. The membrane was incubated with primary antibody (diluted in 2% Marvel in 1x TBST) of appropriate concentration overnight and taken through a series of 3 washes in 1x TBST for 5 mins each. The nitrocellulose membrane was incubated with the secondary antibody (diluted in 2% Marvel in 1x TBST) for 1 hour. Finally, further washes in 1x TBST was carried out as described previously. Primary and secondary antibody concentrations of 1:500 and 1:50,000 were used respectively for Western Blotting. The heregulin blocking peptide was prepared as per manufacturers instructions. 1µl of primary antibody was added to a five-fold excess of blocking peptide (1µg /5ul) in a small volume (50µl) of PBS for neutralisation. The antibody /blocking peptide mixture was incubated at RT for 2 hours following which the mixture was diluted in a 1ml of PBS for Western blot analysis.

To check for protein loading the membranes were reprobed with cytokeratin 8 antibody. Cytokeratin 8 is present in simple epithelial cells and

adenocarcinomas. It is not expressed in squamous epithelia and is a basic type II cytokeratin with a molecular weight of ~ 52kDa. A monoclonal mouse anti-cytokeratin 8 primary antibody (1:200 dilution) and anti-mouse IgG conjugated with HRP was used as a secondary antibody (1:1000 dilution). Blots were taken through a series of three washes in 1xTBST for 5 mins each and then incubated in primary cytokeratin 8 antibody (diluted in 2% Marval in 1xTBST) for 1hour at RT. The blot was then taken through a further 3 washes in 1 x TBST and then incubated with secondary antibody for 1 hour at RT. Finally, further washes in 1x TBST was carried out as described previously.

2.13.3 Detection of protein

The protein was detected using the ECL chemiluminescent detection method. Equal quantities of ECL detection reagents A and B were mixed and poured into a petri dish. The membrane was placed into the petri dish (protein side down) and covered with the ECL mix for 1 min. The blots were then Saran wrapped, and placed protein side up in an x-ray cassette where multiple exposures of autoradiography film was carried out to obtain the best image.

2.14 Antibodies used

Primary antibodies:

- HRG (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc), [conc used for immunohistochemistry 1:200; conc for Western 1:500]
- HER2 (human epidermal growth factor receptor) rabbit anti-human antibody (DakoCytomation California Inc).
- Anti-cytokeratin 8 mouse monoclonal antibody (Zymed Labs, Ca) [conc for Western 1:200]

Blocking peptide:

- Heregulin blocking peptide (Santa Cruz Biotechnology Inc), [100µg peptide in 0.5ml PBS containing 0.1% sodium azide and 100µg BSA]

Secondary antibodies:

- Anti-rabbit IgG antibody (Sigma) conjugated to horse radish peroxidase (HRP) [conc used for Western 1:50000]
- Goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase (DakoCytomation California Inc) [conc used for immunohistochemistry 1:500]
- Anti-mouse IgG conjugated with horseradish peroxidase [conc used for immunohistochemistry 1:1000]

2.15 Sequencing of transmembrane domain

Optimisation and design of transmembrane primer

Primers were designed (using the Primer 3 output programme across the intron/exon boundary that encompassed the whole of the transmembrane domain sequence. The forward and reverse primer sequences were put through a Blast search to ensure that each sequence was specific to the gene of interest.

Reactions were optimised using DNA from human embryonic kidney (HEK 293) cell lines. PCR conditions were: 94°C for 2mins, 35 cycles of 94°C for 10 sec, 60°C for 20 sec, 72°C for 30sec followed by a final elongation step of 72°C for 5min. The tumour samples selected for sequencing of the transmembrane domain are shown in **Table 2.4** below.

Sample No	Histology	Deletion status
33	Advanced tumour T4N1M0	Deletion
38	Advanced tumour T4N0M0	Deletion
65	Advanced tumour T3N0M1	No deletion
66	Advanced tumour T3N2M1	No deletion

Table 2.4: Clinicopathological and deletion status of tumour samples used for sequencing of transmembrane domain

DNA was extracted from the above tumours using Tri reagent as previously described.

Reverse transcriptase–PCR (RT-PCR) with the transmembrane primers on matched colorectal normal/tumour samples

In order to sequence the transmembrane domain of the above matched normal/tumour samples, the region of interest was amplified by RT-PCR using primers that flanked the transmembrane domain (shown in **Table 2.1**). cDNA from the human embryonic kidney cell line HEK 293 was used as a positive control and distilled H_20 as a negative control.

All of the PCR products were resolved on an agarose gel. 5ul of the PCR product was run alongside 0.5ug of 100 bp DNA ladder on an agarose gel. The gel was photographed on a UV transilluminator using Polaroid film (Sigma) with exposure time of 1 sec.

Sequencing of the transmembrane domain

For this purpose the QIAquick PCR purification protocol was used. 5 volumes of buffer PB was added to 1 volume of PCR sample and mixed. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 30-60secs in a microcentrifuge at 13,000rpm. The flow-through was discarded and the QIAquick column was placed back in 2ml collection tube. To wash, 0.75ml of buffer PE was added to the QIAquick column and centrifuged for 30-60 secs. The flow-through was discarded and the column was placed back in the collection tube and centrifuged for an additional 1 minute. The QIAquick column was then placed in a

clean tube and 50ul of buffer EB (10mM Tris-Cl, pH 8.5) was added to the column and centrifuged for 1 min to elute the DNA.

5ul of each sample was run on an agarose gel to check the yield of DNA and to ensure that the product was clean.

To sequence a 200-500bp product, 3-10ng of DNA template was required.

For each sample the amount of DNA present was estimated by comparing the intensity of gel bands against molecular weight markers. Each sample was taken through a PCR reaction using the Big Dye Cycle sequencing kit. Each reaction contained, 2ul of the terminator ready reaction mix, 6ul of dilution buffer (200mM Tris-HCL (pH 9), 5mM $MgCl_2$), 1ul of 5pmole primer and appropriate volume of DNA template for each sample. The final volume was made up to 20ul with distilled water. For each sample the reaction was carried out with both the forward and reverse primer sequence. The PCR products of the sequencing reaction were purified (Big Dye Version 3) and sequenced at the Functional Genomics Unit (University of Birmingham).

The sequencing data obtained for each fragment sequence was put through a Blast search (NCBI website). The sequencing data obtained for each matched normal/tumour sample was analysed. The sequence data was checked to ensure that each peak seen corresponded to the correct nucleotide sequence.

2.16 Isoform- specific reverse transciptase polymerase chain reaction (RT – PCR)

All PCR reactions were prepared in a final volume of 25µl. Each reaction contained 0.75µl of 50mM $MgCl_2$, 2.5µl of 10 x NH4 buffer, 0.1µl of Biotaq DNA

Polymerase, 0.5µl of 100mM deoxynucleoside triphosphate stock, 0.5µl of reverse and forward primer (50pmol conc), 1µl of cDNA sample and was made up to 25µl with distilled water in PCR tubes. Samples were then placed in a PCR thermocycler.

Primer design and RT- PCR conditions

Primer design and optimisation conditions are described in detail in **Chapter 7**. All of the PCR reactions were prepared to a final volume of 25ul as described previously. For each primer pair the PCR conditions were optimised using positive controls. Following optimisation, isoform specific RT- PCR reactions were carried out using 1ul of cDNA from10 different matched normal and tumour colorectal samples.

PCR running conditions were as follows; 94°C for 2mins, 35 cycles of 94°C for 20 sec, 56°C for 30 sec, 72°C for 40sec followed by a final elongation step of 72°C for 5min. The primer sequences and annealing temperatures and are shown in **Table 2.2a**. All of the PCR products were resolved on agarose gel as described previously. The gel concentration varied between 2- 3%, depending on the size of the fragments that needed to be resolved. 5ul of the PCR product was run alongside 0.5ug of 100 bp DNA ladder on an agarose gel.

2.17 Sequencing of Beta 1 Isoform

For this purpose the QIAquick gel extraction protocol was used. The fragments of interest were excised from the agarose gel and weighed. The samples were incubated in 3 volumes of buffer QG to 1 volume of the gel until the gel slices had dissolved. The samples were then placed in a QIAquick spin column and centrifuged to bind DNA. A series of washes were carried out where buffers were added to the QIAquick columns and centrifuged to remove all of the agarose and salts. The samples were eluted in 30ul of EB buffer. The samples were analysed on a gel following elution.

Each fragment (top and bottom band) was taken through a PCR reaction using the Big Dye Cycle sequencing kit. Each reaction contained, 2ul of the terminator ready reaction mix, 6ul of dilution buffer (200mM Tris-HCL (pH 9), 5mM $MgCl_2$), 5ul of DNA template and 1ul of 5pmole primer. The final volume was made up to 20ul with distilled water. For each fragment the reaction was carried out with both the forward and reverse primer sequence. The PCR products of the sequencing reaction were purified (Big Dye Version 3) and sequenced at the Functional Genomics Unit (University of Birmingham).

The sequencing data obtained for each fragment sequence was put through a Blast search (NCBI website) and the top band was found to contain the exon 10 sequence which is specific to *NRG1*- Beta 1.

2.18 Real -time RT- PCR

In comparison to reverse transcriptase - PCR, real-time PCR allows quantitation of cDNA /DNA and collects data whilst the reaction is proceeding. Real-time PCR products are detected via the generation of a fluorescent signal. Samples are analysed using an ABI Prism 7700 Sequence Detection System The machine contains a sensitive optical detection system that monitors the fluorescence in each well of a 96 well-plate reaction at frequent intervals during the PCR reaction.

For the purpose of this analysis SYBR Green, a fluorogenic dye, was used for real-time analysis. SYBR Green binds the minor grooves of double stranded DNA causing fluorescent emission. As double stranded DNA is synthesized, more SYBR Green will bind and fluorescence is increased.

2.18.1 Primer Design

Quantitative real-time PCR primers were designed using the Primer Express version 1.5 programme (Applied Biosystems). Whilst designing primers the following guidelines were used: G-C content was kept in the 30-80% range, runs of an identical nucleotide were avoided (especially true for guanine, where runs of four our more Gs should be avoided), the Tm was kept between 58-60°C, no more than two G and/or C bases were present in the five nucleotides at the 3' end and finally the maximum amplicon size did not exceed >150 basepairs. The primer sequences are shown in **Table 2.3**.

2.18.2 Quantitation of primers

All primers were quantitated using spectophotometry to determine the concentration of the primers. The absorbance was measured at 260nm and the extinction coefficient contribution was calculated for each primer using the values in **Table 2.5**.

Chromophore	Extinction Coefficient	Number	Extinction Coefficient Contribution
A	15,200	1	15,200
C	7,050	6	42,300
G	12,010	5	60,050
T	8,400	6	50,400
Total			**167,950**

Table 2.5: Extinction coefficient contribution

Finally the oligonucleotide concentration (C) in uM was calculated using the equation below:

Absorbance (260nm) = sum of extinction coefficient contributionsx cuvette pathlength x oligoneucleotide concentration / 100.

The primers were made up to a 5µmole concentration and primer dilutions of 50/50(0.75µl), 300/300(9µl) and 900/900(13.5µl) were used for optimisation on positive controls.

2.18.3 Preparation of Real-time RT-PCR samples

All PCR reactions were prepared to a final volume of 25µl using reagents described previously. Each PCR reaction contained 1µl of cDNA, 12.5µl of 2x master-mix (Applied Biosystems) F' and R' primer (volumes used are shown in **Table 2.6** below) and each reaction was made to a final volume of 25µl with H_20.

Primers	Concentration	Volume of F' primer (ml)	Volume of R' primer (ml)
Beta -1	300/300	1.5	1.5
NDF-43	900/900	4.5	4.5
Beta -3	300/300	1.5	1.5
Gamma	900/900	4.5	4.5
KRT-8	300/300	1.5	1.5

Table 2.6: Primer concentrations of *NRG1* isoforms used for real-time PCR analysis

The Beta isoforms as previously mentioned are expressed in breast and pancreatic tissues. The pancreatic cell line BxPC3 and MDA- MB231, breast carcinoma cell line were used as positive controls. HEK- 293 (human embryonic kidney cell line) was used as a positive control for HRG1- Gamma. cDNA was synthesised from in- house RNA extractions of HEK-293, MDA-MB231 and BxPC3 cell lines. cDNA synthesis was carried out using previously described method . Stromal and inflammatory contents may vary between normal and tumour samples therefore the epithelial cell specific gene cytokeratin 8 (KRT 8) was used as an internal control.

Negative controls for each real-time PCR reaction included distilled H_20 and Dnase treated RNA was included for PCR reactions where the primers were not designed across intron/exon boundaries. All reactions were carried out in triplicate on a 96 well reaction plate (ABI). Triplicate positive and negative control were included for each primer reaction.

2.18.4 PCR conditions

PCR conditions were as follows; 95°C for 10mins, followed by 40 cycles of 95°C for 50 sec and 60°C for 1mins. The PCR reactions were performed using ABI Prism 7700 sequence detector (Applied Biosystems).

2.18.5 Optimisation of real-time RT-PCR primers

The real-time PCR products were run on a agarose gel stained in SYBR green dye solution (Applied Biosystems) to check that there were no primer dimers and the Ct values were genuine

Results

Chapter 3

Identification of bacterial artificial chromosome clones (BACs) containing the *NRG1* gene and deletion analysis of *NRG1* in colorectal tumours

3.1 Introduction

Previous LOH studies identified a distinct region of interstitial deletion at the c8p12 locus in early colorectal tumours. Fine mapping of the c8p12 deletion region demonstrated the deletion region lay between markers D8S87 (centromeric) and *WRN* gene (telomeric). The wide range of context dependant functions of the *NRG1* ligands and reported role in solid tumourigenesis suggested *NRG1* was a good candidate gene to study in colorectal tumourigenesis.

From the previous study it was not clear where the telomeric breakpoint lay relative to the candidate gene (*NRG1*). In the colorectal tumour samples examined, markers D8S535, D8S259 and E8.1C were deleted and the *WRN* gene was retained (as shown in **Chapter 1**, **Figure 1.1**). The *NRG1* gene lies telomeric to marker D8S259 which was deleted in 70% of T1/T2 tumours and was retained in node positive cancers (**Table 1.1**). In order to establish whether this site of interstitial deletion encompassed the *NRG1* gene, early (T1/T2N0M0) tumour samples with 8p12 deletion were analysed by comparative genomic hybridisation (CGH) and *fluorescent in-situ* hybridization (FISH).

Fluorescent in-situ hybridisation (FISH)

FISH has proven to be an important tool in the cytogenetic analysis of tumours. As well as allowing the identification of specific chromosome abnormalites, it allows the characterization of commonly deleted regions and the identification of translocation breakpoints. FISH is based on the hybridisation of single stranded

DNA molecules (probe) to target complementary sequence. The detection of fluorescent target in turn allows the number, size and location of specific DNA sequences to be determined microscopically (Tkachuck et al 1991; Reid et al 1999).

Probes used for FISH applications bind to regions of cytogenetic interest and are classified in to three main categories, each with a different range of applications:

Repetitive –sequence probes target chromosomal regions that contain short sequences present in abundance such as alpha satellite probes which target alpha satellite DNA present in the centromere.

Whole chromosome probes have sequence homology at many sites along one chromosome type and enable staining of the entire chromosome.

Locus specific probes target specific sequences ranging from 15 – 500kb on a chromosome and allow study of loci important in genetic disease.

For the purpose of this study, an alpha satellite chromosome 8 centromeric probe was used as an internal control/reference to confirm chromosome localisation of the *NRG1* probe. Probes used for FISH analysis are cloned in a variety of vectors such as plasmids, PAC, BAC and yeast artificial chromosome clones (YAC) (Tkachuck et al 1991; Iourov et al 2005). In this study BAC clones were selected for FISH analysis. BAC vectors can accommodate DNA inserts up to 300kb. BAC DNA exists in supercoiled circular form that permits easy isolation and manipulation with minimal breakage. The other advantages of BAC clones include clonal stability and low rate of chimerism (Shizuyu et al., 1992; Gong et al., 2002, Marra et al., 1997; Strachan & Read.,1999)

This chapter describes the approaches used to identify bacterial artificial chromosome clones (BACs) containing the coding sequence of the candidate gene *NRG1* and the approaches used to verify the sequence and chromosomal location of the BAC clones. Finally, the findings of comparative genomic hybridisation (CGH) and *fluorescent in-situ* hybridisation analysis used to investigate *NRG1* deletion in early colorectal tumours are discussed.

3.2 Results

3.2.1 Identification of bacterial artificial chromosome clones (BACs)

In order to identify BAC clones corresponding to the *NRG1* gene at the c8p12 locus, a computer-based search was performed using the ensembl genomic database (www.ensembl.org). A series of clones was identified from the Ensemble database (Build 35) as shown in **Table 3.1** below.

Ensembl ID	Synonym	start	end
ENSG00000157168	NRG1	31617043	32741561

Intnl clone Name	Accession Number	Build 35 start	Build 35 end
RP11-97N152	AC104000	31524467	31681321
RP11-478B14	AC104029	31596486	31773928
RP11-650B11	AC068931	31728031	31829504
RP11-147C21	AC022833	31816328	31993346
RP11-22F19	AC021909	31907984	32086604
RP11-275E10	AC022850	32082920	32253196
RP11-669B22	AC023948	32146563	32342543
RP11-468C1	AC068359	32307542	32493998
RP11-317J8	AC083977	32443776	32656330
RP11-301H15	AC013561	32530006	32715284
CTD-2329M5	AC103675	32623526	32733075

Table 3.1 Bacterial artificial chromosome clones across the *NRG1* gene: This table represents the BAC clones across the entire genomic sequence of the *NRG1* gene according to ensemble database (Build 35). *(www.ensembl.org)*

A map was constructed of the location of available BACs relative to the *NRG1* gene as shown in **Figure 3.1**.

