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The Role of DNA Methylation in Colorectal Carcinogenesis and Prognosis

Presentation of a thesis by

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and

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RCSI

Work carried out in the Department of Surgery,

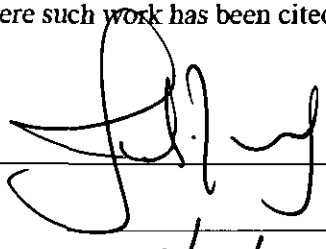
Royal College of Surgeons in Ireland, Connolly and Beaumont Hospital,

Dublin, Ireland.

Supervisor: Mr Eadhbhard Mulligan MD FRCS

Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree MD, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed: 

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CONTENTS

Chapter 7 - Discussion

ABSTRACT

Background

Colorectal cancer is one of the commonest cancers world-wide. Surgery remains the mainstay of treatment however an increasing emphasis is now placed on adjuvant treatment with chemotherapy and radiotherapy incorporating a multi-disciplinary approach to cancer care. Clinical background and pathological staging systems remain the most important predictive and prognostic indicators.

It is well-established that colorectal cancers develop via the adenoma-carcinoma sequence. This sequence is characterised by a step-wise accumulation of genetic mutational changes involving primarily the adenomatous polyposis coli (APC), K-Ras and p53 genes. In addition, the microsatellite instability pathway is responsible for almost all of the hereditary colorectal cancers. Despite ongoing research into these known genetic markers involved in colorectal carcinogenesis, clinical application in diagnosis and prognosis is still limited.

Recently, a novel molecular pathway involving epigenetic mechanisms has been described. This is now recognised to be an additional and distinctive molecular pathway to most neoplasia including colorectal cancers. This pathway is characterised by gene-silencing, most commonly through the process of DNA methylation of promoter regions of genes involved in carcinogenesis.

The aim of this thesis was to examine the role DNA methylation plays in colorectal carcinogenesis, in particular its potential as a prognostic marker in colorectal cancer as well as a predictor of response to neoadjuvant chemoradiotherapy in rectal cancer.

Materials and Methods

Patients with colorectal cancer were identified and samples retrieved from the pathology tissue bank. Immunohistochemical staining was performed using the commercially available 5-Methylcytidine (Eurogentec®, Belgium) and Ki-67 (DakoCytomation®, Denmark) antibodies. Automated image analysis of staining was carried out using the Aperio® (Vista, CA) image analysis platform. Image analysis results were correlated with clinico-pathological data of individual patients including response to neoadjuvant treatment and survival. Ethical approval was obtained from the local hospitals' ethics committees (Appendix V). Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS®) version 17.

Results

i) Role of DNA methylation in the adenoma-carcinoma sequence (Chapter 3)

Methylation analysis along the adenoma-carcinoma sequence (normal, polyps and cancer), identified a significant reduction in methylation between normal mucosa and all dysplasia groups; this was also observed between all polyp dysplasia groups and cancer ($p<0.0001$). No correlation was found between methylation and each advancing dysplasia group of polyps.

ii) The prognostic role of DNA methylation in colorectal cancer (Chapter 4)

A significant reduction in methylation was identified in cancer tissues of 88% of patients analysed compared to normal mucosa ($p<0.001$); in 12% of patients a higher methylation was found in their cancer tissues. No correlation was identified between methylation and proliferative index with clinic-pathological details of patients.

iii) DNA methylation in rectal cancer and its predictive value (Chapters 5 and 6)

Fifty-three patients with locally advanced rectal cancers who received neoadjuvant chemoradiotherapy were analysed for methylation and proliferative index. A significant decrease in methylation was observed in the post-treatment specimens in 38 (72%) patients ($p<0.001$) and an increase in methylation was identified in 15 (28%) patients. Proliferative index also reduced post treatment ($p=0.01$). Pre-treatment methylation was found to correlate significantly with tumour regression grading ($p<0.001$). Pre-treatment methylation was also able to differentiate between eventual complete and partial

pathological responders ($p=0.01$) as well as advancing T-stages ($p=0.005$). No correlation was found between pre-treatment proliferative index with tumour regression or clinic-pathological parameters.

Conclusion

DNA methylation is now established as an alternative and distinctive molecular pathway contributing to colorectal carcinogenesis. The process is an early event along the adenoma-carcinoma sequence and may act as both an initiating event and a driving force towards neoplasia. A genomic reduction in methylation is observed in colorectal cancer tissue compared to normal mucosa. In rectal cancer, neoadjuvant chemoradiotherapy appears to induce an overall hypomethylation status; in addition *it* may have the potential to become an accurate predictor of response to neoadjuvant treatment.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Cancer of the colon and rectum remains one of the commonest cancers worldwide. Traditionally regarded as a disease of the developed nations, its incidence is rising due to an overall increase in life-expectancy. With continuing advances in cancer research, colorectal cancer (CRC) is one of the most studied and well understood cancers in terms of its pathogenesis. One major breakthrough was the identification and description of accumulating molecular events in the adenoma-carcinoma sequence leading to cancer by Vogelstein in 1988[2]. Over the past two decades, major developments in the therapeutic management of colorectal cancer have also occurred. In particular the role of screening for early detection and prevention in the population; new treatment modalities using minimally invasive surgery (MIS) for resection[3]; neoadjuvant treatment with chemoradiation[4] and the acknowledgement of the importance of precise surgical technique in achieving a negative circumferential resection margin (CRM) in rectal cancer[5]. Despite these advances in our understanding of carcinogenesis and developments in treatment, colorectal cancer remains a leading cause of cancer morbidity and mortality, as well as recurrence. Scientists and clinical researchers are continuously searching for new methods to identify those individuals with a high likelihood of developing carcinoma with poor outcome and treatment strategies to improve prognosis.

DNA (Deoxy-ribonucleic acid) methylation is a mechanism by which individual genes can be "switched off". Gene expression is then suppressed, rendering them functionless[6]. This is a novel and expanding area of research which is beginning to receive attention in

the recent years in many malignancies. Recent attention has turned to the role of DNA methylation in colorectal carcinogenesis. It is now recognised that DNA methylation offers an additional and alternative pathway in cancer development[7]. Better understanding of the role it plays in colorectal cancer can potentially be vital in improving the survival of patients.

In this thesis, I aim to examine the role DNA methylation plays in colorectal neoplasia and in particular its contribution to the prognosis of patients.

1.2 Epidemiology of colorectal carcinoma

Colorectal cancer is a common malignancy facing an ever increasing incidence. This disease is presenting an economic challenge for all communities. In Ireland, 2184 new cases were diagnosed in the year 2005 compared to an average of 1899 cases between 1994 to 2005. The age-standardised incidence was 40.5/100,000 and 65/100,000 in females and males respectively[8]. Ireland has been ranked sixth in the incidence of colorectal cancer in Europe. In 2004, colorectal cancer was the second commonest cause of cancer death behind lung cancer. The number of new cases is also set to increase over the next few decades as a result of the aging population and an overall population increase. This malignancy has placed significant burden on individual sufferers.

1.3 Aetiology of colorectal carcinoma

About 75-80% of all colorectal cancers are sporadic in origin and develop without a hereditary predisposition[9, 10]. The major risk factors for development of sporadic colorectal cancer include increasing age, dietary (high fat, low fibre and vegetables), previous history of adenoma or malignant tumour and inflammatory bowel disease[1] (Table 1.1). An estimated 10-30% of colorectal cancers have an inherited basis, however only a small percentage of these have identifiable genetic mutations[10]. This group of inherited cancers consists of recognised familial syndromes with characteristic clinico-pathological and molecular mutations as well as cases that have a family history but do not belong to known inherited syndromes.

Table 1.1 Risk factors for colorectal cancer

Non-preventable	**Preventable risk factors:**
Age > 50yrs	High-fat diet
Previous colorectal cancer	Low fruits and vegetables
Polyps	Lack of exercise
Family history of polyps or colorectal cancer	Obesity
Inflammatory bowel disease	Smoking
	Alcohol

1.4 Pathophysiology

Colorectal cancer is the end result of progression of normal mucosa through various well-defined clinical and pathological pre-cursor benign lesions, known as the Adenoma – Carcinoma Sequence[11, 12]. Accompanying the progression of the adenoma-carcinoma sequence is a step-wise accumulation of genetic alterations that ultimately confer the malignant phenotype to the tumour[13].