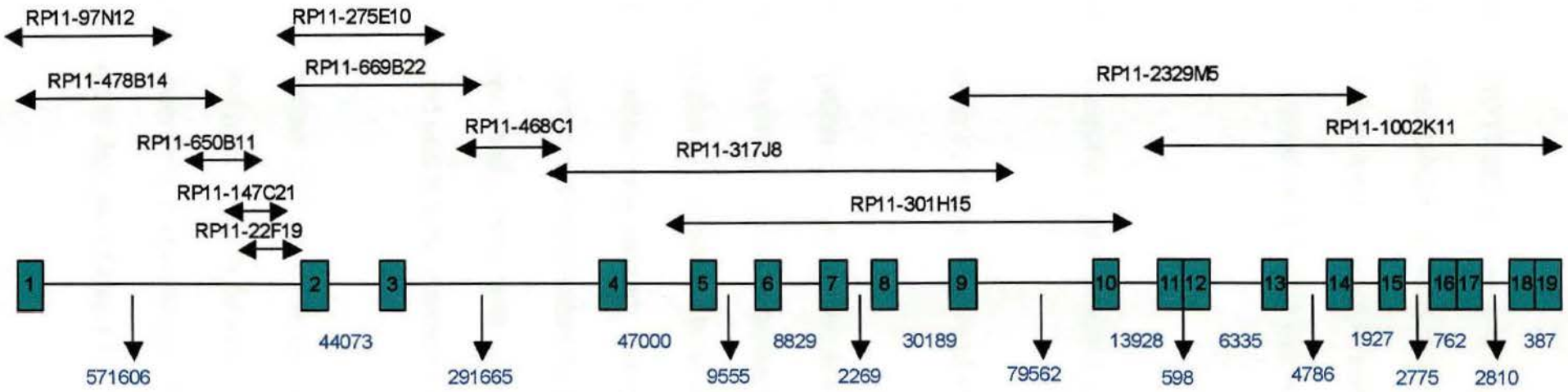


Figure 3.1 Distribution of BAC clones relative to the intron/exon sequence of the *NRG1* gene: This figure illustrates the distribution of the various BAC clones relative to the intron/exon sequence of the the *NRG1* gene.The green boxes represent exons of *NRG1* gene and the black bars represent the introns with the size of each intron represented in blue. The double arrowed bars represent BACs spanning this region of the genome. BAC clones RP11-301H15 and RP11-317J8 lie across exon 7, which represents the common EGF functional domain of the *NRG1* gene and is found in all of the alternatively spliced isoforms.

BAC clones RP11-301H15 and RP11-317J8 were selected for *NRG1* deletion analysis. These BACs covered most of the exons of the *NRG1* gene, and, importantly, cover exon 7 which encodes the functional EGF domain, found in all alternatively spliced isoforms of the *NRG1* gene.

3.2.2 DNA extraction, preparation and detection of fluorescent labelled probes:

This has been previously described in **Chapter 2.**

3.2.3 Verification of chromosome localisation of BAC clones by FISH

To confirm chromosomal localisation of the clones to the c8p12 region, hybridisation of the fluorescent labelled DNA probes was carried out on normal lymphocyte metaphase spread. Metaphase spreads were used as they permit chromosomes to be differentiated by their size, position of centromere and banding pattern and permit localisation to a resolution of ~ 5-10 mbp. Slides were examined under a fluorescent microscope using Smart Capture software.

Despite numerous attempts, the quality of DNA extracted from clone RP11-317J8 was too poor to allow further analysis of this clone. Clone RP11-301H15 (purchased from RPCI) according to the Ensemble genomic database corresponded to the c8p12 locus however, it was found to hybridise to

chromosome 22 (confirmed by size and banding pattern) instead of chromosome 8 as shown in **Figure 3.2** below. This emphasised the importance of screening purchased clones to ensure that they localise to the region of interest.

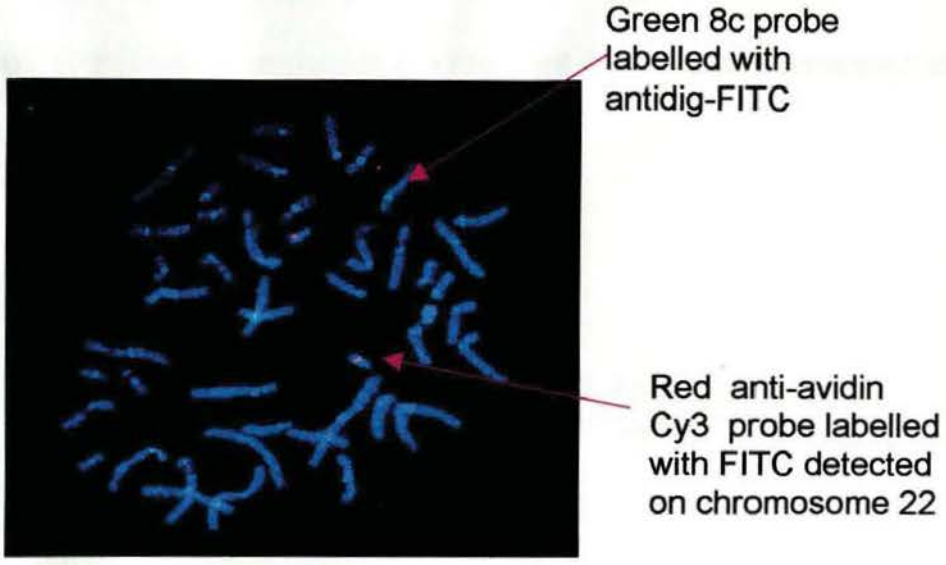


Figure 3.2 FISH of clone RP11-301H15 to chromosome 22: Lymphocyte metaphase spread showing hybridisation of clone 301-H15 to chromosome 22 therefore confirming that this clone does not correspond to the 8p12 region.

In light of the above findings, subsequent BAC clones were screened by reverse transcriptase-PCR (RT-PCR) using STS microsatellite markers mapped to the c8p12 region, providing a quick way of screening clones before FISH analysis.

3.2.4 Screening of bacterial artificial chromosome clones by RT-PCR:

Identification of microsatellite markers

RT -PCR using STS microsatellite markers was used for initial screening of clones. BAC clones to the c8p12 locus (a kind gift from Dr P.Edwards, Cambridge) were used for further analysis. In order to determine whether these BAC clones contained coding sequence of the *NRG1* gene the ensemble database was searched for microsatellite markers that had been mapped to this region which are shown in **Table3.2** & **Figure 3.3**.

Marker	Location
D8S2063	31703894 - 31703988
D8S1810	31811263 - 31811447
D8S2319	31935074 - 31935202
AFMA143YG9	32098397- 32098520
D8S1477	32186709 - 32186878
D8S499	32261497 - 32261631
D8S1125	32487058 - 32487283
D8S375	32577362 - 32577489
D8S278	32667932 - 32668167

Table 3.2 showing list of markers spanning the *NRG1* locus.

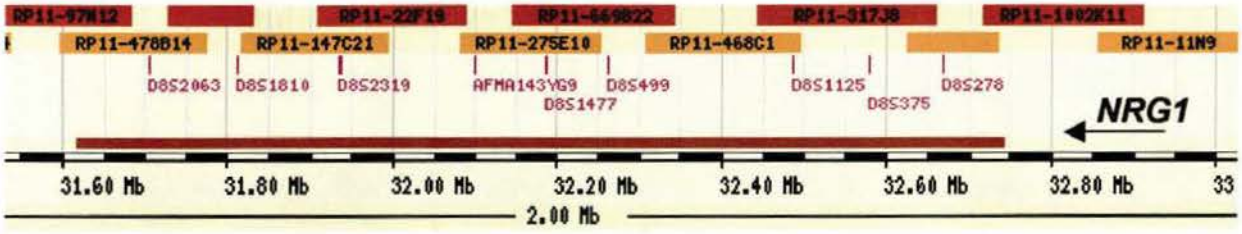


Figure 3.3 Microsatellite markers mapped to *NRG1* gene at the c8p12 locus: Map of the c8p12 region showing the position of microsatellite markers (shown in pink) relative to clones across the *NRG1* gene *(www.ensembl.org).*

Screening of BAC clone RP11-301H15 by PCR using microsatellite marker D8S375

BAC clone RP11-301H15 (gifted by Dr P.Edwards), which covers most of the exons of the *NRG1* gene and more importantly covers exon 7 (which contains the functional EGF domain) was screened by RT-PCR using STS microsatellite marker D8S375, which had been mapped to this region as shown in **Figure 3.4.**

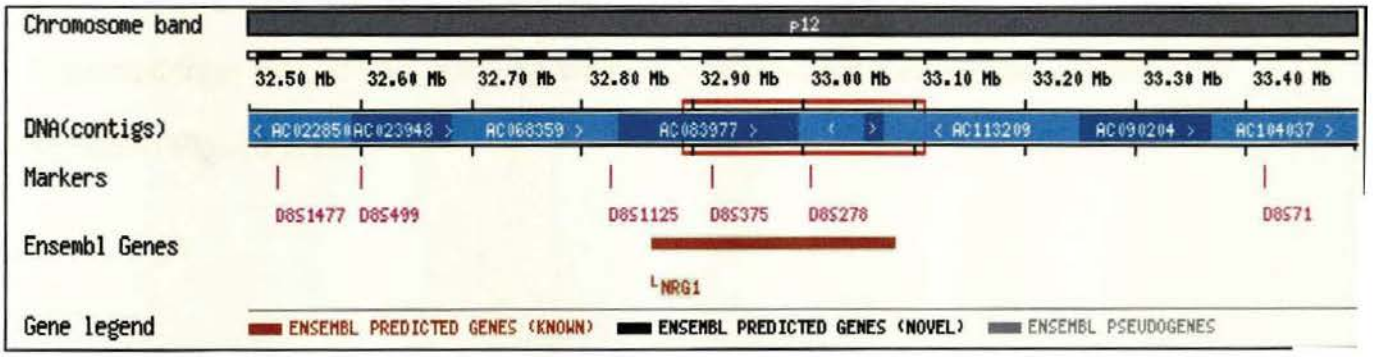


Figure 3.4: Chromosomal location of marker D8S375

Micosatellite Marker	Locus	Primer Sequence 5'- 3'
D8S375	8p12	TTA CAC TAT CTG AAG CAG TGT ACA CAT ATA GGG AAG AAC TCT

Table 3.3 shows the primer sequence used for RT-PCR screening of BAC clones.

(www.ensemble.org)

RT-PCR was carried out on a series of BAC clones with microsatellite marker D8S375. Genomic DNA was used as a positive control and clones RP11-22F19, RP11-97N12, RP11-275E10, Rp11-478B14, RP11-650B11, RP11-669 B22 and H_20 was used as a negative control. Clone 301-H15 was positive for marker D8S375 as shown in **Figure 3.5a**. Although a PCR product was seen for BAC clone RP11-275E10, this was of an incorrect size and could be due to the STS marker primers annealing to similar sequences found in this clone. DNA extracted from BAC clone 301-H15 was also positive for marker D8S375 as shown in **Figure 3.5b**.

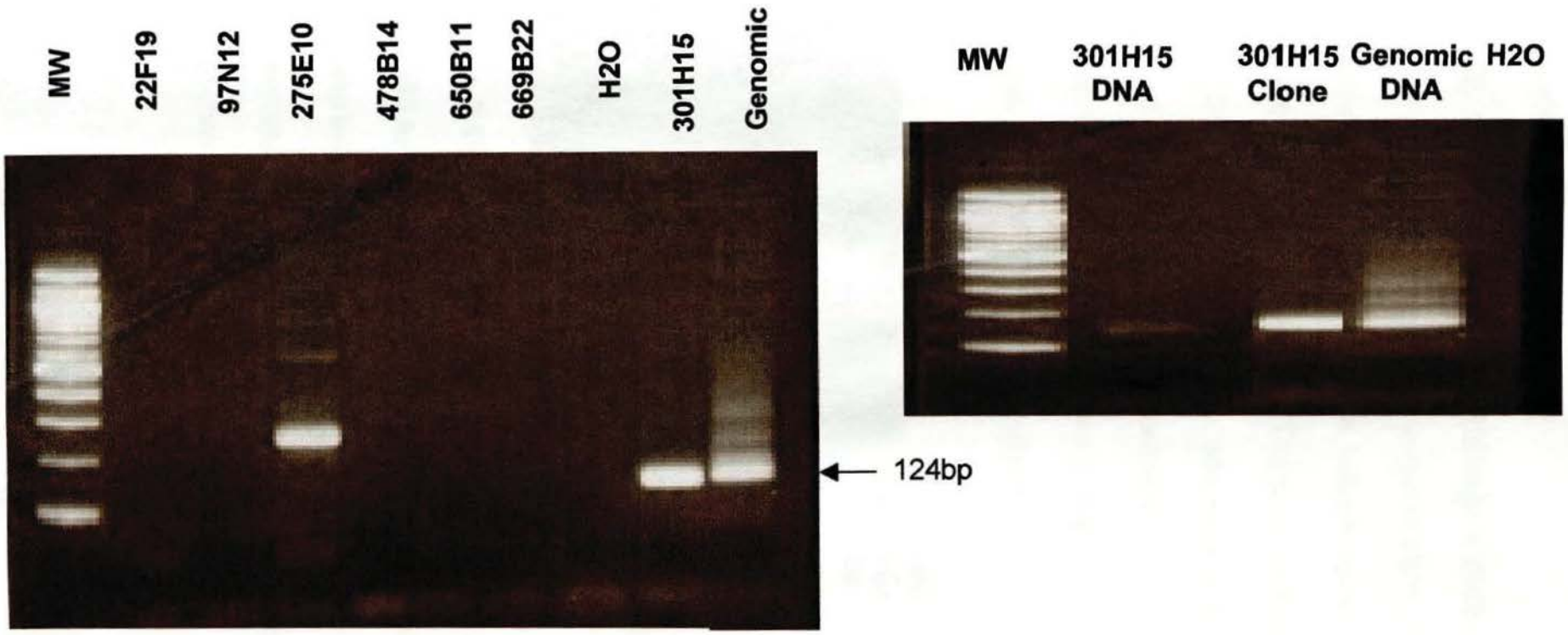


Figure 3.5 a &b: RT-PCR screening of BAC clones using marker D8S375. Figure 3.5a: RT-PCR screening of BAC clones identified a positive clone RP11-301H15 for marker D8S375 **Figure 3.5 b:** RT-PCR using marker D8S375 showing a 124 base pair product for clone RP11-301H15 and from DNA extracted from this clone

3.2.5 Localisation of BAC clone RP11-301H15 by *fluorescent in-situ* hybridisation

Mapping of RP11- 301H15 BAC clone to chromosome 8 by FISH

To confirm the chromosomal localisation of clone RP11-301H15, *fluorescent in-situ* hybridisation was performed on metaphase spreads of lymphocytes, obtained from normal individuals. The position of hybridisation of the fluorescent labelled RP11-301H15 DNA probe, was assessed relative to chromosome 8 centromere specific probe. This confirmed localisation of clone 301-H15 to the short arm of chomosome 8 as shown in **Figure 3.6** and this clone was used subsequently for *NRG1* deletion analysis.

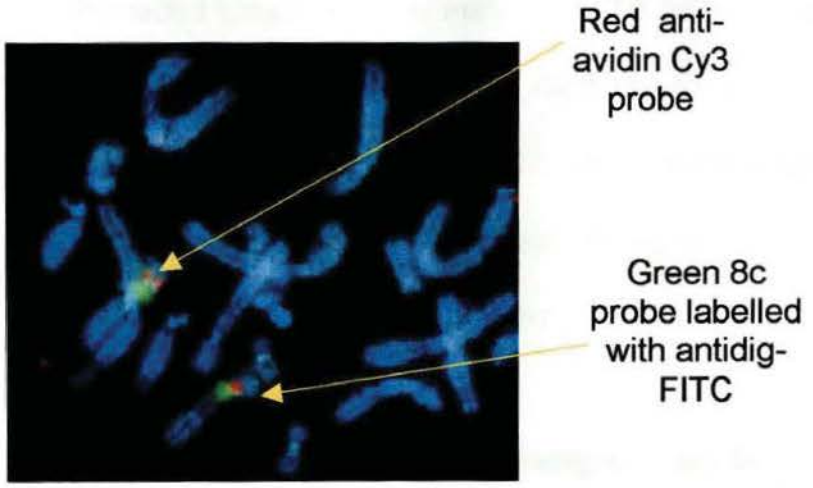


Figure 3.6 Lymphocyte metaphase spread showing hybridisation of clone 301-H15 to chromosome arm 8p12: Normal lymphocyte metaphase spread showing hybridisation of the *NRG1* probe (RP11-301H15 in red) to the short arm of chromosome 8. The *NRG1* probe signal is located towards the centromeric end of chromosome arm 8p.

3.2.6 Clinicopathological and 8p12 deletion status of tumour samples used for deletion analysis

Colon tissue samples were obtained in accordance with local ethical procedures from patients who had undergone surgery for colorectal cancer. The tumour samples were frozen and stored in liquid nitrogen.

All tumour samples were staged according to the TNM classification. All tumours were left sided tumours. Five early (T1/T2N0M0) colorectal tumour samples were selected for deletion analysis. All of the tumour samples used for this analysis had confirmed localised 8p12 deletion between marker D8S87 and WRN gene by previous FISH analysis. All samples had deletion of maker D8S259 as shown in **Table 3.4** below.

Table 3.4: Deletion status of tumour samples used for FISH analysis

Sample	Stage TNM	Marker D8S87	Marker D8S259
61	T1N0M0	D	D
60	T1N0M0	D	D
62	T2N0M0	N	D
63	T1N0M0	N	D
64	T1N0M0	N	D

Pathology: All samples analysed were left sided lesions (Rectal cancers except sample C rectosigmoid carcinoma) Key: D=Deletion, N=no deletion

3.2.7 CGH array in *NRG1* deletion analysis

In order to determine whether *NRG1* was included in the deletion region, an early tumour sample with established c8p12 deletion was analysed by CGH array. This work was done in collaboration with Dr P.Edwards, Cambridge. One of the reasons for choosing CGH array for *NRG1* deletion analysis in previously characterised early tumours was that it would allow us to determine whether our previous 8p12 deletion findings were consistent with data obtained from CGH array. CGH allows the whole genome of the tumour to be analysed for the detection of other chromosomal abnormalities, more importantly identifying chromosomal abnormalities that predominate in early tumourigenesis. CGH array analysis was performed across the whole genome of tumour sample with a series of clones (BAC), with high resolution on chromosome 8.

A series of BAC clones spanning a region of approximately 9.5MB between c8p12 and c8p11.2 loci were used for CGH analysis as shown in **Figure 3.7** The plot of c8p12 is on a log 2 ratio scale and the zero line indicates 2 copies of the chromosome if the ploidy of the sample is normal. The sample showed loss of the 8p12 region from BAC RP11-473A17 (containing the WRN gene) up to BAC CITB-D2343B20 encompassing the *NRG1* locus. BAC's RP11-301H15 and RP11-317J8 both contain coding regions from the gene were used as probes. Deletion of *NRG1* was confirmed as both of these probes gave values below the zero line.

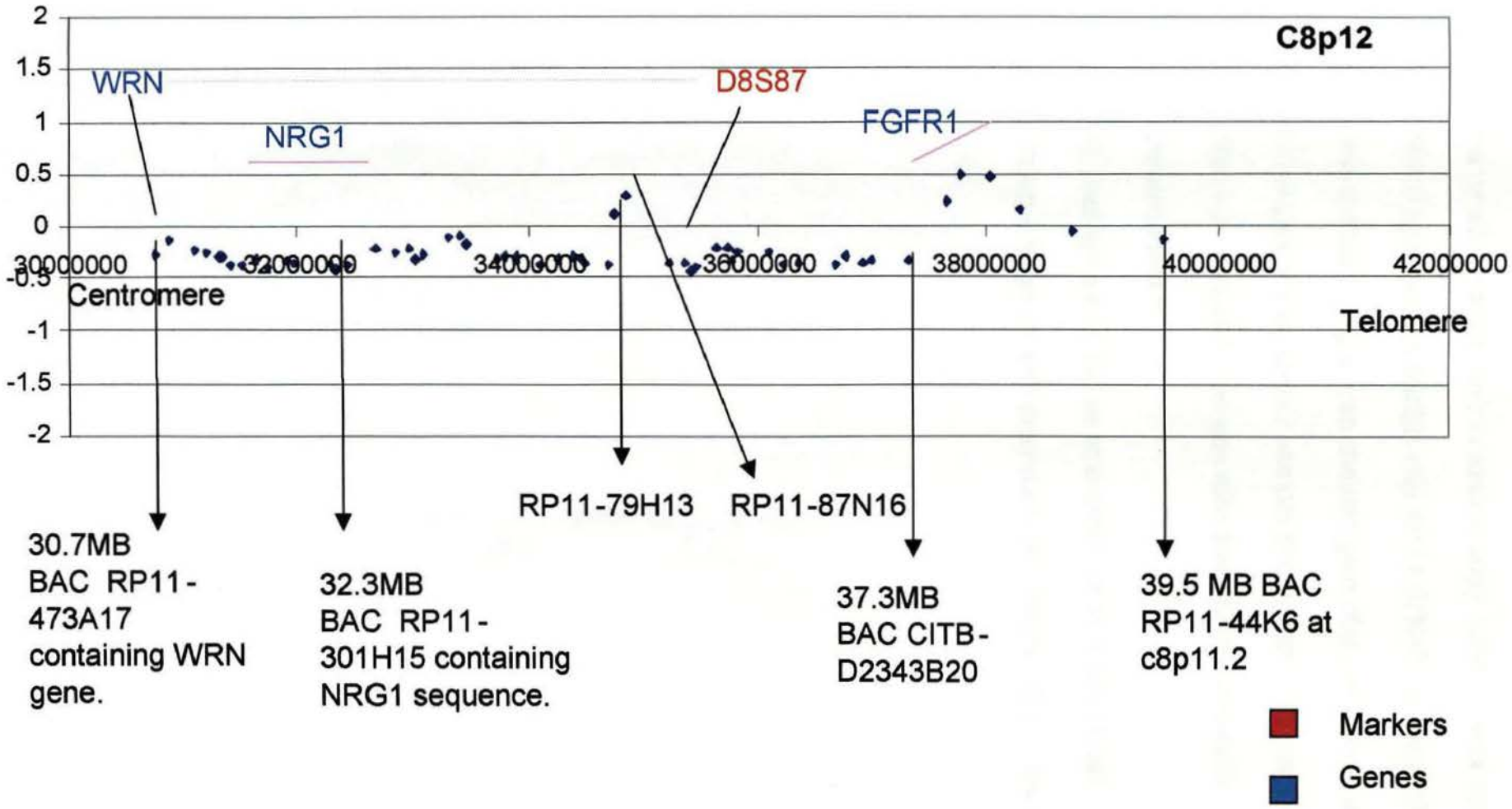


Figure 3.7: CGH array on an early colorectal tumour sample (60).
CGH analysis on an early (T1N0M0) colorectal tumour sample previously examined for c8p12 deletion. The c8p12 plot is on a log2 ratio scale and the zero line indicates 2 copies of the chromosome ie normal ploidy. The pink dotted line represents the localised 8p12 deletion found in colorectal tumours. BAC RP11-301H15 to the *NRG1* gene, lie below the zero line, indicating loss of *NRG1* in this tumour sample.

Within the deletion region an increase in copy number) was seen for 2 BACs, RP11-79H13 (position 34.96MB) and RP11-87N16 (position 35.06MB. The array data of this tumour sample also showed gain of chromosome arm 8q.

The genome plot of this tumour sample showed small regions of gain at the c8p12-c8p11.2. Amplification was also seen of chromosome13, 20 and of the 8q arm of chromosome 8.

The CGH findings prompted us to extend our analysis of deletion on a panel of 5 early colorectal tumours with previously established 8p12 deletion shown in **Table 3.4**.

3.2.8 FISH on panel of 5 early T1/T2 colorectal tumour samples

Quantification of FISH signals:

For each tumour sample, 100 tumour cell nuclei were identified and scored for both Cy3 (*NRG1*) and FITC (8 centromere) signal. The presence of deletion was defined as cells which had fewer Cy3 signals than 8p centromeric FITC signals as shown in **Figure 3.8** below. Cells that appeared intact and were not overlapping were counted. In order to limit false positives, cells with no 8 centromere (FITC) signal were discarded for deletion scoring.

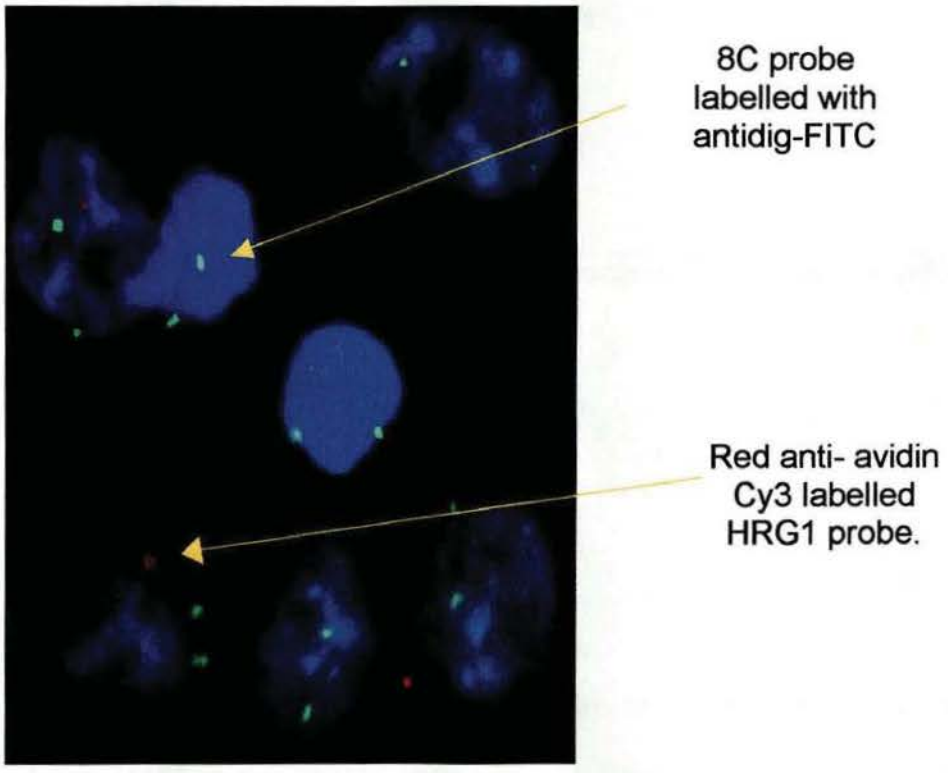


Figure 3.8 *NRG1* deletion analysis- Fluorescent In-situ Hybridisation (FISH) on an early colorectal tumour sample: Colorectal tumour cell nuclei with only

one copy of *NRG1* signal (Cy3 labelled probe). Some tumour cell nuclei showed homozygous loss for *NRG1*.

3.2.9 Analysis of *NRG1* deletion in early and advanced colorectal tumours

The percentage of cells with loss of *NRG1* (< 2 copies of *NRG1* signal/cell) was >50% (range 63-96%) in 4/5 tumour samples (samples B-E) examined as shown in **Table 3.5** below . The percentage loss of *NRG1* per chromosome was defined as:

$$\frac{(\text{Total no of 8C signals} - \text{Total no of signals})}{\text{Total no of 8C signals}} \times 100$$

Samples	Stage	% loss of *NRG1* by cell	No of chromosome 8C / cell				No of *NRG1* signal / cell				
			1	2	3	4	0	1	2	3	4
A (61)	T1N0M0	38	1	45	35	19	5	33	44	14	4
B (60)	T1N0M0	63	0	43	16	41	21	42	34	3	0
C (62)	T2N0M0	94	0	99	1	0	38	56	6	0	0
D (63)	T1N0M0	98	1	95	4	0	77	21	1	1	0
E (64)	T1N0M0	96	1	99	0	0	65	31	3	1	0

Table 3.5: *NRG1* deletion analysis by FISH on early colorectal tumour samples

BAC RP11-301H15 to the *NRG1* gene and 8 centromere probe were used for this analysis. The *NRG1* gene copy and 8 centromere copy no was determined in 100 tumour cell nuclei for each sample analysed. The% loss of *NRG1* per cell was >50% in 4/5 tumour samples.

Analysis of the ploidy status of these tumour samples revealed 2 groups, aneuploid tumours (samples A &B) and euploid tumours (samples C-E). Tumours were defined as being aneusomic if the number of centromeric signals was different from the 2 signals corresponding to disomy in 20% of the tumour cell nuclei . This threshold value was in keeping with previous published reports (Di Vinci A, Cytometry 1999). Aneusomy of chromosome 8 was detected in samples A and B. In sample A, trisomy of chromosome 8 was detected in 35% of tumour cell nuclei, tetrasomy in 19% of tumour cell nuclei and monosomy of chromosome 8 was found in 1% of tumour cell nuclei. In sample B, 41% of tumour cell nuclei were tetrasomic for chromosome 8 and 16% of cells had trisomy of chromosome 8 as shown in **Figure 3.9** below.

Tumour A **Tumour B**

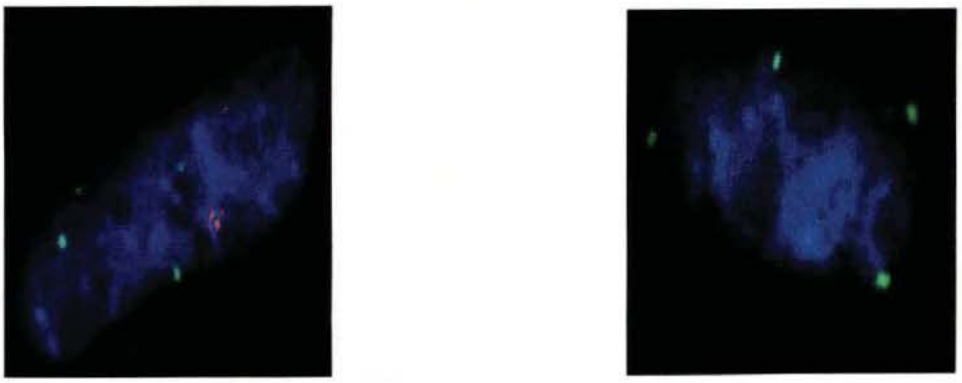


Figure 3.9: Tumour cell nuclei with aneusomy of chromosome 8 centromere. **Sample A:** Tumour cell nuclei with tetrasomy of chromosome 8 centromere and single copy of *NRG1*. **Sample B:**Tumour cell nuclei showing tetrasomy of chromosome 8 centromere and loss of *NRG1*

Homozygous loss of *NRG1* was detected in the euploid tumour group compared to aneuploid group. Comparison of NRG1 copy number between aneuploid and euploid tumours i.e. A&B v C,D&E revealed no significant difference in the number of cells with copy number greater than 2 between the two groups (t-test, equal variances not assumed, p=0.17). The *NRG1* copy number was found to vary directly with 8 centromere signal, such that the number of cells with more than 2 copies of 8C correlated with number of cells with more than 2 copies of NRG1. This was statistically significant (Pearsons correlation coefficient, p=0.02, r=0.93). No significant pathological difference was identified between the 2 tumour groups. All of the tumours analysed were left sided lesions with no lymph node involvement.

3.3 Discussion

CGH array analysis of one tumour sample (Sample 60) showed small regions of gain at the c8p12-c8p11.2. Within the deletion region an increase was seen in copy number of 2 BACs, RP11-79H13 (position 34.96MB) and RP11-87N16 (position 35.06MB). In addition amplification of part of the genome containing the FGFR1 gene (fibroblast growth factor receptor 1 gene) was noted. In response to ligand binding to this receptor a cascade of down stream signals are triggered which influence mitogenesis and differentiation. Amplification of this receptor has been previously documented and expression of this receptor has been reported in colorectal cancer (Adelaide et al., 2003; Courtay-Cahen et al., 2000; Nakao et al., 2004). An increase in copy number of these fragments of DNA may indicate translocation events at this locus. Translocations involving the 8p region have been reported in solid tumours and in haemopathies (Birnbaum et al., 2003).

The array data of this tumour sample also showed gain of chromosome arm 8q, amplification of chromosome13, 20 and loss of 4p and chromosome 18. Similar findings have been reported in colorectal tumours. The putative conditional tumour suppressor gene Deleted in colorectal cancer (DCC) is located on chromosome arm 18q and has been associated with the progression of colorectal cancer (Rodrigues et al., 2007). The MMP-9 gene (matrix matalloproteinases-9 gene) is located on chromosome arm 20q, and increased levels of MMP-9 have been shown to be associated with metastatic colorectal cancer (Nakao et al., 2001; Al-Mulla et al., 1999). Overall CGH analysis revealed loss of the c8p12 locus in early colorectal tumourigenesis.

Despite numerous attempts and adjustments in extraction methods, DNA isolation from clone RP11-317-J8 was not successful. Also screening of BAC clone RP11-301H15 (purchased from RPCI) with FISH revealed hybridisation to chromosome 22. The use of FISH as a first option to verify clones proved to be time consuming as well as technically demanding. In view of this RT-PCR using microsatellite markers was used to provide a quick and easy way of identifying positive clones. BAC clone RP-301H15 (purchased from RPCI) was found to hybridise to chromosome 22, however clone RP11-301H15 (Cambridge) hybridised to the c8p12 region. The most likely explanation for this finding is that an incorrectly labelled clone had been purchased.

As shown in **Table 3.5**, the proportion/number of cells with loss of *NRG1* (< 2 copies of *NRG1* signal/cell) was >50% in 4/5 (80%) of tumour samples. An interesting finding in tumour samples A and B was the presence of subclones of cells with trisomy and tetrasomic chromosome 8. Loss of symmetry in chromosome division is likely to generate subclones with an abnormal number of chromosomes in tumour samples. In addition, chromosome instability within these subclones will allow the existence of different subclones within the same tumour (DiVinci et al., 1999).

Increased range of DNA copy number alterations of both the 8 centromere and the *NRG1* gene was observed in the aneuploid group of tumours. Chromosomal gains and losses are recognised to be a common finding in aneuploid tumours (Giaretti et al., 1994; Reid et al., 1999; Sugai et al., 2000) The *NRG1* copy

number varied directly with 8 centromere signal (statistically significant Pearsons correlation coefficient, p=0.02, r=0.93).

Although the number of early tumour samples analysed were small, overall *NRG1* was consistently deleted and FISH analysis confirmed that *NRG1* was within the localised c8p12 deletion region. Tumour samples used for NRG1 deletion analysis had been previously characterised for 8p12 deletions. In order to verify that the 8p12 deletions were consistent, advanced colorectal tumours with established 8p12 deletion were examined and NRG1 deletion was also identified in these tumours.

We next went on to investigate whether the protein products of the *NRG1* gene were expressed in normal colonic mucosa and in tumours with established *NRG1* deletion.

RESULTS

CHAPTER 4

Expression of NRG-1 protein (heregulin) and erbB2 protein overexpression in colorectal tumours

4.1 Introduction

Following FISH analysis, expression of NRG1 proteins was investigated by immunohistochemical analysis in matched normal/ tumour colorectal samples. Having confirmed deletion of NRG1 in early tumours, we investigated whether deletion of one allele of NRG1 influences protein expression. The NRG1 proteins are alternative spliced products of a single gene that differ in their N-termina, the presence or absence of a transmembrane domain, the type of EGF like domain i.e. α or β and the C-terminus (Falls, 2003, Lemke, 1996). Matched normal/colorectal tumour samples were stained with an antibody which detects the α and β isoforms of heregulin, a protein product of NRG1. At the time of this study antibodies to other isoforms of NRG1 were not available.

The NRG1 proteins, act as ligands for the erbB receptors and bind erbB3 or erbB4, indirectly inducing phosphorylation of erbB2 through the formation of erbB3: erbB2 or erbB4: erbB2 hetrodimers serving as mediators of signal transduction (Venkateswarlu et al., 2002; Carraway et al., 1994; Yarden et al., 1991; Olayioye et al., 2000). Overexpression of erbB3 has been reported in early stage colorectal tumours with a reduction in prevalence being documented in advance stage cancers. Co-expression of erbB3 and erbB2 has also been reported to be significantly higher in early stage tumours. It is thought that erbB2 :erbB4 heterodimerisation may play a role in late stages of colorectal carcinogenesis (Lee et al., 2002). Activation of the MAPK pathway through overexpression of EGF co-receptor ErbB2 (a membrane receptor tyrosine kinase

factor in breast cancer (Lupu et al., 1995; Olayioye et al., 2000) In colon cancer cells, activation of the MAPK pathway through erbB2 has been reported (Venkateswarlu et al., 2002). Establishing overexpression by immunohistochemistry is however, problematic, and this can be over- estimated (Nathanson et al., 2003). Given the different assay systems, antibodies and scoring systems in use, the FDA standardised HercepTest (Jacobs et al., 1999; Perez, 1999) was used to analyse this tumour series for overexpression of erbB2 protein.

4.2 Results

4.2.1 Clinicopathological and 8p12 deletion status of tumours used for immunohistochemical analysis of heregulin expression

Paraffin sections of colorectal adenoma and tumour samples, along with matched normal tissue sections, were obtained in accordance with local ethical procedures from the Histopathology Department (Birmingham University Trust). Tumour samples previously used for NRG1 deletion analysis (highlighted in blue) were included in this analysis as shown in **Table 4.1** below.