Colorectal cancer is an excellent tumour type in which the histopathological and molecular alterations leading to carcinoma can be examined and studied. It progresses through a series of easily recognisable clinico-pathological stages and so provides a wide range of neoplastic lesions that are easily accessible for detailed analysis. Moreover, the standard length of time of progression of normal to adenoma, to advancing dysplasia to carcinoma, is relatively long within colorectal cancer which gives ample time to allow for intervention in the adenoma-carcinoma sequence to halt progression of malignancy. All pre-cursor adenomas and eventual carcinomas have characteristic histological features e.g. differentiation, villous components, that help to determine their behaviour and aggressiveness, and in turn affect their treatment and prognosis.

1.4.1 Polyps

A polyp is simply a mass that protrudes into the lumen of the gut. Polyps can be non-neoplastic such as inflammatory, hamartomatous polyps, or neoplastic, which can be benign or malignant.

Adenomas

Adenomas (adenomatous polyps) are benign neoplasms that arise from epithelial elements. Their significance lies in that they are pre-cursors of invasive colorectal carcinomas. The prevalence of adenoma is about 20% to 30% before the age of 40, rising to 40% to 50% after the age of 60[14]. Males and females are affected equally. Morphologically they can be sessile (flat), or pedunculated (stalked). Based on their epithelial architecture, adenomatous polyps can be subdivided into:-

i) Tubular – tubular glands

ii) Villous – villous projections

iii) Tubulovillous – mixture

Tubular adenomas

These account for upto two thirds of benign large bowel adenomas. Tubular adenomas are usually small <2cm, smooth surfaced and pedunculated. However, the smallest

tubular adenomas can be sessile. The stalk of the adenoma is comprised of fibromuscular tissue and prominent blood vessels. Most tubular adenomas show mild dysplasia, one fifth shows a range of more pronounced dysplastic features which may vary from mild pleomorphism to frank invasive carcinoma. The risk of invasive carcinoma correlates with the size of the adenoma. One percent of tubular adenomas less than 1cm in size display invasive cancer; 10% of 1cm to 2cm adenomas show malignancy but 35% are cancerous if larger than 2.5cm[14].

Villous adenomas

These are typically large (upto 10cm), broad based, elevated lesions that have a cauliflower –like surface. Microscopically, villous adenomas are composed of thin, tall, finger-like processes lined by neoplastic epithelial cells and supported by fibrovascular connective tissue. In contrast to tubular adenomas, invasive carcinoma is more frequeutly observed. Since there is usually no stalk as a buffer zone, invasion is directly into the submucosa or deeper layers of the bowel wall. In polyps less than 1cm in size, the risk of malignancy is 10 times that for tubular adenomas. In polyps >2cm in size, 50% have invasive carcinoma[14].

Tubulovillous adenomas

These adenomas manifest both tubular and villous features (between 25% to 75% villous features). The risk of harbouring invasive carcinoma is intermediate between tubular and villous adenomas and correlates with the degree of villous features.

Histopathologically, colorectal neoplasia begins by widespread hyperproliferation of normal colorectal epithelial cells. One or a few of these proliferating cells then undergo clonal expansion and form a small benign neoplasm termed a tubular adenoma. These adenoma cells then continue to progress through increases in size, acquisition of villous (finger-like) architecture and dysplasia.

Dysplasia is the term used to describe the degree of disordered growth as compared to the normal epithelium which displays uniformity in size, shape and nucleus. Degrees of epithelial dysplasia are judged by the pathologist on the basis of aberrant nuclear changes including enlargement, pleomorphism, loss of polarity, stratification and an increase in mitotic figures[15]. Increasing dysplasia is accompanied by loss of differentiation and mucin production. In colorectal neoplasia, adenomas are classified into mild, moderate and severe dysplasia groups characterised by the degree of these features. When these dysplastic changes are marked and involve the entire thickness of epithelium, but the lesion remains confined to the normal tissue, it is considered a pre-invasive neoplasm referred to as carcinoma-in-situ. Once theses dysplastic adenoma cells develop the ability to overgrow adjacent sister cells and invade through the basement membrane, the

tumour becomes a malignant invasive carcinoma. With continuing progression, carcinoma cells acquire the ability to metastasise to local and extra-regional lymph nodes and ultimately distant sites.