Table 4.1 Histopathological grading of colorectal samples

Advanced tumour samples with 8p12 deletion	Early tumours with 8p12 deletion	Advanced tumour samples without 8p12 deletion	Early tumour samples without 8p12 deletion	Adenomas without 8p12 deletion	Adenomas with 8p12 deletion
T3N0M0	T1N0M0	T3N0M1	T2N0M0	TV, moderate dysplasia	TV, severe dysplasia
T4N0M0	T1N0M0	T3N2M1	T2N0M0	TV, moderate dysplasia	TV, severe dysplasia
T4N1M0	T2N0M0	T3N0M0	T2N0M0	TV, moderate dysplasia	adenoma
T4N0M0	T1N0M0	T3N1M0		TV, moderate dysplasia	adenoma
T4N1M0	T1N0M0			TV, moderate dysplasia	

(T3/T4N0 node negative advanced tumours; T3/T4N1 node positive advanced tumours, T1/T2N0 node negative early tumours; TV tubulovillous adenoma)

4.2.2 Optimisation of heregulin antibody

Primary antibody

A rabbit polyclonal antibody HRG (C-20) (Santa Cruz Biotechnology Inc), raised against a peptide at the carboxy terminus of the protein was used for this analysis. This antibody detects the α and β isoforms of heregulin and was selected as it had previously been tried and tested by different groups (Wang et al., 2001; Aguilar et al., 2001; Shirakabe et al., 2001).

Secondary antibody

A biotinylated goat anti-mouse antibody (Dako, Carpenteria) was used for immunodetection. The avidin-biotin staining method was used to enhance detection of the target antigen by increasing the number of HRP molecules bound to the tissue. The product of reaction was identified using diaminobezidine (DAB).

Controls

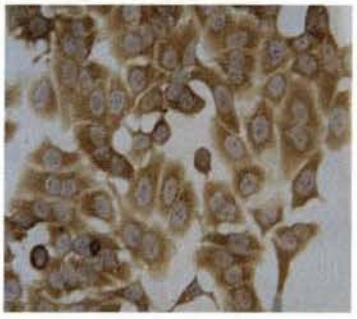
Positive control

The type I transmembrane neuregulins are expressed in breast, heart, liver, kidney, skeletal muscle, prostate, ovary and small intestine (UniProtKB/Swiss-Prot, Le et al., 2002). For the purpose of this study MCF-7 (breast carcinoma cell line) cells were selected for antibody optimisation.

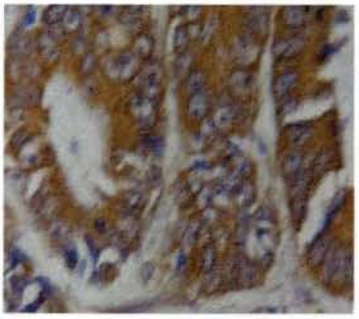
Heregulin Blocking peptide

To test for antibody specificity, heregulin blocking peptide (100ug peptide in 0.5ml PBS containing 0.1% sodium azide and 100ug BSA) was used as a negative control.

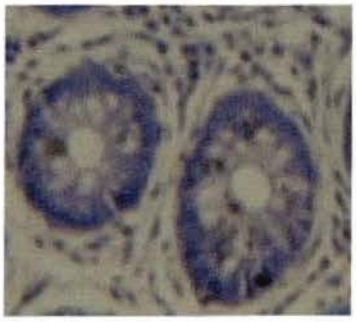
The primary and secondary antibody concentrations were titrated until good staining with little or no background was achieved. Following antibody optimisation, the working concentration of antibodies was applied to a panel of normal colon tissue paraffin sections as illustrated in **Figure 4.1**.



MCF-7 cells were used as a positive control (1:200 primary antibody dilution and 1:500 secondary antibody dilution)



Primary (1:200) and secondary (1:500) antibody



Secondary antibody only (1:500 dilution)

Heregulin blocking peptide

Figure 4.1: **Optimisation of HRG (C-20) antibody**

Top panel: shows optimisation of HRG (C20) antibody on MCF-7 cells. Good staining with minimum background was achieved with primary and secondary antibody concentration of (1:200) and (1:500) respectively. The blocking peptide was used on MCF-7 cells (figure not shown) and on paraffin sections. **Bottom panel**: Expression of heregulin in normal colonic mucosa.

4.2.3 Heregulin protein expression and stage

Heregulin staining was detected in all matched normal samples and this staining pattern was used as an internal control for assessment of tumours. Most normal samples [13/17 (76%)], expressed weakly (1+) but this was seen in only a minority [2/17 (11.7%)] of cancers, the remainder showing higher expression levels. Differences were also apparent between early and advanced tumours. Staining was strong (3+) in 4/9 (44%) advanced tumours compared to 3/17 (17.6%) adenomas and early tumours as shown in **Table 4.2** and **Figure 4.2** indicating an increase expression with tumour progression.

	Intensity score			
Samples	**0**	**1+**	**2+**	**3+**
Normal N=17	0	13	4	0
Adenomas N=9	0	3	5	1
Early Tumours N=8	1	1	4	2
Advanced tumours N=9	0	0	5	4

Table 4.2: Heregulin protein expression in adenomas, early and advanced colorectal tumours. This table shows intensity of heregulin immunostaining in normal colonic mucosa, adenomas, early and advanced tumours. An increase in protein expression was noted with progression from normal mucosa to advanced tumours.

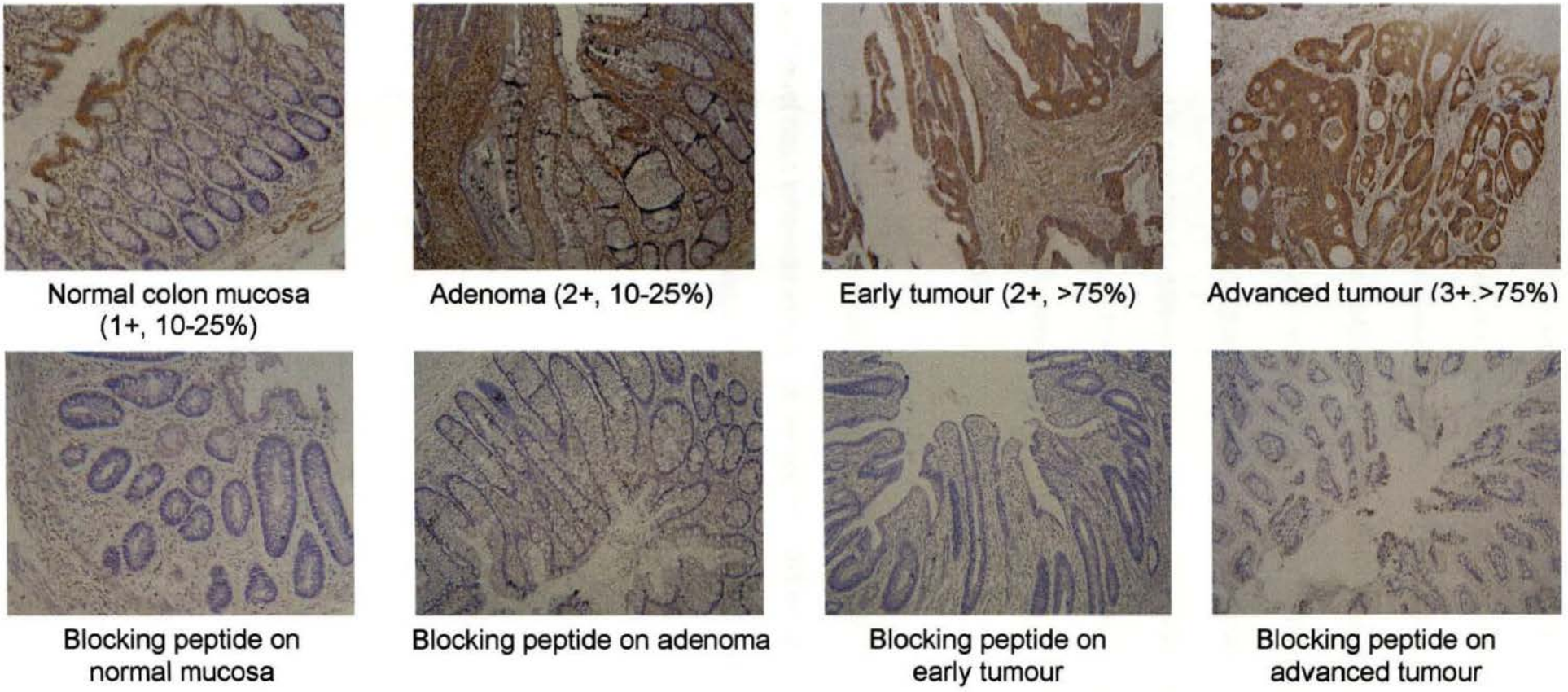


Figure 4.2: Heregulin immunostaining in normal mucosa, adenoma, early and advanced colorectal tumour samples. In the top panel, heregulin expression is seen with progression from normal colonic mucosa to advanced tumours. Increased staining is seen in advanced tumours. The figures in brackets include intensity and proportion of tumour stained. The above tumour samples were negative for 8p12 deletions. Control samples are shown in the bottom panel.

The frequency of intensity score of 3+ was compared between normal, adenomas, early and advanced tumour groups using the Fishers exact test. The score was clearly more frequent in more advanced tumours in the table, and this was significant ($p=0.01$). Advanced tumours on average expressed more heregulin. The mean score per group was compared using the Kruskal-Wallis test. After excluding samples with intensity score zero there was a statistically significant difference ($p<0.0001$). The mean score in each group, with the standard error of the mean in brackets, was as follows
Normal = 1.24 (0.11), Adenoma = 1.78 (0.22), Early = 2.14 (0.26), Advanced = 2.44 (0.18).

In normal mucosa, heregulin was expressed predominately in the luminal epithelial cells whereas in tumour samples a diffuse staining pattern was seen in both luminal and epithelial cells and cells located deep in the crypts, as shown in **Figure 4.3**.

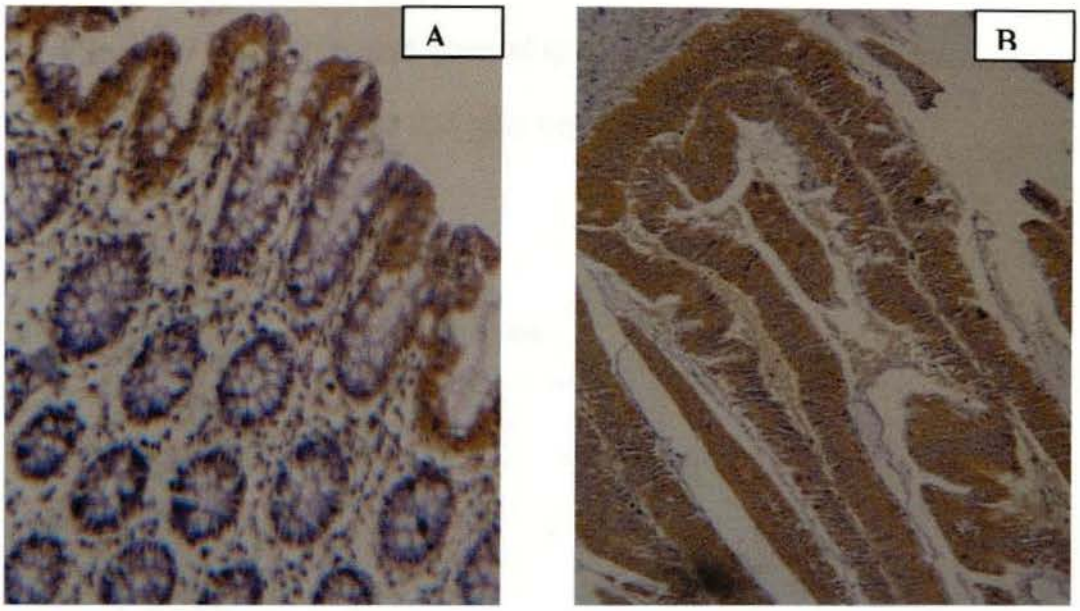


Figure 4.3: Immunolocalisation of heregulin protein in matched normal colonic mucosa and advanced colorectal tumour. In normal colonic mucosa (A) heregulin was seen predominately in the cytoplasm of luminal epithelial cells. Minimum staining was noted in the cells located deep in the crypts. In matched tumour sample (B) a more diffuse staining pattern was noted.

4.2.4 Deletion status and heregulin expression

Some variation in protein expression between early and advanced tumours according to the deletion status (loss of one allele of NRG1) was seen as shown in **Table 4.3.** However, this was not statistically significant.

Samples	8p12 Deletion status	Intensity score			
		0	1+	2+	3+
Normal	No Deletion	0	13	4	0
Adenomas N=4	Deletion	0	2	1	1
Adenomas N=5	No Deletion	0	1	4	0
Early tumours (T1/T2) N=5	Deletion	1	1	2	1
Early tumours (T1/T2) N=3	No Deletion	0	0	2	1
Advanced tumours (T3/T4) N=5	Deletion	0	0	2	3
Advanced tumours (T3/T4) N=4	No Deletion	0	0	3	1

Table 4.3: Heregulin expression in adenomas, early and advanced colorectal tumours with and without deletion.

In the advanced tumour group, comparing the frequency of score 3+ between deletion/no deletion tumours was not significant. (p=0.25, Fishers exact).

If the mean intensity score was compared between the same groups (using the Mann-Whitney test) it was higher in those with deletion (2.60 v 2.20) but remained non significant (p=0.52). In adenomas and early tumours, a frequency of score 1+ or less was no different between those with and without deletion (p=0.20, Fishers exact). The mean intensity score was higher in those with no deletion (2.00 v 1.67) but this was not significant (p=0.48)

4.2.5 ErbB2 protein overexpression

The FDA approved Hercep Test and scoring system was used to investigate overexpression of erbB 2 protein (refer to **Chapter 2 Methods and Materials**). The Hercep Test was applied to the same cohort of tumour samples shown in **Table 4.1**. This work was carried out in collaboration with Dr.P.Taniere (Consultant Pathologist, University Hospital, Birmingham).
The Hercep- Test revealed over-expression of erbB2 protein in 3/9 advanced tumours and in only 2/17 adenomas and early tumours as shown in **Table 4.4.** The intense membranous staining seen in advance tumours was not restricted to the basolateral membrane were erbB2 receptors are normally situated (Vemeer et al., 2003) as shown in **Figure 4.4**.

Samples	ErbB2 OVER- EXPRESSION			
	0	1+	2+	3+
Normal N=17	17	0	0	0
Adenoma N=9	8	0	1	0
Early tumour N=8	7	0	1	0
Advanced tumour N=9	5	1	2	1

Table 4.4: ErbB2 protein overexpression. Using the Hercep Test guidelines over-expression of erbB2 was detected in 3/9 advanced tumour (score ≥ 2+) and in only 2/17 adenoma and early tumours.

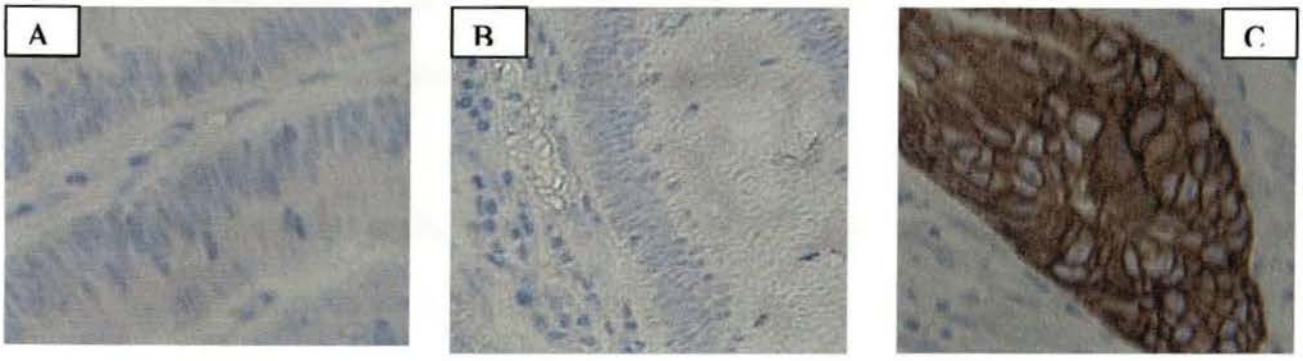


Figure 4.4: Immunostaining of normal colonic mucosa (A), early (B) and advanced (C) colorectal tumours with Hercep Test. Over-expression of erbB2 protein was not detected in normal mucosa and in the early tumour sample. Over-expression was detected in the advanced tumour as shown by the intense brown membranous staining seen in the tumour epithelial cells.

Statistical analysis of erbB2 protein overexpression, comparing the frequency of score of <2 and greater than or equal to 2 was statistically non-significant . (p=0.30, Fishers exact).

4.3 Discussion

Immunohistochemical analysis of NRG1 isoform heregulin, overall identified increased expression in advanced tumours compared to normal mucosa, adenomas and early tumours. This was statistically significant (p=0.01). Comparison of heregulin expression between tumour groups with and without deletion using a number of statistical tests revealed no significant difference This may be a result of the small number of tumour samples analysed. Lack of availability of tumour samples with previously characterised 8p12 deletions prevented further analysis of the influence of deletion on NRG1 protein expression. It is therefore not possible to comment whether deletion of one allele of NRG1 had any effect on protein expression.

Although, we identified erbB2 protein overexpression in advanced colorectal tumours (3/9 advanced vs. 2/17 early tumour and adenomas), small sample numbers prevented any meaningful analysis. Therefore heregulin and erbB2 protein overexpression were investigated in a large cohort of colorectal tumours.

Results

Chapter 5

Multiple tissue array analysis of heregulin protein expression and erbB2 protein overexpression in colorectal tumours

5.1 Introduction

Immunohistochemical analysis of heregulin protein (chapter 4) identified increased expression in advanced tumours compared to normal mucosa, adenomas and early tumours, which was statistically significant (p=0.01). Although erbB2 protein overexpression was seen in advanced colorectal tumours (3/9 advanced vs 2/17 early tumour and adenomas), this was statistically non-significant.

To extend the above finding, a multiple tissue array (MTA) of 250 colorectal tumours was used for immunohistochemical analysis (antibody optimisation previously described in **Chapter 4**) to establish the prevalence of heregulin protein expression and erbB2 protein overexpression in colorectal tumours. Clinical and pathological data were collected in accordance with ethical guidelines.

5.2 RESULTS

5.2.1 Multiple tissue array analysis of heregulin protein expression and erbB2 protein overexpression in colorectal tumours

A MTA of 250 colorectal tumours was analysed for heregulin and ErbB2 overexpression and results were obtained for 165 cases. 73% of tumours were left-sided lesions (descending colon, sigmoid, rectosigmoid and rectal tumours). Heregulin was expressed in 73%, 58%, 60% of Dukes A, B and C tumours respectively, while erbB2 protein over-expression was seen in only 6% of Dukes stage A, 11.3% of Dukes stage B and 7.7% of Dukes C tumours as shown in **Table 5.1**.