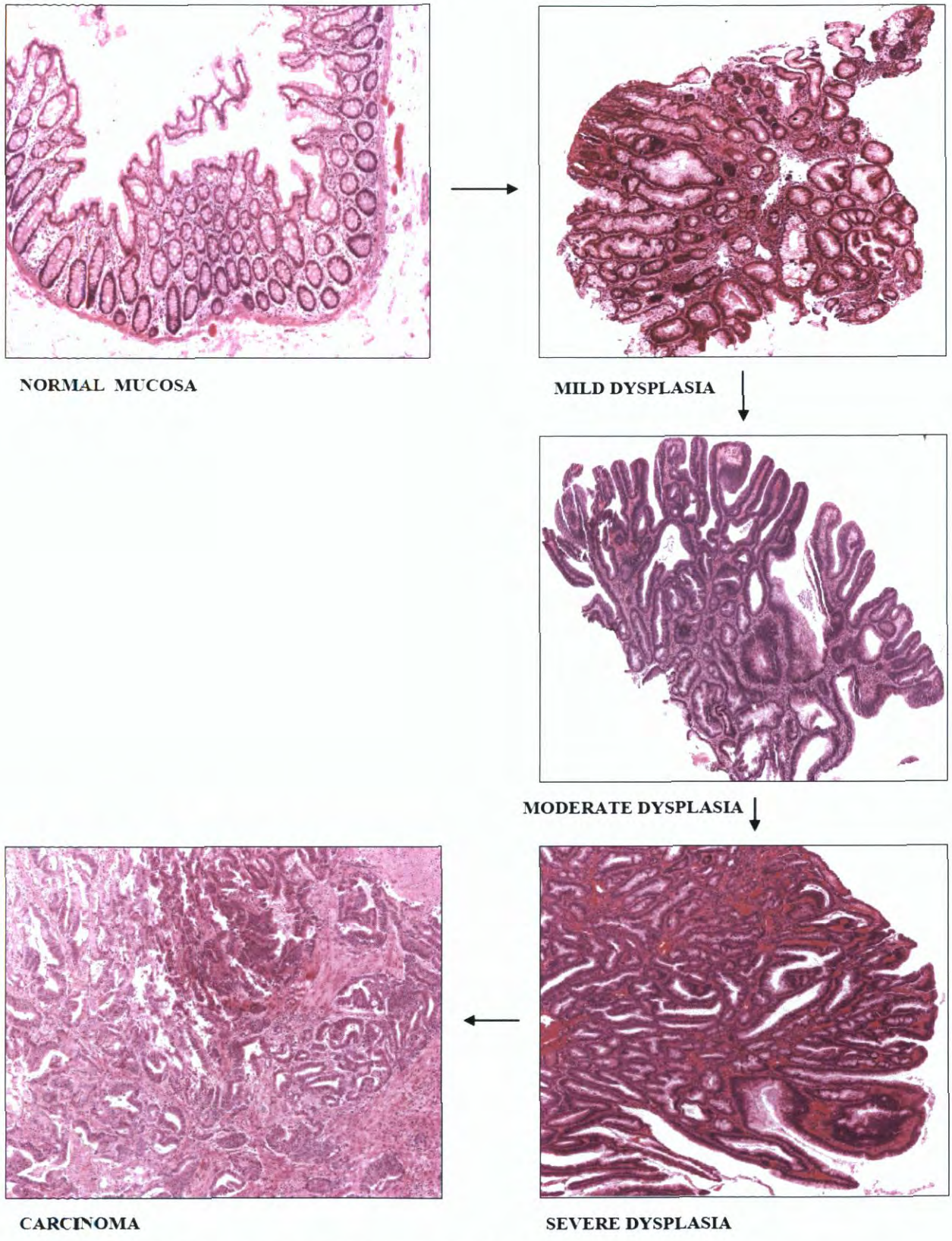


Figure 1.1 Adenoma - carcinoma sequence Histological representation

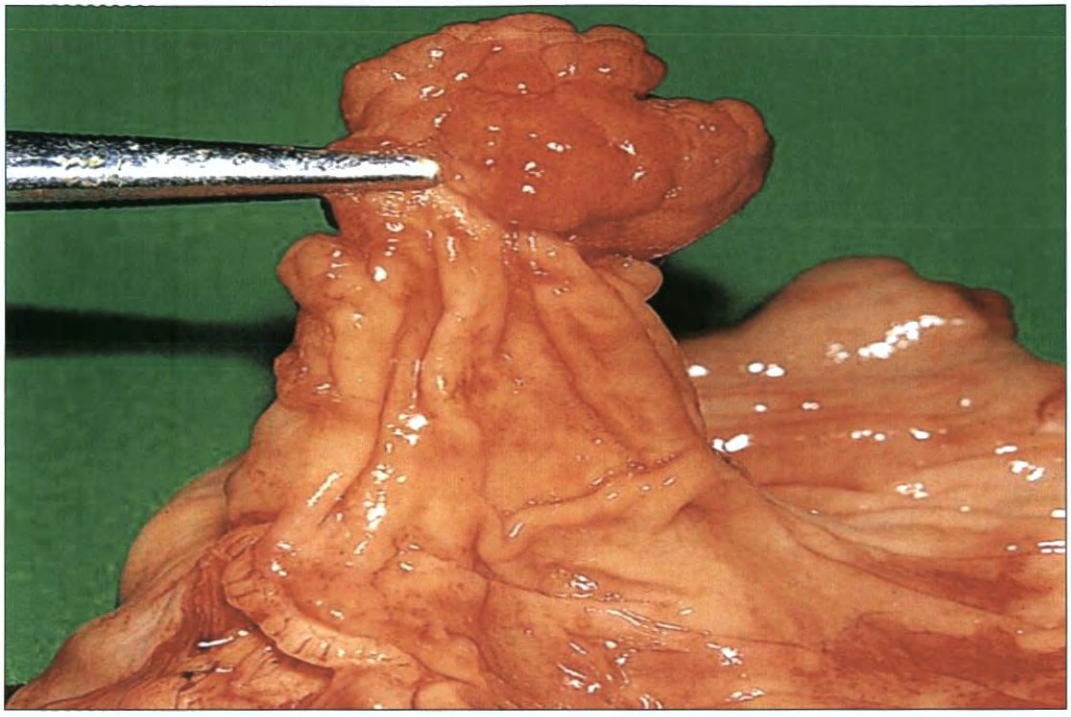


Figure 1.2 Adenomatous polyp

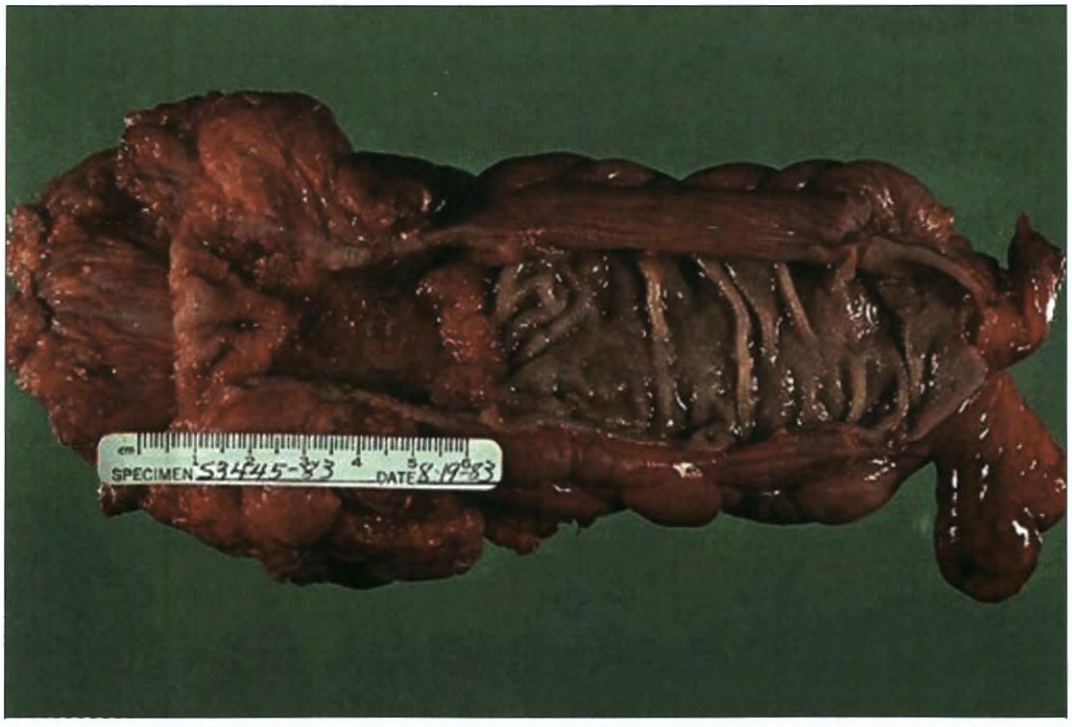


Figure 1.3 Invasive adenocarcinoma

1.5 Molecular classification

Colorectal cancer displays considerable genetic heterogeneity in terms of its make-up. Vogelstein[16] proposed the traditional chromosomal instability (CIN) model in 1990 and for many years this was presumed to be the sole and only molecular pathway and that colorectal carcinogenesis was thought to be homogeneous. However, it is now understood that at least two additional molecular pathways are involved that contribute to colorectal cancer development and increasingly different distinct subsets of colorectal cancer are being classified based on their molecular varieties. (Table 1.2)

Genomic instability is the term used to describe the cellular environment developed by cancerous cells to enhance further mutational events[7]. The two main models of genomic instability are the chromosomal instability and the microsatellite instability pathways[17]. Recently described, the methylator pathway is now understood to be an important alternative pathway to cancer formation involving epigenetic mechanisms[7].

Table 1.2 Suggested molecular pathways in colorectal carcinogenesis

Molecular pathway	Key (epi)genetic events
Chromosomal instability	APC, K-Ras, p53 mutations
Microsatellite instability	Mismatch repair gene mutations
DNA methylation	Suppression of gene expression

1.5.1 Molecular pathway I - Chromosomal instability

Vogelstein proposed a genetic model for colorectal carcinogenesis in 1990 after discovering the genetic alterations contributing to the initiation and progression of colorectal cancer in an analysis of 172 colorectal neoplastic lesions[2]. In his genetic model, four salient features were emphasised:

i) CRC arises as a result of mutational activation of oncogenes coupled with mutational inactivation of tumour suppressor genes.

ii) Mutations in at least 4 to 5 genes are required for the formation of a malignant tumour.

iii) Although there is a preferred order of mutational changes, accumulation of these changes rather than their order of appearance is responsible for determining the tumour's biological properties.

iv) Mutant tumour suppressor genes are capable of exerting effect in the heterozygous state in some cases.

This genetic model consists of a variety of genetic alterations including point mutations of DNA, allelic losses as well as changes in expression and activity of individual genes. Different alterations are found in varying proportions at different stages of the adenoma-carcinoma sequence providing an indication of the importance of each event along the sequence.

The chromosomal instability pathway is responsible for approximately 70%-85% of all colorectal cancers and the key genetic events involve 4 genes namely adenomatous polyposis coli (APC), K-Ras, p53 and deleted in colon cancer (DCC) genes[18].

The APC gene functions as a tumour suppressor gene and is located on chromosome 5q21. The APC protein plays a role in cell adhesion through its binding to β-catenin which in turn controls the *Wnt*-signalling pathway[19], which is responsible for cellular differentiation and proliferation. At the same time it binds to microtubule bundles and promotes cell migration and adhesion. β-catenin is a member of the cadherin-based cell adhesive complex, which also acts as a transcription factor if the protein is translocated into the nucleus. Normally, when β-catenin is not bound to E-cadherin to participate in cell adhesion, a cytoplasmic complex (APC, Axin, GSK-3β and β-catenin) forms, this leads to the phosphorylation and degradation of β-catenin. This pathway normally regulates growth, apoptosis and cell differentiation. Through binding to β-catenin, APC down-regulates the Wnt-signalling pathway which in turns inhibits cellular proliferation, a major role of APC as a tumour suppressor gene[18]. Allelic losses of chromosome 5q have been observed in 40% of colorectal cancers and adenomas.