Multiple Tissue Array (MTA)

Stage	+ / +	+ / -	- / +	- / -	Total no of tumour samples per stage
A	1	10	0	4	15
B	7	34	1	29	71
C	5	42	1	30	78
D	0	0	0	1	1
Total	13	86	2	64	165

Key:
- +/+ Heregulin &Her-2 positive
- +/- Heregulin positive & Her-2 negative
- - /+ Heregulin negative & Her-2 positive
- - /- Heregulin & Her-2 negative

Table 5.1: Heregulin protein expression and erbB2 overexpression in MTA of colorectal tumours. In total, 60% (99/165) of tumours expressed heregulin protein and overexpression of erbB2 was identified in 9% (15/165) of all cases.

Heregulin protein expression and tumour stage

Heregulin expressing (positive) tumours were compared with tumours negative for heregulin expression for each tumour stage. No statistical difference was identified (p=0.45, fishers exact).

ErbB2 protein overexpression and tumour stage

Tumours with overexpression of erbB2 protein were compared with tumours negative for erbB2 overexpression for each tumour stage. Again, no statistical difference was identified (p=0.86, fishers exact).

Heregulin and erbB2 positive tumours – Is there a correlation?

13% (13/99) of heregulin positive tumours overexpressed erbB2 protein compared with 3% (2/66) of heregulin negative tumours. The frequency of erbB2 positive tumours was compared between heregulin positive vs. heregulin negative samples. This was found to be significant p=0.027, chi sq=4.889.

A further group of tumours was identified 38.7% (64/165) which were negative for heregulin and erbB2 protein overexpression as shown in **Table 5.1**.

5.2.2 Recurrence and tumour stage

Of the 165 tumours investigated, recurrence (local/metastatic) was identified in 22% (37/165) of patients. Recurrent disease was detected in 3% (1/37) Dukes A, 35% (13/37) Dukes B and 62% (23/37) Dukes C patients. Although there was a trend towards greater recurrence with more advanced tumour stage (A=6.67%, B=18.31%, C=29.49%) this was not significant p=0.10 (chi sq).

Heregulin expression and ErbB2 overexpression of primary colon tumours in patients with and with out recurrence

Recurrent disease was detected in 37 of 165 patients with colorectal cancer. Heregulin expression and erbB2 overexpression in the primary colon tumour samples of patients who developed recurrence are shown in **Table 5.2** below.

Stage	+ / +	+ / -	- / +	- / -	Total no of tumour samples per stage
A	0 (1)	1 (9)	0 (0)	0 (4)	1 (14)
B	0 (7)	7 (27)	0 (1)	6 (23)	13 (58)
C	1 (4)	10 (32)	0 (1)	12 (18)	23 (55)
D	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	1 (12)	18 (68)	0 (2)	18 (45)	37 (127)

▪**Key**:
▪ +/+ Heregulin &Her-2 positive
▪ +/- Heregulin positive & Her-2 negative
▪ - /+ Heregulin negative & Her-2 positive
▪ - /- Heregulin & Her-2 negative

Table 5.2: Heregulin and erbB2 protein overexpression of primary colon tumours. Recurrent disease positive cases are shown in black and recurrent disease negative cases are shown in blue.

Heregulin protein expression and recurrence

Comparing recurrence vs. no recurrence for heregulin positive tumours in all Dukes stages did not identify any significant correlation/relationship (p=0.30, Chi sq=1.02). The same was true for heregulin positive tumours in each stage.

ErbB2 protein overexpression and recurrence

Comparing recurrence vs. no recurrence for erbB2 positive tumours in all Dukes stages did not identify any significant correlation/relationship (p=0.19, fishers exact).

5.2.3 Disease free survival (DFS)

The hospital database and pathology records were used to calculate disease free survival (DFS). For each patient, date of operative procedure, date of recent clinic visit and date of diagnosis of recurrent disease if present was recorded. Follow up data was collected till June 2005 on patients who had undergone surgical resection between June 1997 and Feb 2002.

Disease Free survival (DFS) and tumour stage

The DFS data was obtained for 140 patients of which 12 were Dukes A, 61 were Dukes B and 67 Dukes C. The median disease free survival was 36.2 months. In the Dukes A group the median DFS was 63.1months (range 40-95 months). In the Dukes B and C groups the DFS was < 5 months in 15% (9/61) and 42% (28/67) of cases respectively. Comparison of the frequency of survival of <

5months between the Dukes B and C groups was statistically significant p=0.001, Chi sq=11.36.

Heregulin and erbB2 status of tumours with DFS of < 5months

The DFS for all stages and the heregulin and erbB2 status are shown in **Table 5.3 below.**

Stage	+ / +	+ / -	- / +	- / -	Total no of tumour samples per stage
A	0 (0)	0 (9)	0 (0)	0 (3)	0 (12)
B	1 (5)	3 (26)	0 (1)	5 (20)	9 (52)
C	2 (2)	16 (19)	1 (0)	9 (18)	28 (39)
D	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	3 (7)	19 (54)	1 (1)	14(41)	37 (103)

Table 5.3 DFS ,heregulin and erbB2 status in all tumour stages. The heregulin and erbB2 status of tumours with a DFS of < 5months are shown in black and tumour groups with a DFS of > 5months are shown in blue. In patients who had developed recurrent disease, DFS = Date of diagnosis of recurrence – Date of operation. In patients who had no evidence of recurrence, DFS= Date of last clinic visit – Date of operation.

The number of tumours (combining stages B &C, excluding stage A) that were heregulin positive was no different between those with a DFS of > 5 months and those with a DFS of < 5 months (p=0.76 Fishers exact). When each stage was analysed separately, no significant difference was identified (Dukes stage B Fishers exact p=1.00, Dukes stage C Fishers exact p= 0.39).

The above analysis was also carried out for erbB2 protein overexpression and no statistical difference was identified between the number of tumours that were erbB2 positive tumours between those with a DFS of less than 5 months and those with DFS greater than 5 months (Fishers exact p= 0.74).
This was also true when each stage was analysed separately (Dukes stage B Fishers exact p=1.00, Dukes stage C Fishers exact p= 0.64).

5.3 Discussion

Previous immunohistochemistry analysis (**Chapter 4**) had identified increased expression of heregulin protein in advanced tumours (p=0.01) compared to normal mucosa, adenomas and early tumours. Similar findings have been reported in papillary thyroid cancer (Fluge et al., 2000). MTA analysis identified heregulin protein expression in 60% of tumours but no difference between number of heregulin positive tumours and stage (p=0.45) was identified.

Overexpression of erbB2 protein was identified in 9% (15/165) of tumours. A study by Nathanson et al. (2003) using the HercepTest reported a 3.6% (5/139) prevalence of erbB2 overexpression, and Ramanathan et al. (2004) in reported overexpression of HER-2/neu in (8.0%, 11/138) of advanced colorectal cancer. Our findings were in keeping with the above studies. The prevalence of erbB2 overexpression in colon cancer ranges from 0-83% (Ross et al., 2001, Caruso et al., 1996) and this variation in data can be explained by technical variability, choice of primary antibody and differences in slide interpretaion. With the

exception of HerceptTest no standardised system exists to determine erbB2 protein overexpression by immunohistochemistry.

Although overexpression of erbB2 protein in colon cancer is low, erbB2 has been reported to be widely expressed at nomal levels in colon cancer (Breuleux, 2007). The absence of erbB2 expression in the remaining tumours cannot be confirmed as the HerceptTest assay detects only overexpression of erbB2 protein. No statistical difference was noted between stage and erbB2 protein overexpression. Similar findings have been reported by Kim et al. (2004) in tissue microarray of colorectal tumours.

MTA analysis of tumour samples identified a significant correlation between heregulin expression and erbB2 protein overexpression in tumours (p=0.027, chi sq=4.889). A further group of tumours were identified in which expression of heregulin and erbB2 protein overexpression was not detected.

A trend towards recurrence and advance tumour stage was noted. Liver metastasis (38%, 14/37) was the most frequent type of recurrence followed by local recurrence seen in 19%, (7/37) of cases. No statistical difference was identified with recurrence and heregulin expression and erbB2 overexpression in all tumour stages.

Disease free survival was worse in advanced tumour stage (DFS < 5 months Dukes B 15% vs. Dukes C 42%) p=0.001. No association was identified between

disease free survival and heregulin expression for all Dukes stages. The association of erbB2 overexpression with prognosis in colorectal is controversial with some studies reporting poor prognosis in erbB2 overexpressing tumours. In our study, we did not identify any association between erbB2 overexpression, tumour stage and disease free survival. Several studies have reported no prognostic significance of erbB2 overexpression in colorectal tumours (Lee et al., 2002; Osako et al., 1998; Kim et al., 2004; McKay et al., 2002).

In summary MTA analysis identified a significant correlation between heregulin expression and erbB2 protein overexpression in colorectal tumours (p=0.027, chi sq=4.889). We further investigated heregulin expression by Western Blotting in a panel of normal mucosa, adenomas, early and advanced tumours, in samples previously used for immunohistochemical analysis.

RESULTS

CHAPTER 6

Western blot analysis of the NRG1 protein (heregulin) in colorectal tumours

6.1 Introduction

Immunohistochemical analysis demonstrated significant ($p=0.01$) increased expression of heregulin protein in advanced colorectal tumours compared to early tumours and adenomas. The mean protein expression (intensity score) was higher in adenomas and early tumours with no deletion compared to adenoma and early tumours with deletion. The reverse finding was noted in advanced tumours, where the mean intensity score was higher in tumours with deletion. Although these immunocytochemistry findings were non-significant, semi-quantitative western blotting was used to further examine heregulin protein expression in these colorectal tumours.

6.2 Results

6.2.1 Clinicopathological and 8p12 deletion status of colorectal tumour samples used for western blotting

Samples used for Western Blot analysis are shown in **(Table 6.1a).** It was not possible to carry out western blot analyses on all of the samples used previously for immunocytochemistry, due to tumour tissue not being available.

Sample No	Histology	Deletion status
4	normal mucosa	No deletion
10	normal mucosa	No deletion
11	Tubulovillous adenoma, mild dysplasia	No deletion
2	Tubulovillous adenoma, mod dysplasia	No deletion
7	Tubulovillous adenoma, severe dysplasia	Deletion
70	Early tumour T2N0M0	No deletion
64	Early tumour T1N0M0	Deletion
60	Early tumour T1N0M0	Deletion
6	Advanced tumour T3N0M0	Deletion
16	Advanced tumour T4N0M0	Deletion
33	Advanced tumour T4N1M0	Deletion
38	Advanced tumour T4N0M0	Deletion
46	Advanced tumour T4N1M0	Deletion
65	Advanced tumour T3N0M1	No deletion
66	Advanced tumour T3N2M1	No deletion
67	Advanced tumour T3N0M0	No deletion

Table 6.1a:Histopathological staging and deletion status of samples used for protein analysis (T1/T2 early tumours, T3/T4 advanced tumours, N0 node negative, N1 node positive, M0 no distant metastases, M1 distant metastases)

Heregulin expression has been reported in human colorectal, ovarian, pancreatic, heart, liver, kidney and breast tissue (Le et al., 2002). In view of this cell lines shown in **Table 6.1b** were selected.

Cell Line	**Tissue from which cell line derived.**
HT29	Colon Carcinoma
HEK293	Human embryonic kidney
Colo320	Sigmoid colon Carcinoma
Panc1	Pancreatic ductal Carcinoma
BxPC3	Pancreatic Carcinoma
HS578T	Breast normal tissue
HS174T	Colon adenocarcinoma
SW480	Colon adenocarcinoma
Caco -2	Polarized cell line
HCT116	Colorectal carcinoma
MCF-7	Breast carcinoma

Table 6.1b: Cell lines used for western Blot analysis .

6.2.2 Optimisation of heregulin antibody

The heregulin primary antibody HRG (C-20) detects α and β1 and β2 isoforms of *NRG1* protein. The molecular weight of NRG1-alpha isoform detected with this antibody is 44kDa. The molecular weight of *NRG1* isoforms are shown in **Table 6.2** below.

NRG1 Isoforms	Amino acid length	Molecular weight (Da)
NRG1-Gamma	211	22755
NRG1-Beta 1	645	71157
NRG1-Beta 2	637	70200
NRG1-Beta 3	241	26143
NRG1- SMDF	296	31686
NRG1- NDF 43	462	50748
NRG1- GGF II	422	45141
NRG1- Alpha	640	70392
NRG1- Alpha 1a	648	71331
NRG1- Alpha 2b	4662	50879
NRG1- Alpha 3	247	26590

Table 6.2 Molecular weight of NRG1 isoforms

The HRG (C-20) antibody was optimised on a panel of breast, pancreatic and human embryonic kidney cell lysates shown in **Figure 6.1**. Co-precipitation with the heregulin blocking peptide was used as negative control (as described in **Chapter 2**). A primary antibody concentration of 1:500 and blocking peptide working concentration of 1µg (5µl) of peptide, 1 µl of primary antibody diluted in 1ml of PBS was used.

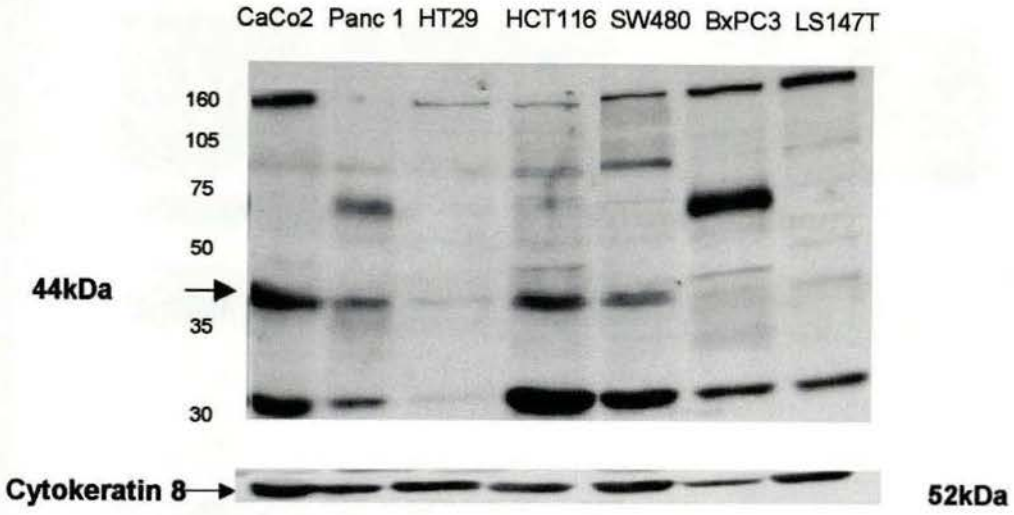


Figure 6.1: Optimisation of HRG (C-20) antibody on cell lines. Although this antibody detects NRG1-alpha a 44kDa protein, molecular weight bands of 160kDa, ~90kDa, 70kDa and 30kDa were also detected. This antibody also detects NRG1-beta 1 & 2 isoforms, which are ~71 and 70kDa respectively and are seen in pancreatic cell lines (Panc 1 and BxPC3). The protein bands of 160 and 90kDa could possibly represent non-specific antibody binding. To check for protein loading the membranes were reprobed with cytokeratin 8 antibody. Equal protein loading was detected as shown by the cytokeratin 8 blot below.

Optimisation of the primary antibody on cell lines showed protein bands of 160 and 90 and 30 kDa band. It was not clear whether these bands were specific to primary antibody reaction. In light of this, heregulin blocking peptide was used as a negative control on cell lines and in tumour samples **Figure 6.2a** and **6.2b** respectively.

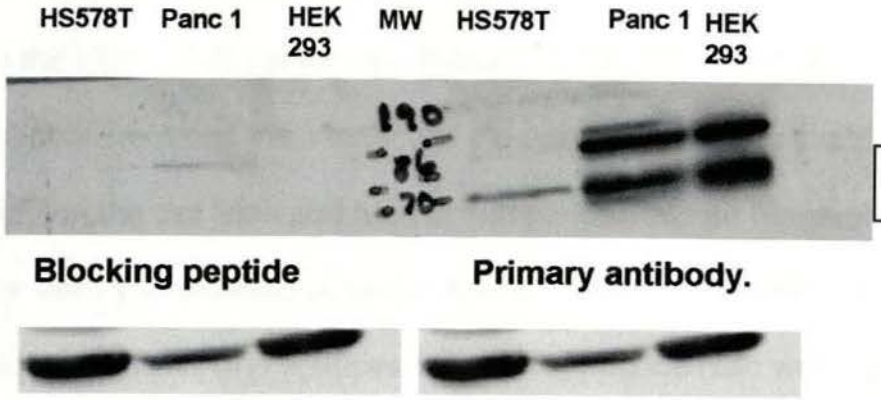


Figure 6.2a

Blocking peptide **Primary antibody.**

Cytokeratin 8

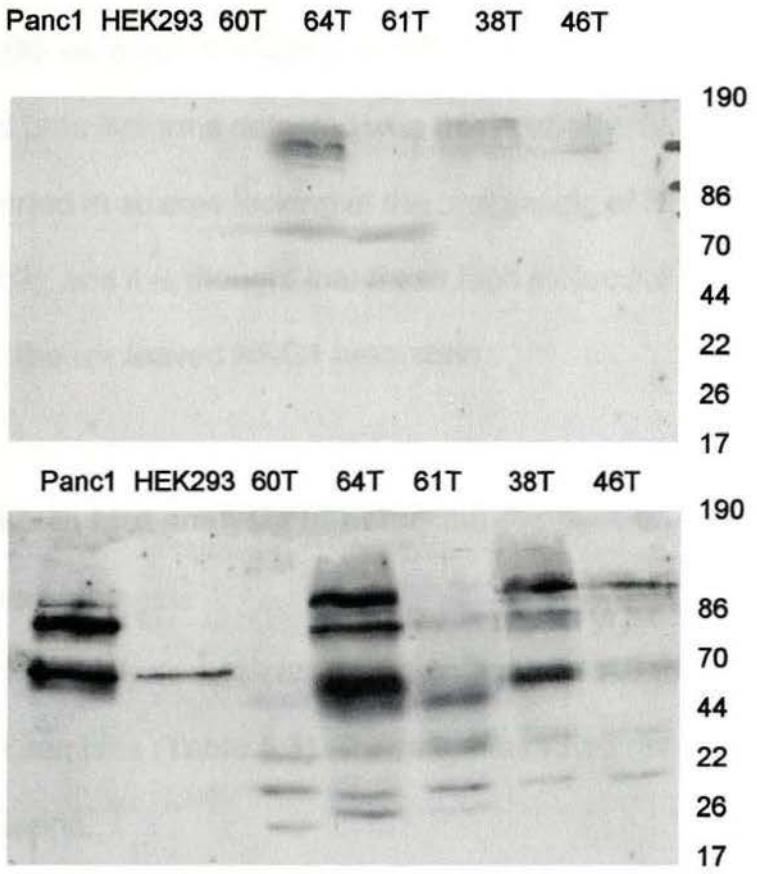


Figure 6.2b

Blocking peptide

Primary antibody

Figure 6.2: Western Blot on cell lines and tumour samples using primary antibody concentration (1:500) and heregulin blocking peptide (working conc: 1µg (5µl)of peptide, 1 µl of primary antibody diluted in 1ml of PBS). Bands of reduced intensity were seen with blocking peptide in both cell lines Fig 6.2a and tumour samples Fig 6.2b. The cytokeratin 8 antibody was used to check protein loading. (Figure not shown for tumour samples)

As shown in **Figure 6.2 a and b**, bands between 85 kDa and 190kDa were noted with the HRG (C-20) antibody. Immunoblotting with the heregulin blocking peptide, reduced the intensity of the bands seen using the HRG (C-20) antibody on the cell lines and tumour samples examined however some faint bands were still detected possibly due to the blocking peptide not binding to all available epitopes (the epitopes to blocking peptide ratio was higher). This confirmed that the multiple bands of different molecular weight detected with the heregulin antibody were specific to this reaction. The bands greater than 85 kDa were not in keeping with the molecular weight of other NRG1 alpha and beta isoforms detected with this anitbody. Similar findings have been reported in studies looking at the processing of NRG1 proteins (Montero et al., 2000), and it is thought that these high molecular weight bands may represent the uncleaved NRG1 proprotein.