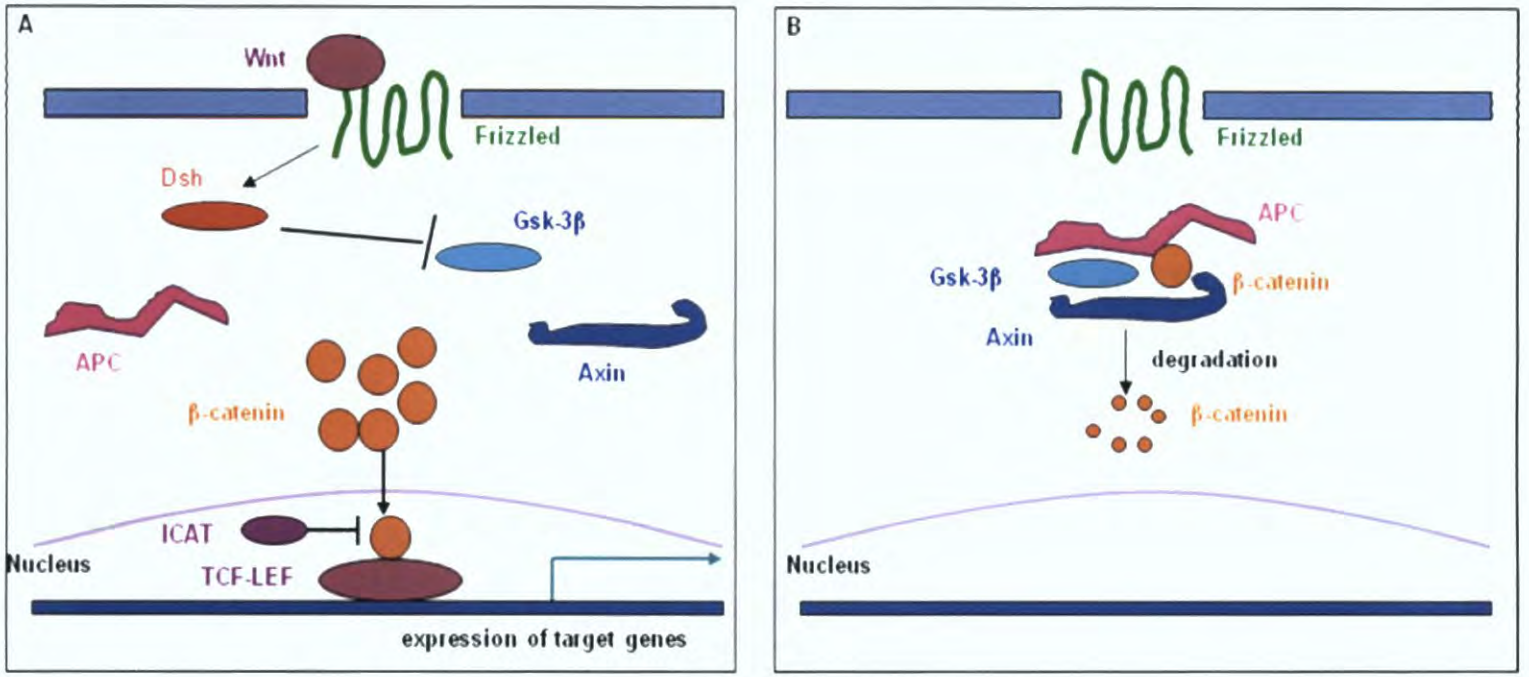


Figure 1.4 Wnt- signalling pathway. A- Wnt- signalling stabilises β-catenin through inhibiting GSK-3β. Accumulated β-catenin is transported into the nucleus which leads to the expression of target genes. B – APC associates with β-catenin and in co-operation with axin, leads to phosphorylation of β-catenin by GSK-3β. The resultant β-catenin then undergoes proteasome-dependent degradation, resulting in inhibition of the Wnt-signalling pathway. (Adapted from Cadigan KM, Liu YI. Wnt signaling: complexity at the surface. *J Cell Sci* 2006;119(Pt 3): 395-402.)

K-Ras (12p12) is a proto-oncogene involved in signal transduction. Activating K-Ras mutations are found in 50% of colorectal cancers and in similar percentage in intermediate and late stage adenomas. However, these mutations are found in <10% of small (<1cm) adenomas.

Chromosome 18q21.1 codes for the deleted in colou cancer (DCC) gene. DCC is implicated to be involved in cell adhesion. Allelic losses of DCC genes are found in 70% of colorectal cancers, 47% of large adenomas and <10% of small and intermediate adenomas. This suggests that 18q losses predominantly occur in the later stages of the adenoma-carcinoma sequence[13]. P53 is well known tumour suppressor gene involved in a variety of human carcinomas including breast, lung, bladder and brain. It is located on chromosome 17q13, and mutations of this chromosome are found in 75% of colorectal cancers[13].

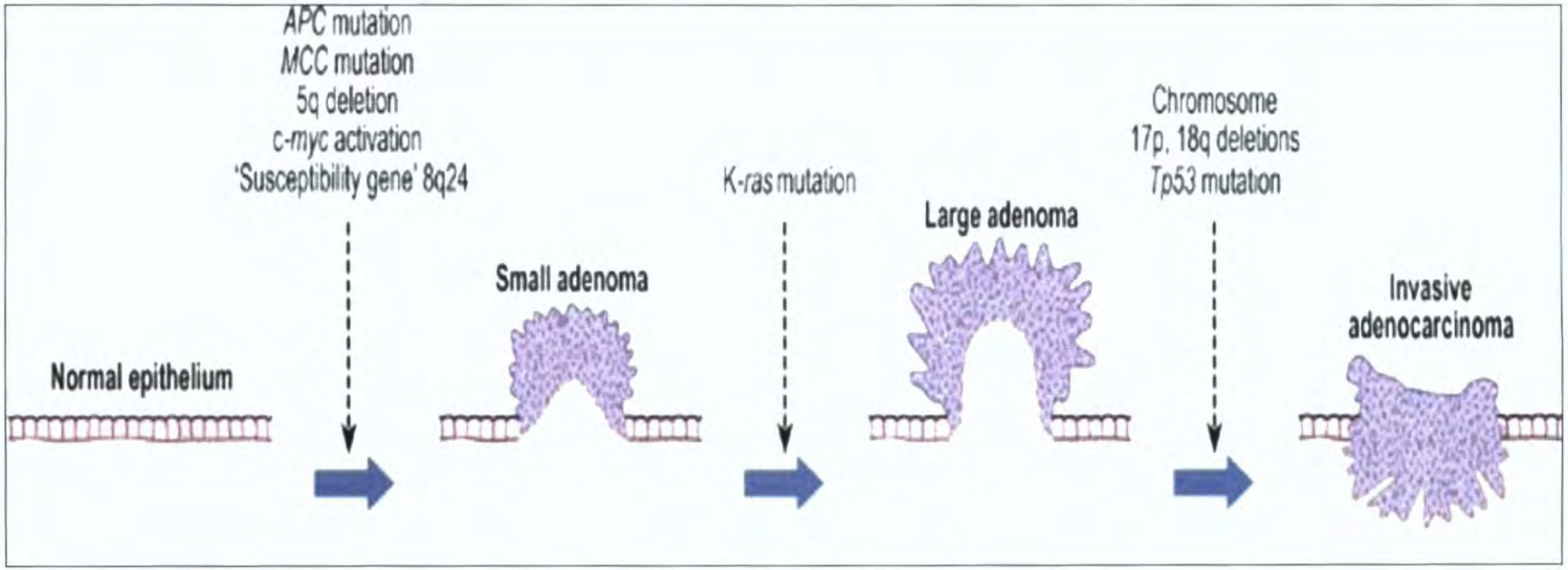


Figure 1.5 Vogelstein's multistep genetic mutations along adenoma-carcinoma sequence (Adapted from General and Systemic Pathology 5th edition, Underwood and Cross)

1.5.2 Molecular pathway II - Microsatellite instability

The second chief mechanism for genomic instability is the microsatellite instability (MSI) pathway also known as the mutator pathway. MSI is responsible for 10-15% of all sporadic colorectal cancers and is observed in almost all adenocarcinomas from patients with hereditary non-polyposis colon cancers. Microsatellites are a type of DNA that consists of tandem repeats of one to five base pairs, repeated many times. Numerous microsatellites are interdispersed throughout the human genome and are particularly susceptible to replication errors by DNA polymerase. These errors are repaired by DNA mismatch repair (MMR) proteins under normal circumstances. However when MMR proteins are deficient, such as following a germline mutation, microsatellites are prone to replication errors, commonly due to insertion of a wrong base by DNA polymerase during replication.. Microsatellite instability is further classified into MSI-high (MSI-H), MSI-low (MSI-L) and MSI-stable (MSS) depending on the number of microsatellites identified.

The mismatch repair (MMR) system consists of at least 7 proteins, of these hMLH1 (human MutL Homolog 1) and hMSH2 (human MutS homolog 2) are essential components of the MMR system and germline mutations of either of these genes represent over 90% of hereditary non- polyposis colon cancer (HNPCC) cases. The majority of MSI-H tumours are however, sporadic, and result from epigenetic inactivation or silencing of the mismatch repair proteins through DNA methylation, with hMLH1 being the main target of inactivation.