6.2.3 Western blot analysis of heregulin expression in matched normal / tumour samples

Western blot analysis was carried out on a panel of 3 matched normal/tumour colorectal samples (**Table 6.3**). These tumours had not been characterised for 8p12 deletions.

Table 6.3

Tumour samples	Early/Advanced tumour
58T	Early
7T	Advanced
5T	Advanced

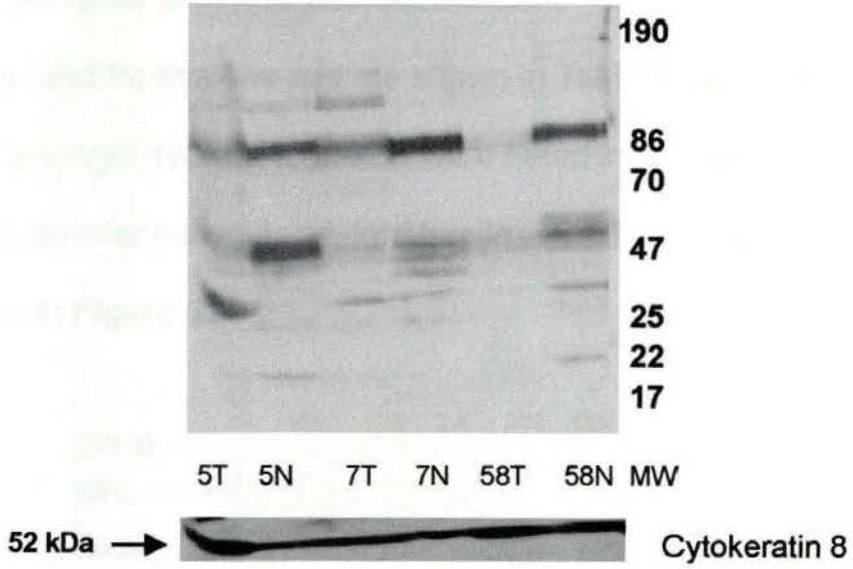


Figure 6.3: Western blot of heregulin protein expression in matched normal/tumour samples: Multiple bands were noted in matched normal/tumour samples. In tumour samples the bands were not as intense as in the matched normal samples despite equal gel loading.

Immunocytochemistry showed reduced intensity of staining in normal colorectal tissue compared with tumours. This is not what was found by western blotting. It is difficult to correlate the immunocytochemistry findings with western blotting. We next went on to investigate whether the immunohistochemistry findings in tumour samples with and without deletion were identified on Western blot analysis.

6.2.4: Expression of heregulin in a panel of adenomas, early and advanced tumour samples with and without deletion

Samples used for this analysis are shown in **Table 6.1a**. A range of bands of varying molecular weight 190kDa to 39kDa were noted in normal mucosa and adenoma samples. Smaller molecular weight bands were seen in early and advanced tumours as shown in **Figure 6.4**.

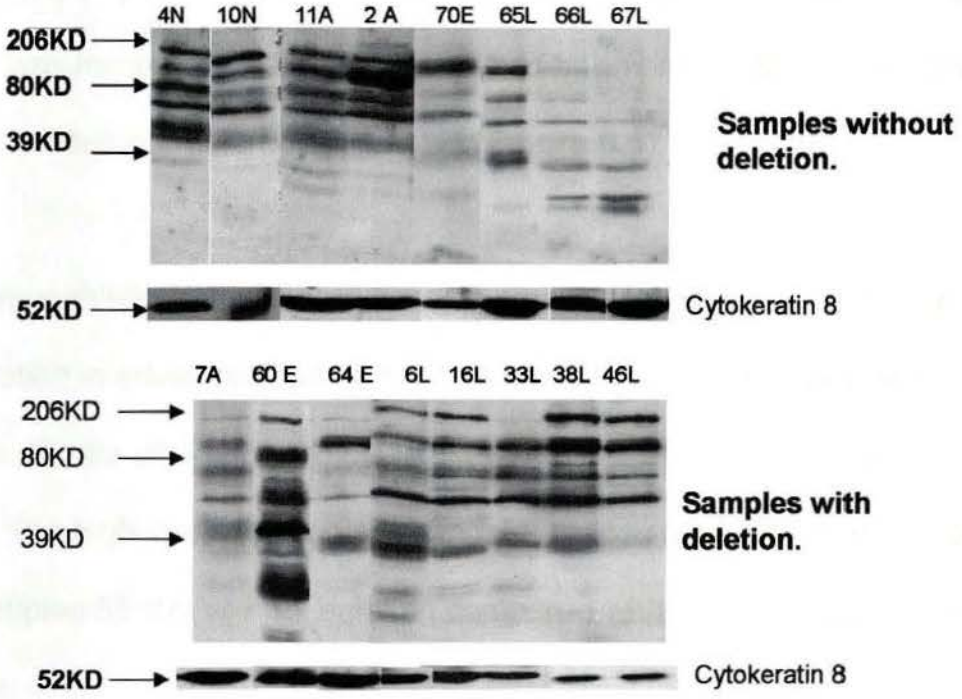


Figure 6.4: Western Blot of tumour samples with and without deletion. Multiple fragments of different molecular weight were noted in all samples. In advanced tumours with deletion the intensity of the high molecular weight bands were reduced and increased fragments of low molecular weight were noted. N= Normal mucosa, A= Adenomas, E = Early (T1/T2 N0M0) tumour L = Advanced(T3/T4 N0/N1 M0/M1) tumours.

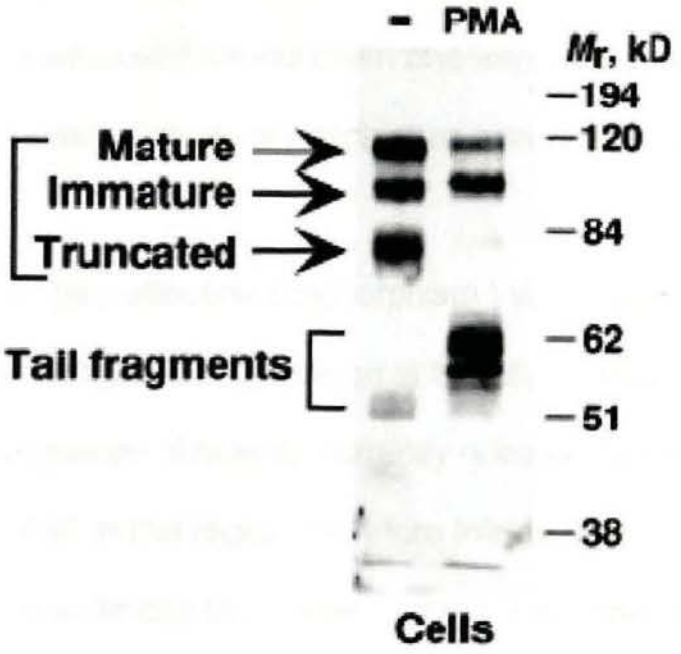
Similar findings were also noted in early tumours with and without c8p12 deletion. With progression towards advanced tumour stages, bands ranging from ~86-25kDa were noted in the advanced tumour samples. Even though only a small number of tumour samples was analysed, bands of low molecular weight were more apparent in advanced tumours without deletion compared with advanced tumours with deletion **(Figure 6.4)**. In advanced tumours with no deletion (samples 65 – 67) the intensity of the ~190kDa band was faint and the intensity of the other fragments (ranging from ~86-17kDa) was increased. Similar findings had been previously noted in the matched normal/tumour colorectal samples **(Figure 6.3)**.

Although immunohistochemical data showed significant increased expression of heregulin protein in advanced tumours, this finding was not consistent with Western Blot analysis. Bands of varying molecular weight and intensity were noted throughout all samples. The high molecular weight bands were less apparent in advanced tumours (samples 65-67) without deletion compared with the advanced tumour group with deletion.

6.3 Discussion

Western blot analysis of heregulin protein using the C-terminal antibody revealed multiple bands of varying molecular weight in colorectal tumours. It was difficult to explain the findings of Western blot analysis in matched normal/tumour samples and in tumour samples with / without deletion. A review of current literature on NRG1 has shown similar findings on Western blot analysis (Montero et al., 2000).

Immunoprecipitation and Western blot studies have shown that cleavage of transmembrane neuregulins, generates multiple cell bound truncated fragments detected with an anti-endodomain antibody **(Figure 6.5)**.



Montero et al 2000

Figure 6.5: Processing of pro-NRG. The figure above shows cleavage of pro -NRG. The processing of the neuregulin proteins has been shown to be stimulated by activation of PKC (protein kinase C) with PMA (phorbal myrisate acetate) which led to several truncated cell-bound fragments being generated.

Type I transmembrane *NRG1* ligands such as heregulin are cleaved to release bioactive EGF domain. In advanced tumours, high and low molecular weight fragments were noted but in advanced tumours without deletion (samples 65-67), low molecular weight bands were predominant . We investigated whether mutations at the cleavage site (transmembrane domain) were responsible for the Western Blot findings in advanced tumours with and without deletion by sequencing of the transmembrane domain of matched normal/tumour samples with and without deletion.

A SNP (single nucleotide polymorphism) was identified in the transmembrane domain of sample 65 (no deletion at the c8p12 locus). Review of the transmembrane domain sequence (Ensembl, currently release 43- Feb 2007) did not identify any reported SNP in this region, therefore this represents a novel, previously unreported mutation. It is difficult to comment on the relevance of this mutation as it was detected in the patients normal and matched tumour sample.

This SNP identified in the coding sequence of the transmembrane domain was a missense mutation leading to change in the amino acid sequence. Sequence analysis of transmembrane domain did not explain the range of high and low molecular weight fragment see on Western Blotting. Further studies will need to be undertaken on a large panel of tumours to establish if this missense mutation accounts for differences observed on Western blot analysis.

These bands may also represent alternative spliced isoforms of NRG1. Since limitations in the range of NRG1 antibodies available prevented further analysis of

NRG1 proteins, we went on to investigate the possibility that the Western blot reflects changes in relative expression levels of alternatively spliced isofoms using qualitative and semi-quantitative PCR.

RESULTS

CHAPTER 7

Analysis of *NRG1* isoform expression in colorectal tumours.

7.1 Introduction

One challenge with investigating *NRG1* gene products in tumourigenesis is the possibility of changes in the splicing profile during the process of tumour formation. Since no single antibody can reliably detect all isoforms, it is possible that some of the changes seen in Western blotting may result from a change in splicing patterns between the isoforms detected by the antibody (alpha and beta isoforms) and those not detected (NRG1-gamma, GGF, NDF43, SMDF). We therefore established specific RT-PCR assays, to investigate alternative spliced products of *NRG1* in colorectal tumours.

7.2 RESULTS

7.2.1 Clinicopathological status of tumour samples used for NRG1 isoform analysis:

A cohort of ten advanced (T3/T4, N0/N1M0) colorectal tumour samples ~~and~~ were selected. Matched normal samples were included to investigate for any change in isoform expression between normal and tumour samples as shown in **Table 7.1** below).

Samples	Histology
18N	T3N0M0
18T	T3N0M0
24T	T3N1M0
24N	T3N1M0
28T	T3N0M0
28N	T3N0M0
33T	T4N1M0
33N	T4N1M0
35T	T3N0M0
35N	T3N0M0
38T	T4N0M0
38N	T4N0M0
46T	T4N1M0
46N	T4N1M0
12T	T4N1M0
12N	T4N1M0
29T	T4N0M0
29N	T4N0M0
4T	T3N0M0
4N	T3N0M0

Table 7.1: **Histopathological staging of tumour samples used for NRG1 isoform analyses**

7.2.2 Quality of synthesised cDNA

B-actin RT-PCR was carried out to validate the quality of the cDNA synthesised. All of the PCR products were resolved on an agarose gel as shown in **Figure 7.1**.

Figure 7.1: B-actin RT-PCR on a panel of matched normal/tumour samples.The quality of the cDNA synthesised from extracted RNA of matched colorectal tumour samples was assessed by RT-PCR. Primers to the house keeping gene B-actin were used. 5ul of the PCR product was run alongside 0.5ug of 100 bp DNA ladder on a agarose gel. As shown above for all of the samples strong intensity bands were seen indicating that cDNA synthesis was of good quality. The pancreatic cell line cDNA (BxPC3) and H_20 were used as positive and negative controls respectively.

7.2.3: Design of primers to distinguish between different *NRG1* Isoforms

Oligonucleotide primer sequences, which flank the alternative spliced region, were designed. The mRNA sequence of each isoform was obtained from the NCBI database. The exon and intron sequence of the *NRG1* gene (shown in **Figure 7.2a**) was obtained from genomic database (NCBI and ensembl website). Primers were designed (using the Primer 3 output programme, http://frodo.wi.mit.edu/cgi-bin/primer3) across intron- exon boundaries of those exons that distinguished between the different isoforms. Each primer

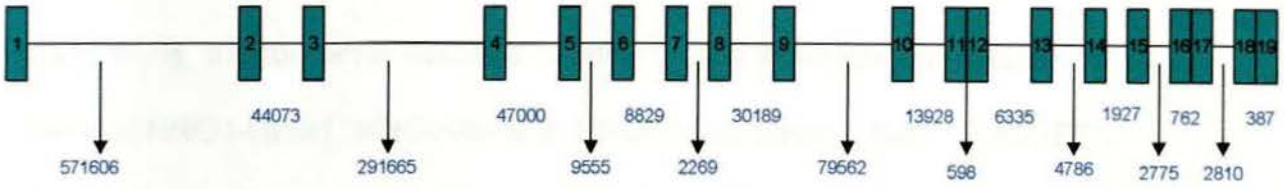


Figure 7.2a: Exon / intron sequence of the *NRG1* gene

This figure illustrates the intron/exon sequence of the the *NRG1* gene.The green boxes represent exons of *NRG1* gene and the black bars represent the introns with the size of each intron represented in blue. Exon 7represents the common EGF functional domain of the *NRG1* gene and is found in all alternatively spliced isoforms.

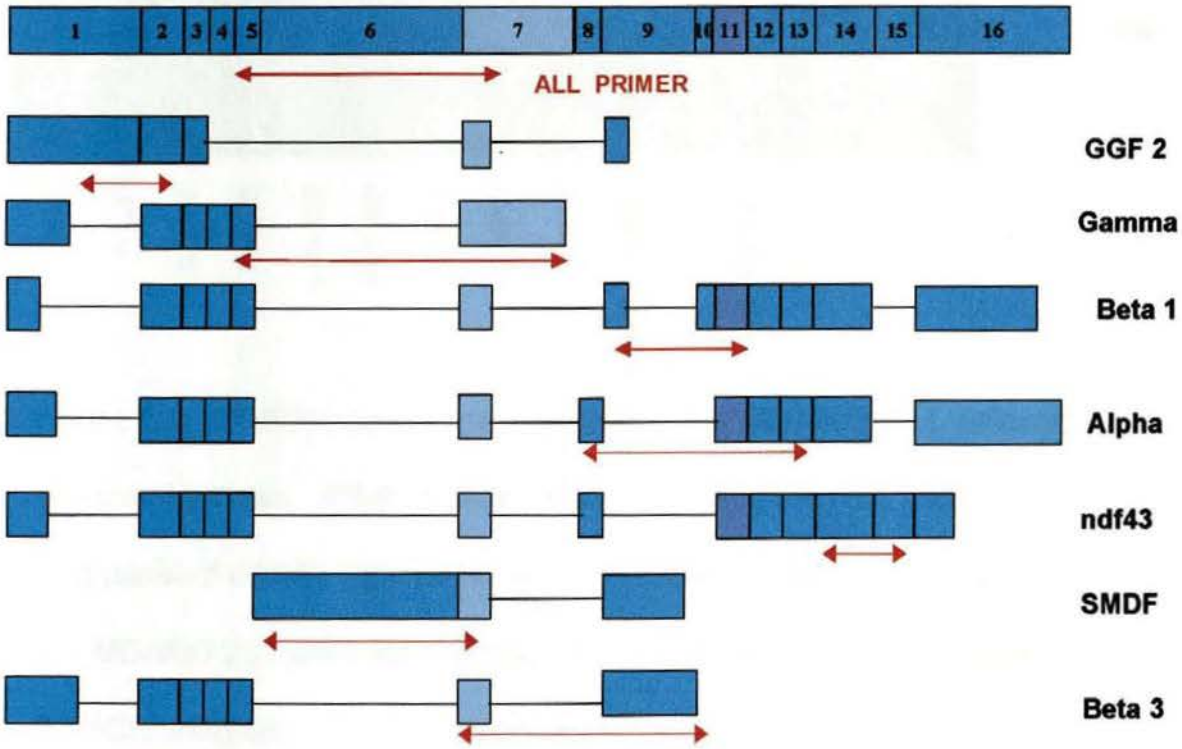


Figure 7.2b: Primer design for NRG1 isoforms. Isoform specific primers were designed to distinguish between NRG1 isoforms. The bars illustrate amplicon size and position. The light blue box (exon 7) represents the common EGF domain and the transmembrane domain is shown in dark blue.