MSI-H tumours correlate with a distinct set of clinico-pathological features:

- Proximal location
- Diploid DNA content
- Favourable Duke's stage and better prognosis
- Abundant mucinous differentiation and Crohn's-like infiltrates

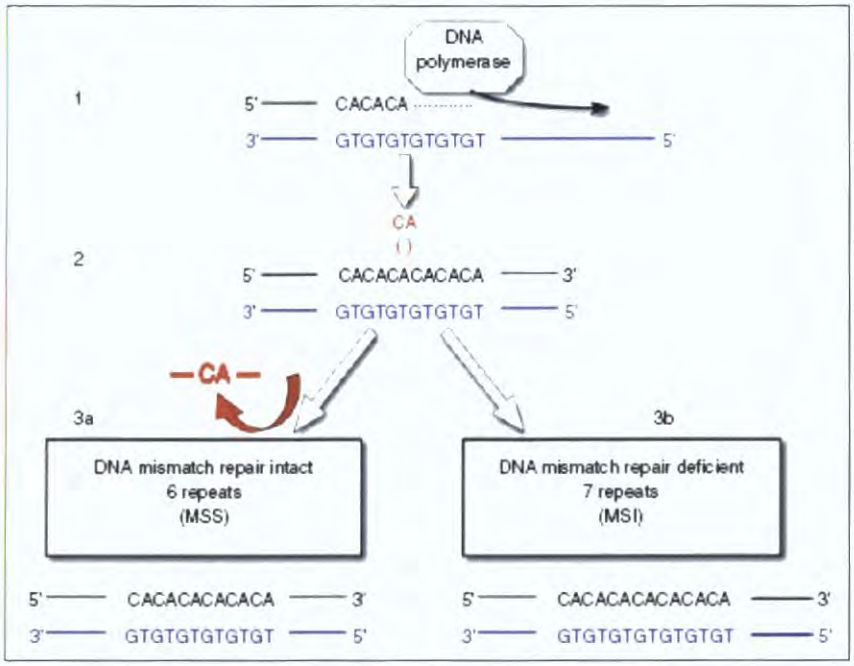


Figure 1.6 Mechanism of microsatellite instability. 1. Normal DNA replication. 2. A CA repeat is erroneously built into the newly synthesised strand of DNA. 3a. Error repaired by mismatch repair proteins – microsatellite stable. 3b. Error not repaired – microsatellite instability.

1.6 Hereditary colorectal carcinoma

1.6.1 Familial Adenomatous Polyposis

Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colon Cancer (HNPCC) are the two most recognised familial syndromes and account for about 5% of all colorectal cancers. Hamartomatous polyposis syndromes are less common familial syndromes and account for approximately 0.1% of all colorectal cancers. These include Juvenile polyposis, Peutz-Jegers syndrome and Cowden syndrome[10].

FAP is responsible for less than 1% of all colorectal cancers and has an incidence of 1 in 10,000. The primary genetic defect is mutation of the Adenomatous Polyposis Coli (APC) gene located on chromosome 5q21. The APC gene is a tumour suppressor gene and its encoded protein is essential in cell adhesion, signal transduction and transcriptional activation. Numerous types of mutations have been reported with insertions, deletions and nonsense mutations responsible for the majority, giving rise to a truncated malfunctioning APC gene product[20].

FAP is inherited in an autosomal dominant manner and clinically patients develop multiple (>100) adenomatous polyps in their teenage years. If left untreated, progression to cancer is inevitable with a mean age of malignancy developing in their mid-thirties. In addition, FAP can be associated with extra-colonic manifestations (Gardener's syndrome) including benign polyps of the small intestine and stomach, desmoid tumours, osteomas,

congenital hypertrophy of the retinal pigment epithelium and malignant tumours such as hepatoblastoma, duodenal, periampullary and brain tumours[20].

First degree relatives of affected patients are recommended for genetic screening for APC gene mutation or endoscopic screening if genetic testing is not available at the age of 12. Once polyps are identified, prophylactic surgery should be recommended to interrupt the adenoma – carcinoma sequence. Common surgical options include total colectomy with ileo-rectal anastamosis or proctocolectomy with ileo-anal anastomosis and ileo J-pouch formation[21].

Figure 1.7 Familial adenomatous polyposis with hundreds of adenomatous polyps

1.6.2 Hereditary Non-Polyposis Colon Cancer

Hereditary Non-Polyposis Colon Cancer (HNPCC) or Lynch syndrome is the commonest form of syndromic familial colorectal cancer responsible for 1-5% of all cases. The incidence of polyps is less than the familial adenomatous polyposis (FAP) syndrome however they occur at an earlier age than the normal population. Germline mutation in several DNA mismatch repair (MMR) genes is the genetic defect identified in patients with Lynch syndrome. Germline mutations of either the hMLH1 (human MutL Homolog 1) or hMSH2 (human MutS homolog 2) genes account for almost 90% of cases. DNA mismatch repair proteins are required to repair errors in DNA replication particularly involving short repeat sequences termed microsatellites. Deficiency in mismatch repair proteins therefore result in a distinct phenotype known as microsatellite instability (MSI), or the mutator phenotype[10].

Lynch syndrome[22] consists of a set of distinctive clinico-pathological features:

- Earlier age of onset than general population - Average 45 years
- Proximal colon involvement (70% proximal to splenic flexure)
- Excess of synchronous and metachronous tumours
- Histologically poorly differentiated with excess mucoid and signet-cell features; excess of tumour infiltrating lymphocytes within the tumour
- Increased risk of extra-colonic malignancies – Endometrial and ovarian with the highest risks

- Accelerated interval to development of carcinoma – 2-3 years

The Amsterdam Criteria (Table 1.3) was developed by the International Collaborative Group on Hereditary Non-Polyposis Colon Cancer (HNPCC) in 1991 to standardise the diagnosis. The Bethesda guidelines were subsequently developed to aid the use of MSI testing in the diagnosis of Lynch syndrome[23]. Individuals with identified germline mutations should undergo regular colonoscopic screening aimed at identifying polyps or dysplastic elements in patients at risk of developing overt carcinoma with the potential to interrupt the dysplasia to carcinoma sequence.

Table 1.3 Amsterdam criteria and Bethesda guidelines. CRC: colorectal cancer; FAP: Familial adenomatous polyposis; HNPCC: Hereditary non-polyposis colon cancer; MSI: Microsatellite instability

Amsterdam I	At least 3 1st degree relatives affected
	One is a 1st degree relative of other 2
	At least 2 successive generations affects
	One case <50 yrs
	FAP excluded
Amsterdam II	As for Amsterdam I except CRC maybe substituted by endometrial, small bowel, stomach, ovary, pelvi-ureter cancers
Bethesda	CRC < 50yrs
	Multiple CRC or HNPCC related cancers
	CRC with MSI-related histology and < 60yrs
	CRC or HNPCC-related cancer in at least one 1st degree relative < 50yrs
	CRC or HNPCC-related cancer in at least two 1st or 2nd degree relatives, any age

Microsatellite instability (MSI) testing

Patients with colorectal cancer with clinico-pathological features suggestive of Hereditary Non-Polyposis Colon Cancer and who fulfil either the Amsterdam criteria or Bethesda guidelines should undergo the commercially available geuetic testing for germline mutations or microsatellite instability testing of tissues to further confirm the diagnosis.

The National Cancer Institute in USA has published a list of five markers (Bethesda markers) which include 2 mononucleotides – BAT25, BAT26 and 3 dinucleotides - D2S123, D5S346 and D17S250 to diagnose microsatellite instability on fresh or paraffin embedded tumour tissues. Tumours with no instability in any of the markers are considered to be microsatellite stable (MSS). On the other hand, when one reference marker is mutated, a tumour is considered to have low microsatellite instability (MSI-L) and if two or more markers are altered, a tumour is considered to have high microsatellite instability (MSI-H). The disadvantages with MSI testing in tissues are that a good quality of DNA is required for analysis as well as an experienced pathologist; this test also does not provide any information on the particular mismatch-repair gene that is affected. Alternatively, immunohistochemistry (IHC) can be used by raising antibodies against hMLH1 and hMSH2 to look for losses of these protein products in tumour tissue. Immunohistochemistry is a more rapid and less expensive method for identifying mismatch repair gene alterations[24]. Immunohistochemistry has shown a direct correlation with 100% specificity to MSI-H tumours and 96.7% for MSS and MSI-L

tumours. Immunohistochemistry is useful in mutations that result in truncation of the resultant protein such as nonsense, splice site mutations and frameshift. However in missense mutation, immunohistochemistry may not be diagnostic as the protein maybe partly expressed and therefore still detected. Immunohistochemistry also requires good quality immuno-staining and experienced pathological analysis.