7.2.4: Optimization of isoform specific primers

Identification of positive controls for NRG1 **isoforms**

In this study, the following isoforms of NRG1 were investigated; NRG1-Gamma, NRG1-Beta1, NRG1-Beta 3, NRG1-Glial Growth factor 2 (GGF2), NRG1-Sensory motor derived factor (SMDF), NRG1-Neu- diffrentiation factor (ndf43) and NRG1-Alpha. A literature search was carried out and RT-PCR with oligonucleotide ALL primer pair (which detects most of the NRG1 isoforms) was performed on a panel of in house cell line cDNA's to identify positive controls for the ALL amplicon as shown in **Figure 7.3**

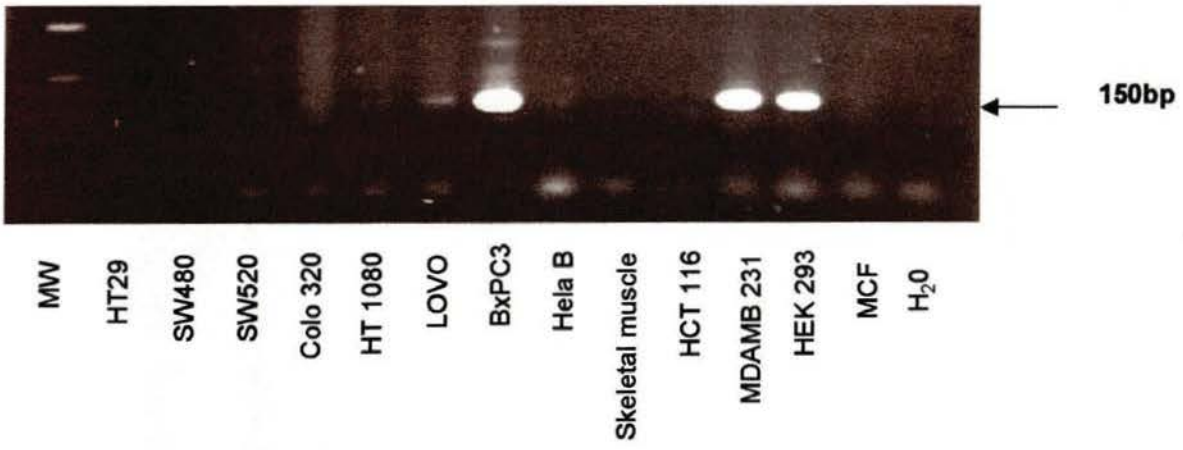


Figure 7.3: RT-PCR on a panel of cell line cDNA using ALL primer oligonucleotides. RT-PCR was carried out using the ALL primer pair on a panel of cDNAs from cell lines. The cell lines BxPC3, HEK 293 and MDAMB 231 were identified as positive controls for isoform-specific RT-PCR analysis.

Human brain cDNA was used as a positive control for SMDF, GGF2 that are predominately expressed in neuronal tissues. The Beta isoforms are expressed in breast and pancreatic tissues so pancreatic cell line BxPC3 and MDA- MB231 breast carcinoma cell line were used as positive controls. HEK-293 (human embryonic kidney cell line) was used as a positive control for HRG1- Gamma.

7.2.5 Analysis of NRG1 isoform expression by RT-PCR

Isoform specific RT-PCR experiments were carried out on 10 matched normal/tumour colorectal samples as shown in **Figure 7.4**.

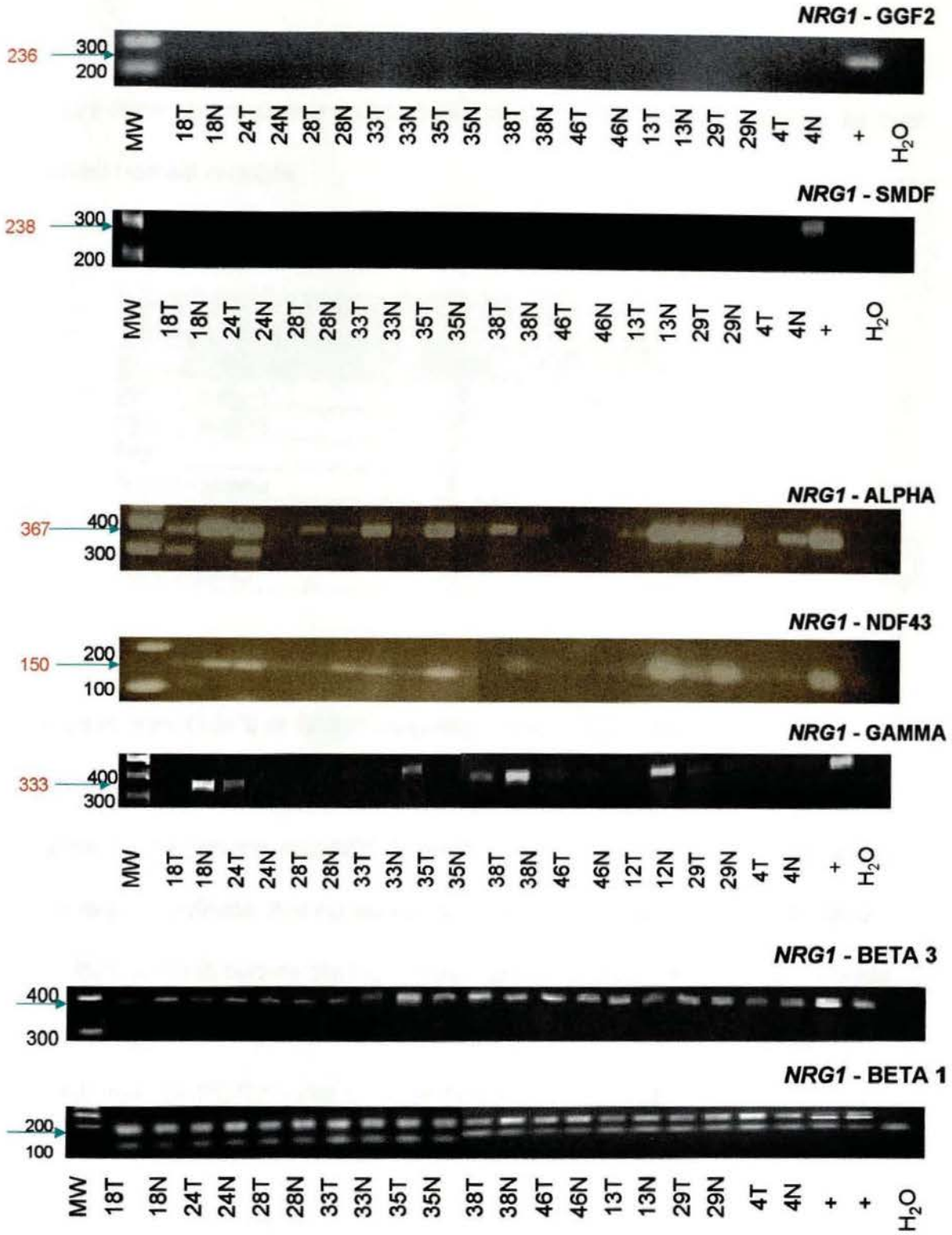


Figure 7.4: Isoform specific RT-PCR on 10 matched normal/tumour colorectal samples. Isoform specific RT-PCR confirmed that Beta 1 and Beta 3 were expressed in colorectal samples. No qualitative change in isoform expression was noted in the samples. The NRG1-alpha isofom was detected in 9/10 matched normal/tumour samples. Differential expression of isoform gamma and ndf-43 was noted. In 2/10 matched pairs, ndf-43 was only expressed in normal colonic mucosa. In 3/10 matched samples gamma isoform was expressed in normal and tumour samples and only expressed in tumour sample

The results of this exercise, summarised in **Table 7.2** demonstrated that the tumours reviewed expressed essentially similar NRG1 isoform profiles as their matched normal mucosa.

Expression of NRG1 isoforms in 10 matched normal/tumour samples		
Isoforms	Normal	Tumour
NRG1- Beta -1	10	10
NRG1- Beta -3	10	10
SMDF	0	0
NRG1-Gamma	6	6
NRG1-GGF2	0	0
NRG1-Alpha	9	9
NRG1-ndf-43	9	8

Table 7.2

In no case was GGFII or SMDF detected, while all tissues examined expressed beta-1 and beta-3. 9 of 10 of each series expressed alpha isoforms, while gamma and NDF43 were detectable in the majority of cases. These results indicate that no absolute shut-down or *de novo* expression of any NRG1 isoform occurs during tumour formation but this does not exclude the possibility of changes in the relative expression levels of each, since conventional RT-PCR cannot provide quantitative results.

Although the NRG1-Beta 1 isoform was expressed in all of the matched normal/tumour samples, 2 bands were noted in all of the samples (see **Figure 7.4 & 7.5)**.

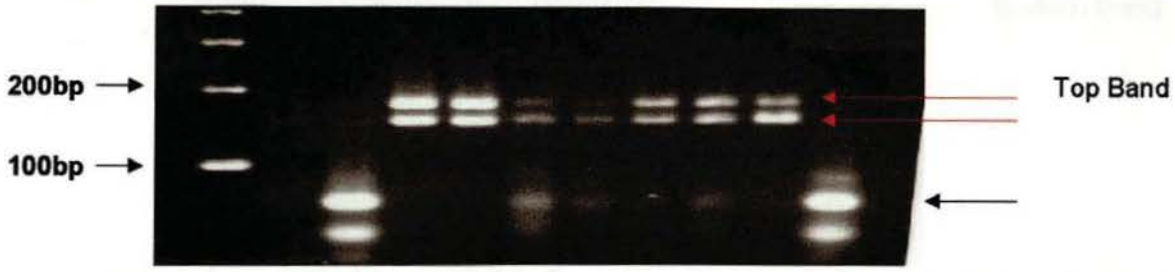


Figure 7.5: NRG1-beta 1 expression in matched normal/tumour colorectal samples. Following the isoform-specific RT-PCR analysis, PCR products of the beta 1isoform of matched normal/tumours samples were resolved on a 3% agarose gel to allow further separation of the fragments of interest.

In order to confirm Beta 1 isoform expression, sequencing of these fragments was performed.

Sequencing of Beta 1 Isoform:

For this purpose the QIAquick gel extraction protocol was used (described in Chapter 2). The samples were analysed on a gel following elution (as shown in **Figure 7.6**).

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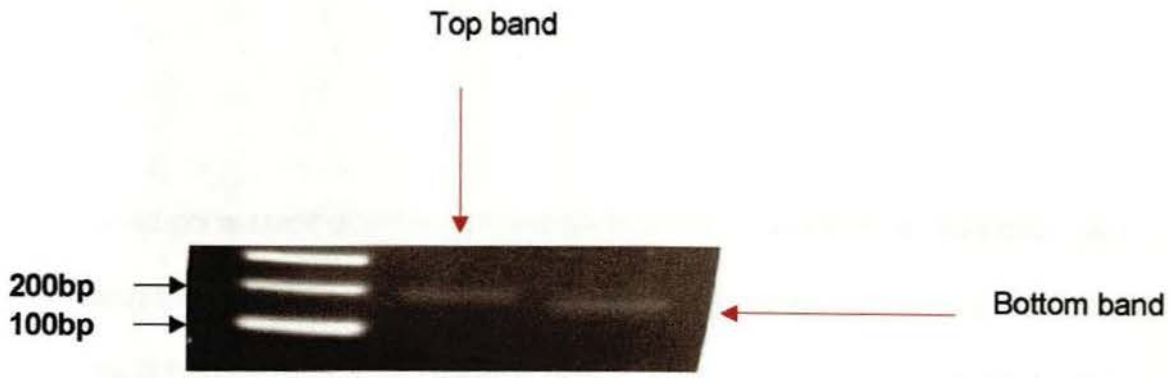

Figure 7.6: **Analysis of NRG1- Beta 1 isoform PCR products.** Following DNA extraction the 5ul of each fragment of interest was run on a 2% agarose gel to check the yield. Samples were run against 0.5ug of 100bp DNA ladder.(100bp marker has a DNA mass of 48ng)

Each fragment (top and bottom band) was taken through a PCR reaction using the Big Dye Cycle sequencing kit. The PCR products of the sequencing reaction were purified (Big Dye Version 3) and sequenced at the Functional Genomics Unit (University of Birmingham).

Sequencing Data

The sequencing data obtained for each fragment sequence was put through a Blast search (NCBI website) Sequence analysis demonstrated that the upper band contained exon 10 sequence and was therefore the expected 174bp NRG1-Beta 1 PCR amplicon. Sequence analysis of the lower band identified a clone similar to the NRG1-alpha isoform.

Over all no consistent qualitative change in isoform expression was detected. To extend these data we also performed a partial review, by semi-quantitative real-time RT-PCR, of the expression levels of beta-1, beta-3 and NDF43 in a series of 5 early and 5 advanced cancers, compared with matched normal mucosa. Semi- quantitative real- time PCR was used to further evaluate the above findings. Real-time PCR was also used to examine whether secretory or transmembrane isoforms were predominately expressed in colorectal tumours.

7.3 Semi-quantitative Real-time RT-PCR analysis of NRG1 isoform expression in colorectal tumours

Having confirmed expression of *NRG1* isoforms Beta1, Beta 3, Gamma and ndf43 in colorectal tumours, the expression levels of these isoforms were investigated by semi-quantitative real-time RT-PCR in a series of 5 early and 5 advanced cancers, compared with matched normal mucosa (**Table 7.3**). Early tumours were also selected to investigate for any correlation between tumour stage and expression of *NRG1* isoforms.

Tumour	Stage
18	T3N0M0 (advanced)
35	T3N0M0 (advanced)
24	T3N1M0 (advanced)
38	T4N0M0 (advanced)
29	T4N0Mx (advanced)
60	T1N0M0 (early)
54	T2N0M0 (early)
73	T2N0M0 (early)
53	T2N0M0 (early)
58	T2N0M0 (early)

Table 7.3: Histopathological staging of tumour samples used for real-time analysis

7.3.1: Design of primers to distinguish between different NRG1 Isoforms

The Beta-1 and ndf-43 isoform specific primers were designed across exon/ intron boundaries (across exon 10, unique to Beta-1 and exon 15, unique to ndf-43). Primers to isoform Gamma were designed across the sequence of exon 7 unique to this isoform. This primer was not designed across exon / intron boundary as the amplicon would have been too large for real-time analysis. DNase treated RNA was used as a negative control for each matched normal/tumour sample. Similarly, the Beta-3 isoform primer was designed across exon 9. Although this sequence (exon 9) is present in *NRG1* isoforms SMDF and GGFII, real-time RT-PCR with Beta –3 oligonucleotide primers can only detect the Beta-3 amplicon, as both GGFII and SMDF were not expressed in colorectal tumours. Due to the nature of the sequence it proved too difficult to design a NRG1-alpha specific real time RT-PCR assay.

7.3.2: Optimization of isoform specific primers

The C_T (cycle threshold) values obtained for each primer pair are shown in Table 7.5 a & b. The PCR products were run on a gel to check that the Ct values were not a result of primer-dimer formation (Figures 7.6 a,b ,c and d).

Optimization of Beta –1 and ndf-43 isoform specific primers.

Primer	Sample	primer conc	C_T	Average C_T
Beta 1	BXPC3	50/50	39.12	
Beta 1	BXPC3	50/50	39.25	39.25
Beta 1	BXPC3	50/50	37.14	
Beta 1	BXPC3	**300/300**	**23.51**	
Beta 1	BXPC3	**300/300**	**23.44**	23.44
Beta 1	BXPC3	**300/300**	**23.19**	
Beta 1	BXPC3	900/900	22.76	
Beta 1	BXPC3	900/900	22.65	22.65
Beta 1	BXPC3	900/900	22.39	
Beta 1	H20	300/300	40	
Beta 1	H20	300/300	38.28	38.28
Beta 1	H20	300/300	40	
ndf	BXPC3	50/50	40	
ndf	BXPC3	50/50	40	40
ndf	BXPC3	50/50	40	
ndf	BXPC3	300/300	23.14	
ndf	BXPC3	300/300	23.07	23.07
ndf	BXPC3	300/300	22.62	
ndf	BXPC3	**900/900**	22.59	
ndf	BXPC3	**900/900**	22.47	22.47
ndf	BXPC3	**900/900**	22.34	
ndf	H20	300/300	40	
ndf	H20	300/300	40	40
ndf	H20	300/300	40	

Table 7.4a: The C_T values obtained from different primer dilutions are shown in the table above. The figures in red represent the primer dilutions used for real-time PCR analysis. For Beta-1 isoform, although the C_T values for the 900/900 primer dilution were better, no significant difference in the intensity of PCR product was noted on the gel for 300/300 and 900/900 primer dilutions. Therefore a primer dilution of 300/300 was chosen. A primer dilution of 900/900 for ndf-43 primer revealed product of increased intensity on the gel which corresponded with C_T values.

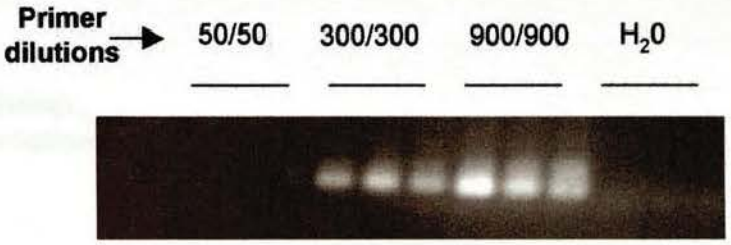

Fig: 7.7a

Fig: 7.7b

The PCR products of Beta-1 primer **(Fig 7.7a)** and ndf43 primer **(Fig 7.7b)** optimisation were run on an Agarose gel stained in SYBR Green dye (1in 200 dilution)

Optimization of Gamma and Beta-3 isoform specific primers.

Primer	Sample	Primer	C_T	Average C_T
Gamma	BxPC3	50/50	40	
Gamma	BxPC3	50/50	40	40
Gamma	BxPC3	50/50	40	
Gamma	BxPC3	300/300	32.12	
Gamma	BxPC3	300/300	33.23	32.816667
Gamma	BxPC3	300/300	33.1	
Gamma	BxPC3	900/900	28.11	
Gamma	BxPC3	900/900	28.74	28.536667
Gamma	BxPC3	900/900	28.76	
Gamma	H20	300/300	40	
Gamma	H20	300/300	39.61	39.87
Gamma	H20	300/300	40	
Beta 3	Brain	50/50	30.24	
Beta 3	Brain	50/50	31.1	30.276667
Beta 3	Brain	50/50	29.49	
Beta 3	Brain	300/300	22.04	
Beta 3	Brain	300/300	23.07	22.74
Beta 3	Brain	300/300	23.11	
Beta 3	Brain	900/900	22.72	
Beta 3	Brain	900/900	22.91	25.166667
Beta 3	Brain	900/900	29.87	
Beta 3	H20	300/300	40	
Beta 3	H20	300/300	40	40
Beta 3	H20	300/300	40	

Table 7.4b: The C_T values obtained from different primer dilutions are shown in the table above. The figures in pink represent the primer dilutions used for subsequent real-time PCR analysis. For Gamma and Beta-3 primer pairs the C_T values correspond with the findings on the gel.