Individuals with positive microsatellite instability (MSI) analysis from tumour tissue should then undergo formal genetic testing for germline mutations in hMLH1 and hMSH2. Currently several methods exist including direct sequencing, denaturing gradient gel electrophoresis with sequencing of aberrant fragments. Genetic testing is time consuming and expensive and should only be performed if there is a high suspicion from family history, or positive tumour testing for MSI.

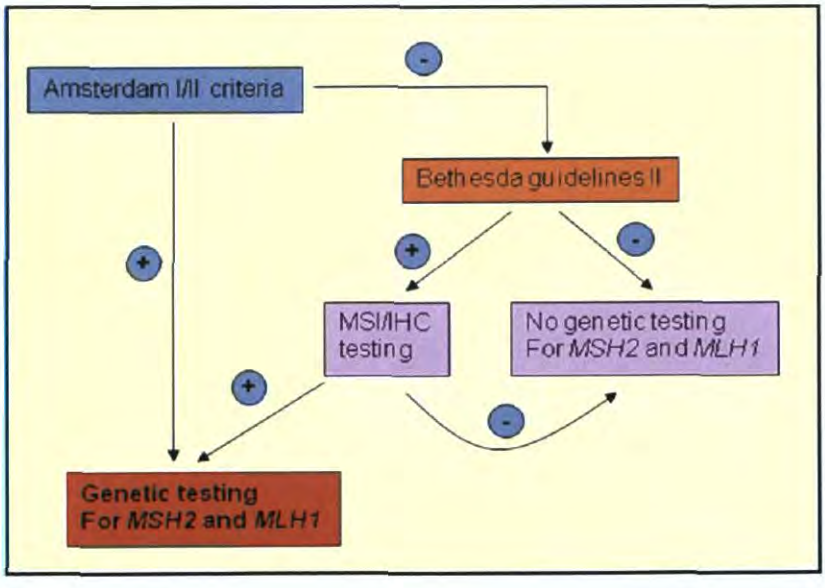


Figure 1.8 Referral guidelines for genetic testing for microsatellite instability

1.6.3 Positive family history

10-30% of colorectal cancer cases have a positive family history but do not meet the diagnostic criteria for inherited syndromes. These individuals appear to have an increased risk of colorectal cancer compared to the normal population and this risk increases with the number of relatives affected[21] (Table 1.4).

Table 1.4 Lifetime risk of colorectal cancer in 1st degree relatives of patient

Risk in general population	1 in 50
One 1st degree relative affected	1 in 17
One 1st degree relative and one 2nd degree relative	1 in 12
One 1st degree relative (<45yrs)	1 in 10
Two 1st degree relatives	1 in 6

1.7 Presentation of colorectal carcinoma

Symptoms of colorectal cancer are generally non-specific, unlike the association of a breast lump to breast cancer or a mole to melanoma, no single symptom alerts an individual to colorectal cancer to the same degree and symptoms are often ignored. As a result, colorectal tumours tend to be diagnosed at a late stage and commonly with metastases. Presenting symptoms are site-related with right-sided lesions commonly presenting with symptoms of iron-deficiency anaemia and left-sided lesions with altered bowel habits (Table 1.5). Advanced lesions may present acutely with perforation or obstruction, while others may present with symptoms of metastatic spread before the primary lesion causes symptoms.

Table 1.5 Presenting symptoms of colorectal cancer

Right-sided lesions	**Left sided-lesions**
Iron deficiency anaemia	Altered bowel habit
Right- sided mass	Rectal bleeding; mucus passage
Abdominal pain	Lower abdominal pain
Weight loss	Tenesmus
Small bowel obstruction	Large bowel obstruction/ Perforation

1.8 Screening

The well-defined pathological and genetic stages of development of colorectal cancer provide an ideal target for interception. It takes on average 5-10 years for malignancy to occur from the commencement of neoplasia of normal mucosa. This means that screening methods, can potentially identify the pre-cancerous lesions – adenomas, as well as early-stage curable cancer, and subsequently remove them to prevent cancer altogether. After removal, patients are placed in a follow-up surveillance program to identify future recurrence.

Colorectal cancer screening is challenging however, mainly due to low compliance rates of patients and cost issues[25]. In addition, current available screening modalities have limited sensitivity and specificity rates. Nonetheless, a successful screening program in colorectal cancer can significantly reduce the mortality and burden it places on the population.

1.8.1 Screening test – Stool occult blood and DNA testing

Stool testing aims to identify colorectal cancer patients before symptoms start to appear. Testing relies on blood and other tissue components shed by the tumour into faeces. Faecal occult blood testing is the most successful screening modality at present. Faecal DNA testing has also been investigated.

Faecal occult blood (FOB)

This is a guaniac-based test for faecal blood first developed in the 1970s. Testing relies on the pseudoperoxidase of haemogloblin. Guaniac turns blue after oxidation by oxidants or peroxidases in the presence of an oxygen donor such as hydrogen peroxide. Haemoglobin must be degraded to haem in the gastrointestinal tract for the guaniac test to function properly. One disadvantage of the guaniac test is that the reactiou can occur with other peroxidases in faeces such as fruits, vegetables and red meat therefore dietary restrictions are necessary to avoid false positive results. A recent meta-analysis of randomized controlled trials has shown that screening with using FOB can reduce colorectal cancer mortality by 25%[26]. It is suggested that screening with FOB can prevent 1 in 6 deaths from colorectal cancer.

In the United Kingdom, the National Health Service instituted the national bowel cancer screening program in 2006 to be phased in over 3 years. Home testing kits are sent out to individuals from 60 to 69 years every 2 years. Positive test patients are invited to undergo colonoscopy.

The faecal occult blood test only has a sensitivity rate of 50% to 60%[10]. False positives will be subjected to unnecessary further investigations such as colonoscopy. Another problem is the low compliance rate. Participation rates from the studies carried out were 64% at the first screening which reduced further in subsequent rounds[10].

Faecal immunochemical tests

Immunochemical tests to detect faecal blood are undergoing evaluation. Antibodies are directed against human globin epitopes which detect colonic blood with greater sensitivity. A comparison of guaniac and immunological test in a high-risk population in France has found a 2-fold increase in sensitivity rate[27]. Other studies have found no change in sensitivity but an improved specificity value with immunological blood testing[28]. Further studies are therefore required to better demonstrate the usefulness and benefits of the immunochemical test over the guaniac test.

Faecal DNA testing

Genetic mutations in colorectal cancer can be identified from DNA exfoliated into stool from the tumour[29]. The optimal set of molecular markers to be examined remains to be determined. Studies have found a better sensitivity using the faecal DNA compared to the guaniac test in the average-risk population[30]. The main disadvantage remains to be the cost of such tests, prohibiting their wide-spread use in the population.

1.8.2 Screening test – Endoscopy

Flexible sigmoidoscopy

This uses an endoscope to directly examine the large bowel upto a distance of about 60cm from the anal verge. Case controlled- trials from the USA have shown a reduction in colorectal cancer mortality from a combination of rigid and flexible sigmoidoscopy of 60% - 80%[31, 32]. Three large randomized controlled trials have been carried out to assess cancer detection rates, which range from 0.2% - 0.7%[33-35]. Advantages of sigmoidoscopy are less time – consuming, sedation may not be required, bowel preparation is easier and quicker and morbidity is minimal. However, the obvious disadvantage is that only the left colon is assessed. Specificity rates are high and range from 98% - 100% but sensitivity of the entire colon is expectedly low (35% - 70%) as due to the significant number of right sided lesions occurring without presence of left-sided tumours[32].

Colonoscopy

Colonoscopy allows the detection of polyps and cancer throughout the whole colon. Sensitivity and specificity rates are high for both polyps and cancer. Colonoscopy with polypectomy has been shown to reduce the expected incidence of colorectal cancer by 76% - 90% compared with reference populations in the large US National Polyp Database study[36].