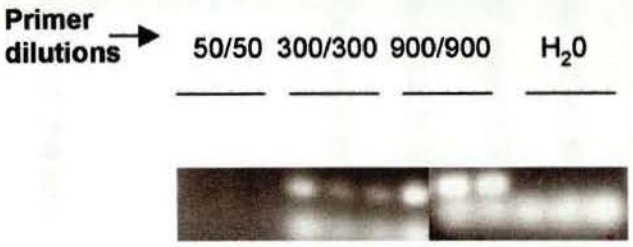

Fig: 7.7c

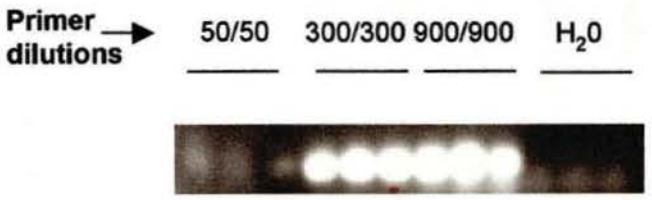

Fig: 7.7d

Figure 7.7c&d: The PCR products of Gamma **(Fig 7.7 c)** and of Beta –3 **(Fig 7.7 d)** primer optimisation were run on an Agarose gel stained inSYBR Green dye (1in 200 dilution)

7.3.3 Analysis of NRG1 isoform expression by Real-time RT-PCR

Cytokeratin-8 (KRT-8) an epithelial cell marker was use as an internal control. The expression of cytokeratin 8 was consistent between normal and tumour samples (Data not shown). The *NRG1* isoform expression in each matched normal and tumour colon sample was standardised to KRT8 gene expression. The expression of each *NRG1* isoform in tumours was normalised to the mean expression of each *NRG1* isoform in matched normal mucosa. Expression of *NRG1* isoforms is given by $2^{\wedge-\Delta\Delta Ct}$, where - $\Delta\Delta C_T = \Delta C_T$ tumour $- \Delta C_T$ normal .

Figure 7.8, shows how the isoform levels were altered in tumours compared with matched normal tissue. 4 of the 5 early tumours showed overall decreased expression of at least 2 of the 3 transcripts reviewed, in many cases by a factor of 5 to 10, although in one case (53T) transcript levels were significantly increased compared with normal tissue, by up to 35-fold in the case of beta-1. In advanced cancers, the results were more heterogeneous with reductions of 10- to 100-fold in some cases and inductions in the 10- to 20- fold range in others, but no consistent pattern emerged between the cases investigated.

Real-time analysis of NRG1 isoform expression in colorectal tumours.

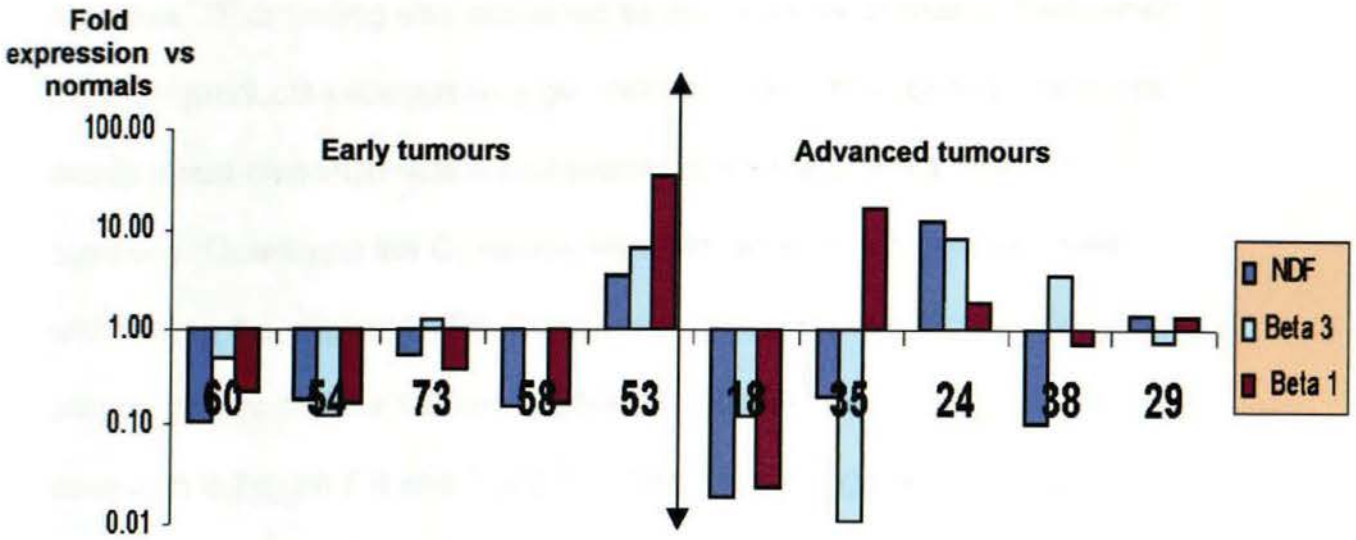

Table 7.5: Expression levels of NRG1 isoforms

Tumour	Beta 1	NDF	Beta 3
60	0.22	0.11	0.49
54	0.17	0.18	0.13
73	0.36	0.50	1.19
58	0.17	0.16	0.96
53	35.02	3.32	6.28
18	0.02	0.02	0.12
35	16.34	0.19	0.01
24	1.80	12.50	8.09
38	0.67	0.10	3.53
29	1.32	1.34	0.74

Figure7.8: The figure above shows real-time RT-PCR quantitation of NRG1 isoforms mRNA expression levels in 10 matched colorectal tumours. Samples 18, 35, 24, 38 & 29 are advanced tumours and samples 60,54, 73, 53,& 58 are early tumours. To normalise for the quality and quantity of cDNa, the expression of each isoform was normalised to the cytokeratin 8 gene. The expression level of each isoform for each tumour sample was normalised to the mean value obtained for the matched normal mucosa. The expression level was calculated using $2^{Ã\text{-}ddCT}$ where $ddC_T = dC_T$ of tumour sample- dC_T of normal. The expression levels are shown on a logarithmic scale. No clear trend towards transmembrane or soluble isoforms being expressed during different stages of colorectal tumour progression was identified.

Real-time analysis of Gamma Isoform: The C_T values obtained for this isoform were similar for both the positive and negative control triplicate reactions. This finding was explained by primer-dimer formation seen when the PCR products were run on a gel. Although the SYBR Green mastermix used for real-time PCR was subsequently purchased form a different company (Quantace) the C_T values were similar to previous results seen when using the original SYBR Green mastermix (Applied Biosystems). To prevent primer dimmer formation different concentrations of MgCl were used as shown in Figure 7.6 and Table 7.7. Despite altering the PCR reaction conditions, the C_T values did not correspond with the gel findings.

Primer	Sample	C_T values	MgCl conc (mM)
Gamma	H_20	32.41	2
Gamma	H_20	31.49	2.5
Gamma	H_20	31.36	3
Gamma	H_20	31.68	3.5
Gamma	H_20	31.15	4
Gamma	H_20	31.24	4.5
Gamma	H_20	31.36	5
Gamma	H_20	31.2	5.5
Gamma	BxPC3	30.17	2
Gamma	BxPC3	29.89	2.5
Gamma	BxPC3	30	3
Gamma	BxPC3	31.52	3.5
Gamma	BxPC3	31.67	4
Gamma	BxPC3	31.87	4.5
Gamma	BxPC3	30.01	5
Gamma	BxPC3	30.09	5.5

Table 7.6: Real-time PCR with Gamma primers (900/900dilution) using different concentrations of MgCl

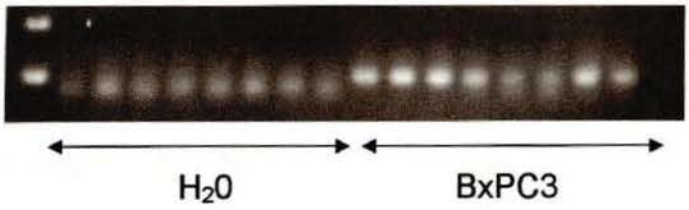

Figure 7.9: Gamma real-time PCR products were run on a gel stained with SYBR Green solution (1 in 200). The first 8 lanes following the MW marker show the PCR products obtained using H20 (negative control) with increasing concentrations of MgCl as shown in Table 7.7. In the remaining lanes the PCR product for positive control is shown.

This is one of the limitations of SYBR Green dye, as it binds any double stranded DNA and cannot distinguish between specific and non-specific products accumulated during the PCR reaction. As the C_T values were not reliable this prevented further real-time analysis of the NRG1 gamma isoform.

7.4 Discussion

The expression of NRG1 isoforms was reduced in the early tumours, compared with advanced tumours. This change was consistent in 4/5 (80%) of early tumours. In the advanced tumour, group upregulation of atleast one isoform was noted in 3/5 (60%) tumours. Unlike the early tumour group, no consistent change in isoform expression was noted in advanced tumours, Deletion of one allele of NRG1 was found in 4/5 advanced (except tumour 29) and 2/5 early tumours. Tumour samples 53, 58 and 73 had not been previously investigated for deletion of NRG1. In early tumour group with deletion (60T and 54T), decreased expression of all NRG1 isoforms was noted compared with matched normal controls. This was not the case in advanced tumour group with deletion where upregulation of atleast one isoform was noted in 3/4 (75%) tumours.

Real-time RT-PCR isoform analysis confirms increased expression of NRG1 isoforms in advanced tumours in the presence of deletion. Limitations in primer design prevented complete analysis of NRG1 isoform expression by real-time RT-PCR. Although transmembrane (Alpha, Beta-1and ndf-43) and secretory (Gamma, Beta-3) isoforms are expressed in colorectal tumours it

was not possible to establish which forms are predominately expressed due to study limitations.

Chapter 8

Discussion

8.1 Discussion

Loss of heterozygosity studies identified a distinct region of interstitial deletion at c8p12 locus in early (T1) cancers (Chughtai et al 1999). Interstitial deletions normally correspond to tumour suppressor genes, but the behaviour at c8p12 in colorectal tumours is more complex. Deletions at c8p12 were more frequent in early than advanced colorectal tumours (Chughtai et al 1999). Further studies (Adams, 2004) defined more closely this interstitial deletion, implicating a cluster of genes close to marker D8S259 in early colorectal cancer. One explanation for these findings at c8p12 is that both tumour suppressive and oncogenic effects reside at this locus. These activities could reside in two closely-linked genes or a single gene capable of showing both actions. We investigated *NRG1*, one of the genes in this region in colorectal tumours.

In this study deletion (loss of one allele) of *NRG1* was identified in early tumours with previously characterised 8 p12 deletions. Although the number of tumour samples studied were small, analysis of the ploidy status of these tumours revealed two groups; aneuploid and euploid tumours. Homozygous loss of *NRG1* was detected in the euploid tumours compared to the aneuploid group. An increased range of DNA copy number alterations of both the 8 centromere and *NRG1* gene was observed in the aneuploid group of tumours. The NRG1 copy number varied directly with the 8 centromere signal and was statistically significant. Chromosomal

gains and losses have been previously reported in aneuploid tumours (Reid et al., 1999; Sugai et al., 2003).

Investigation of NRG1 protein (heregulin) identified significantly increased expression in more advanced cases compared with adenomas and early tumours. In this study, small sample numbers prevented any meaningful analysis of whether *NRG1* deletions had any effect on NRG1 protein expression. However a recent study by Prentice et al. (2005) did not identify any correlation between NRG1 deletions and protein expression in a multiple tissue array of breast tumours. MTA analysis did not identify any correlation between stage and heregulin expression in colorectal tumours examined. Although increased expression of heregulin has been reported with tumour stage in tissue microarray of invasive breast tumours (Prentice et al., 2005), limited information regarding tumour grade in this study prevented further analysis.

Overexpression of erbB2 protein was identified in 9% (15/165) of colorectal tumours, which was in keeping with published reports (Ramanathan et al., 2004). In this study, no statistical difference was noted between tumour stage and erbB2 protein overexpression. Similar findings have been reported by Kim et al. (2004) in tissue microarray of colorectal tumours. However, MTA analysis of tumour samples identified a significant correlation between heregulin expression and erbB2 protein overexpression in tumours ($p=0.027$).

Although the erbB2 receptor proteins are reported at the basolateral membrane in normal airway epithelium (Vemeer et al., 2003), both MTA and previous immunohistochemical analysis (Chapter 4), did not show this pattern of distribution in colon tumour epithelium. In normal mucosa, epithelial cell polarity and tight junctions maintain ligand-receptor segregation. Disruptions of tight junctions and epithelial cell polarity e.g. in response to tissue damage, allow ligand-receptor interaction and repair of damaged tissues. One possible explanation as to why a more diffuse pattern of membranous staining was seen in advanced tumours is that loss of normal tissue architecture will lead to disruption of epithelial cell polarity and tight junctions, thereby allowing receptor- ligand interation.

Western Blot analysis did not show increased expression of heregulin in advanced tumours compared with adenomas and early tumours, instead multiple bands of varying molecular weight were noted. Although these bands could represent different *NRG1* isoforms, studies investigating the processing of *NRG1* proteins have reported similar findings and these bands of varying molecular weights are thought to represent cleavage products of the pro-protein (Monterro et al., 2000). As already mentioned, cleavage of transmembrane proteins is necessary to allow protein signalling and release of bioactive EGF domain. Therefore it is possible that the increased number of low molecular weight protein bands seen in advanced tumours, are due to processing of NRG1 pro-protein.

RT-PCR analysis of NRG1 alternatively spliced isoforms did not identify any difference between which forms were predominantly expressed in matched normal and tumour samples. Semi-quantitative real-time RT-PCR, in early tumours showed overall decreased expression of at least 2 of the 3 transcripts reviewed, in many cases by a factor of 5 to 10, although in one case *NRG1*-beta 1 transcript levels were significantly increased in the tumour compared with normal tissue. In advanced cancers, the results were more heterogeneous with reductions of 10- to 100-fold in some cases and inductions in the 10- to 20- fold range in others, but no consistent pattern emerged between the cases investigated.

Having identified the region close to marker D8S259 as the focus of this deletion in early tumours, *NRG1* (which resides close to D8S259) was an attractive target for further study, since the transmembrane isoforms contain both mitogenic EGF-related extracellular domains and pro-apoptotic intracellular domains (Montero et al., 2008; Le et al 2002). While the EGF action may dominate over apoptosis in unpolarised cells, in well-polarised epithelia heregulin, at least, is delivered to the apical membrane, segregating it from its basolateral receptor, and acts as a wound healing mitogen when tissue damage leads to loss of polarity and allows interaction with ErbB receptors (Vermeer et a., 2003). Cleavage between the two domains by TACE (Montero et al., 2000) in a well-polarised tissue would however reveal the pro-apoptotic function of the intracellular domain. Recent studies indicate that TACE activation is a consequence of beta-catenin-independent Wnt signalling, in turn promoted by the loss of soluble Wnt antagonists and the re-organisation of Wnt

response pathways early in tumourigenesis (Caldwell et al 2004, Caldwell et al 2008). It is plausible that NRG1 loss is selected in well-polarised early tumours to evade apoptosis by this route. Thus, deletion allows the tumour to form, but the subsequent absence of the oncogene function prevents further development of the tumour. Tumours carrying c8p12 deletions can still progress however, although our evidence indicates that the deletion extends centromerically in more advanced cases, which may reflect a compensating loss of a further tumour suppressor. DUSP26 (MAP kinase phosphatase-8) is one of the genes in this region that encodes a dual specificity phosphatase that associates with p38 MAPK, inhibiting apoptosis and could have a potential role.

Future studies

Due to time restrictions, it was not possible to corroborate the findings of each analysis undertaken in a larger cohort of tumours. Below I have discussed some experiments which would strengthen the data.

Although FISH analysis showed loss of *NRG1* in early tumours, CGH array analysis on a large cohort of early and advanced tumours would establish whether loss of *NRG1* is a more frequent event in early tumours compared with advanced tumours. Loss of one allele of *NRG1* was detected in the advanced tumours with previously established c8p12 deletion but a clear difference in tumour behaviour was detected between the two groups of tumour samples. In early tumours, NRG1 deletion (loss of one allele) resulted in reduced expression of *NRG1* protein but in advanced tumours

increased expression was noted. These findings warrants further investigation on a larger panel of early and advanced tumours with established 8p12 deletion.

Fluorescent immunocytochemistry studies would allow apico-basal polarity of the ligand (heregulin) and erbB2 receptors to be established in early and advanced colorectal tumours. As previously discussed loss of polarity and disruption of tight junctions allows receptor –ligand interaction to take place. Findings of loss of polarity in advanced tumours compared to early tumours will provide evidence of oncogenic activity by heregulin in advanced tumours.

Other Studies

Establish a murine model to investigate NRG1 in colorectal tumourigenesis:

The data reported in the thesis supports rather than refutes the original hypothesis that NRG1 plays a role in early and late colorectal tumourigenesis. In order to test this hypothesis further, however it will be necessary to perform functional studies *in vivo.*

Pan-knock out studies of NRG1 in mice have shown the importance of neuregulin in development of the nervous system and heart during embryogenesis (Falls., 2003; Meyer et al., 1997) but this phenotype prevents more detailed study in adult tissue. Meaningful functional studies in animal models would therefore require conditional knock-out. Investigating NRG1 in a min-mice model will allow the effects of knock-out / knock-in approaches.

Mice carrying defects in APC, such as APC^{min}, allow assessment of the impacts of tumour suppressor genes upon early events in tumourigenesis . The current study was restricted to human tissues and the behaviour of NRG1 in APC-mutant mice is unknown, but this could be assessed using similar methodology. If it behaves in a similar manner in mice, conditional deletion of NRG1 in an $APC^{+/-}$ background would increase the number of adenomas formed if the hypothesis that it acts as an early tumour suppressor is correct. Such a study could be extended to examine the contributions of individual isoforms by conditional deletion of their unique exons. The converse experiment, conditional over-expression of NRG1, may also be informative. The $APC^{+/min}$ mice rarely progress to cancer. If NRG1- encoded factors contribute to progression, their overexpression may promote progression, which can be scored histologically.

The combined data from these two approaches would provide robust evidence for a functional role in colorectal tumourigenesis which, in turn, could provide the basis for developing therapeutic approaches that target NRG1.

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