Colonoscopy is clearly the 'gold-standard' for the detection of polyps and cancer at present. All patients with a positive faecal occult blood test or sigmoidoscopy should be referred for colonoscopy subsequently. However, the disadvantages are that it is time-consuming, requires a skilled endoscopist, more expensive and more inconvenient to the patient (whole bowel preparation). These reasons explain why colonoscopy has not been adopted as the first line screening procedure of choice in all countries.

1.8.3 Screening test – Radiological

Double-contrast barium enema (DCBE)

The diagnostic sensitivity and specificity of DCBE is inferior to colonoscopy[37]. The detection rate for polyps is low which makes it an unattractive option for screening[38]. Furthermore, a positive DCBE examination necessitates a subsequent colouoscopy which adds patient discomfort and time.

Computed tomographic colonography (CT Colonography)

Two and three dimensional reconstructions of the colonic lumen are available with this procedure. Studies using this procedure for detecting colorectal polyps and cancers have showed a high sensitivity for detecting large polyps (>10mm) [39, 40]. However, for smaller sized polyps, the results are not satisfactory compared to conventional colonoscopy.

This procedure still has several disadvantages including radiation exposure and the need for radiological expertise. More studies and improvements are required before CT colonoscopy could be considered as an effective screening tool.

Table 1.6 Colorectal cancer screening methods

Screening method	Sedation	Perforation rate	Biopsy/ polypectomy, or both	Sensitivity for detection (%)		Advantages	Disadvantages
				Large polyps (>1cm)	Cancer		
Faecal occult blood	No	NA	No	50%-60%		User-friendly Cheap	Low sensitivity
Faecal immunochemical test	No	NA	No	60%		More sensitive than FOB	Cost
Flexi-sigmoidoscopy	Rarely	1:10 000	Yes	35-70 (left colon only)		Less time (vs colonoscopy) Convenient bowel prep Low morbidity High specificity Allows polyp removal	Left colon exam only Low sensitivity
Colonoscopy	Usually	1:2000	Yes	98	97	Full colon examined Allows polyp removal	Skilled endoscopist needed Expensive Full Bowel prep needed Time-consuming
Double-contrast barium enema (DCBE)	No	1:10 000	No	48	83-94	Widely available	Radiation Requires colonoscopy if positive Low sensitivity and specificity vs colonoscopy
CT colonography	No	5:10 000	No	59-85	97	Sensitive and specific for large polyps (>1cm)	Expensive Radiation exposure Expertise needed Low sensitivity for smaller lesions

1.9 Diagnosis and investigations in colorectal cancer

Colonoscopy with biopsy is currently the gold standard for diagnosis of colonic polyps and colorectal cancer. Biopsy of the lesion must be followed by definitive pathological confirmation by a pathologist. Colonoscopy offers the advantage of removal of polyps by polypectomy, an essential method in preventing colorectal cancer development. Double-contrast barium enema is an alternative investigation if colonoscopy is unsuccessful or incomplete although it does not allow biopsies to be taken for histological examination. It is essential however for the purpose of searching for the presence of synchronous lesions (4%)[41].

Computed tomographic colonoscopy is a novel and emerging diagnostic tool. It is particularly useful in detecting large polyps (>10mm) and uses a computer generated 2 or 3 dimensional reconstruction of the colon. Besides its potential as a screening tool, its role in clinical implications is increasing. It offers a second line investigation after an incomplete colonoscopy; useful in examining the colon proximal to a stenosing lesion; and I patients not suitable for conventional colonoscopy e.g. High risk of bleeding[42].

1.10 Staging of colorectal cancer

Many imaging modalities are routinely used to determine the extent of spread of the malignant lesion both at a local and distant level. The gold standard of care in modern colorectal cancer staging is computed tomography of thorax, abdomen and pelvis with intravenous and oral contrast to evaluate local and distant spread.

For rectal cancers, early lesions can be assessed accurately by endorectal ultrasound (ERUS) in determining invasion into the rectal wall. A meta-analysis compared ERUS, MRI and computed tomography in the staging of rectal cancers. They showed ERUS to be the most accurate imaging modality for local staging of rectal cancers.[43] (Table 1.7). However recently, magnetic resonance imaging (MRI) has become the investigation of choice to assess the rectal wall boundaries and mesorectal fascia, which are crucial indicators of circumferential resection margin involvement and hence determinants of the need for preoperative radiotherapy. Studies suggest high resolution MRI could accurately predict tumour at the potential surgical radial resection margin if the tumour was within 1mm of the mesorectal fascia with a sensitivity of 88% and specificity 78%[44, 45]. A prospective European multicentre study carried out by the MERCURY group confirmed that high resolution magnetic resonance imaging can accurately predict whether the surgical resection margins will be clear or affected by tumour, to allow appropriate management[46, 47]. Both endorectal ultrasound and MRI are now routinely used together in the staging of rectal cancer if available.

Table 1.7 Sensitivity and specificity of MRI and TRUS in local rectal cancer staging

[43] TRUS: Trans-rectal ultrasound; MRI: Magnetic resonance imaging

	Imaging modality	Sensitivity	Specificity
***Muscularis propria* invasion**	TRUS	94%	86%
	MRI	94%	69%
Peri-rectal fascia invasion	TRUS	90%	75%
	MRI	82%	76%
Adjacent organ invasion	TRUS	70%	97%
	MRI	74%	96%
Lymph node involvement	TRUS	67%	78%
	MRI	66%	76%

Positron emission tomography (PET) now has an established role in detecting recurrent disease in colorectal cancer. A meta-analysis demonstrated a 97% sensitivity and 76% specificity with the use of PET scanning in diagnosing recurrent colorectal cancer[48]. Advancing technology has allowed PET scanning to be combined with computed tomography (PETCT). This combination is valuable in detecting residual disease after initial therapy and diagnosing recurrent colorectal cancer[49, 50]. In a study by Even-Sapir, PETCT was shown to have an improved sensitivity and specificity over PET alone (sensitivity 98% vs 82% and specificity 96% vs 65% respectively)[50]. Biochemical markers such as liver function tests and carcinogenic embryonic antigen (CEA) are useful

in detecting hepatic spread and recurrent disease and are therefore essential that these are obtained pre-operatively to facilitate post-operative surveillance.

1.11 Prognosis in colorectal cancer

The principal purpose of staging is to provide an accurate indication of prognosis for patients and to guide therapy.

Table 1.8 Prognostic indicators in colorectal cancer [15]

Radiological	**Histological**
Metastasis	Tumour penetration
Large mass	Lymph node
Circumferential margin involvement (Rectal)	lymphovascular invasion
	Grade
	Mucin production
	Circumferential margin involvement (Rectal)

The TNM classification system described by the Union Internationale Contra la Cancer (UICC) and the American Joint Committee on Cancer (AJCC) is one of the most widely used staging systems for a large range of cancers. Each tumour is assigned a "T" (tumour) stage indicating the progressive degree of invasion (T0 to T4) of the tumour into the bowel wall; "N" stage indicates local lymph node involvement and "M" stage

indicates whether distant metastasis is present. Dukes' staging was the first colorectal staging system developed in 1932 specific to colorectal cancer[51], this was later modified by Astler-Coller in 1954 to include subdivisions[52]. (Table 1.9 - 1.11, Figure 1.9)

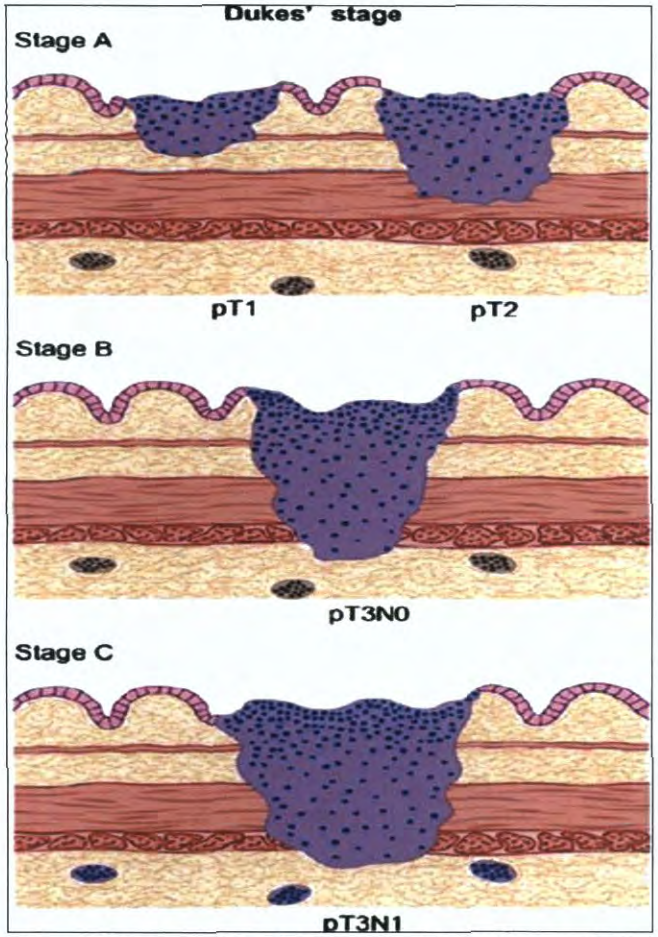


Figure 1.9 Invasion of tumour with Dukes and T-staging(adapted from General and Systemic Pathology 5th edition, Underwood & Cross)

Table 1.9 TNM staging for colorectal cancer

T- stage	
Tis	Carcinoma in situ
T1	Invades submucosa
T2	Invades muscularis mucosa
T3	Into subserosa or non-peritonealised pericolic/ perirectal fascia
T4	Into other organs or perforates
N-regional lymph nodes	
N0	No regional lymph nodes metastasis
N1	1 to 3 regional lymph nodes
N2	>3 regional lymph nodes
M- distant metastasis	
M0	No distant metastasis
M1	Distant metastasis

Table 1.10 Dukes' staging and prognosis for colorectal cancer

Dukes stage	Invasion	5 year overall survival
Dukes A	Confined to bowel wall	90%
Dukes B	Through the bowel wall	70-85%
Dukes C	Lymph node positive	30-60%

Table 1.11 Group staging of AJCC/TNM system with prognosis for colorectal cancer [1]

Stage	T	N	M	5 yr overall survival
Stage I	T1, T2	N0	M0	80-95%
Stage IIA	T3	N0	M0	72-75%
Stage IIB	T4	N0	M0	65-66%
Stage IIIA	T1, T2	N1	M0	55-60%
Stage IIIB	T3, T4	N1	M0	35-42%
Stage IIIC	Any T	N2	M0	35-27%
Stage IV	Any T	Any N	M1	0-7%

1.11.1 Molecular markers in prognosis of colorectal cancer

Despite the extensive research and knowledge gained in the molecular carcinogenesis of colorectal cancer, to date, a sensitive and clinically proven molecular marker of prognosis is still not available. The most promising marker is perhaps microsatellite instability. Sporadic microsatellite tumours have been shown to arise from promoter methylation of the hMLH1 (human MutL Homolog 1) gene while in the hereditary non-polyposis colon cancer (HNPCC) syndrome, microsatellite instability is is due to germline mutations in one of the DNA mismatch repair genes[53, 54]. In a meta-analysis, microsatellite instability has been shown to have a significant survival advantage compared to microsatellite stable tumours[55]. Further prospective studies are however still required to confirm its role as a routine clinical prognostic marker. (Table 1.12)

Table 1.12 Potential genetic biomarkers of colorectal cancer

Genetic marker	Function	Disadvantage
APC	Cell proliferation and differentiation	Non-specific
K-Ras	Signal transduction	Low sensitivity
P53	Tumour suppressor gene	Arises late in carcinogenesis
18q	Cell adhesion	Non-specific to colorectal cancer
Microsatellite instability	Defective DNA repair	Arises late in carcinogenesis

1.12 Treatment modalities in colorectal cancer

Significant developments have been made in the treatment of colorectal cancer in the last 20 years. Surgery is the main therapeutic modality in cancer management with chemotherapy and radiotherapy as adjuvant therapies proven in certain circumstances to improve either recurrence, disease-free survival or overall survival or both. We have seen advances in each of these 3 modalities in colorectal cancer and a strong emphasis is now in place in most colorectal cancer treatment centres for a multi-disciplinary team (MDT) approach including one, two or three of these modalities. Accurate pre-operative staging of patients remains an important step in the management algorithm and facilitates the formation of an optimal care plan individualised for each patient.

1.12.1 Treatment in colon cancer

Despite ongoing advances in the use of radiotherapy and chemotherapy, surgery continues to form the basis of treatment in colon cancer. Major developments have also been made in the area of minimally invasive surgery (MIS). Traditional cancer resection principles remain important and are continually applied. Novel concepts in colon cancer surgery include recent work done on extensive mesocolic resection[56]. The surgical procedure of choice depends on the location of the tumour as well as the essential knowledge of tumour spread occurring via lymphatics distributed along well-defined venous drainage systems as well as the need to determine resection extent on the blood supply anatomy of the remaining colon.

In colon cancers, radiotherapy use is limited to the adjuvant settings for advanced disease often with palliative intent. In R1 and R2 resections with macroscopic or microscopic residual tumour left after resection, directed radiotherapy boost may be given to the area (usually marked by staples) post-operatively to target residual tumour cells. In cases where the tumour is too large to be resected or extensive residual tumour is left behind after resection, palliative radiotherapy can be given for symptomatic control. The effect of irradiation on adjacent organs such as small bowel makes the use of radiotherapy in colon cancer limited.

1.12.2 Treatment in rectal cancer

Over the last 30 years, three landmark discoveries have been made in rectal cancer management leading to a much improved local recurrence and hence survival rates in rectal cancer. These include:

a) Circumferential resection margin – based on an increased understanding of the pathological spread of rectal tumours [57]

b) Total mesorectal excision – an emphasis on precise surgical dissection of rectal tumours [58]

c) Pre-operative radiotherapy

Circumferential resection margin

Pathologist Philip Quirke has shown that a positive circumferential resection margin (CRM), defined as tumour seen within 1mm of the edge of the resected specimen, and depth of extramural invasion are independent and significant indicators of poor prognosis and the likelihood of local recurrence[57]. The importance of the circumferential resection margin is highlighted by the findings from this study that a positive margin is associated with a 12 fold increase in local recurrence rates compared to patients with a negative resection margin. A positive circumferential resection margin is the result of incomplete surgical resection of the primary tumour – the main culprit behind local tumour recurrence[59].

Local recurrence (LR) in rectal cancer is therefore an indicator of the completeness of surgical excision. It results in severe morbidity with debilitating pelvic pain, ureteric obstruction, fistulae formation and poor bowel and urinary function[60]. Prognosis is extremely poor once LR occurs, with a 90% chance of death from the disease. Treatment is mostly palliative once local recurrence is diagnosed. Median time to diagnosis is 16-18 months with a median survival of 7-11 months in those treated with surgery alone and only about 6-7 months in those treated with pre-operative radiotherapy[61]. The 5-year survival is less than 5%.

Total mesorectal excision

Almost at the same time as Quirke's publication of the circumferential resection margin, the concept of total mesorectal excision (TME) was introduced by RJ Heald in 1982. This technique stresses the importance of meticulous sharp dissection out with the mesorectal fascia - "Holy Plane" – the plane between the visceral fascia of the mesorectum and the parietal fascia of the pelvis[62]. The mesorectal fascia contains all the draining lymphatics and veins of the rectum; total mesorectal excision aims to completely excise, under direct vision, the surrounding lymphovascular envelope and mesorectum of the tumour all the way to the pelvic floor.

Prior to the introduction of total mesorectal excision, the local recurrence rate following traditional anterior resection and abdomino- perineal resection (APR) was 20-30% with 5-year survival rates ranging from 30-68%, differing between centres and surgeons. Heald published his own series of 519 patients over a 9 year period in 1998 – Total mesorectal excision was able to achieve an overall local recurrence rate of 6% at 5 years and 8% at 10 years giving an overall specific survival of 68% and 66% respectively[63]. For patients who underwent a curative anterior resection, the local recurrence was as low as 2% at 5 and 10 years with a survival rate of 80%. These results are of marked contrast to historical figures. Following publication of his results, training programs were held in conjunction with colleagues in Sweden, Norway, Stockholm and Holland. Results have all revealed improved outcomes following the adoption of total mesorectal excision[64, 65]. It has become clear that quality surgical technique is the key to achieving a low local

recurrence. With optimal pre-operative assessment and selection of patients, followed by high quality surgery involving total mesorectal excision, local recurrence rates of <10% can be achieved for cancers of the mid and low rectum[60, 66].

Pre-operative radiotherapy

The use of radiotherapy has increased substantially in the last 10-20 years. Prior to the introduction of total mesorectal excision, the poor local recurrence rates of over 30-40% alluded to above prompted the addition of radiotherapy to surgery with the aim of improving these figures.

Randomised trials comparing radiotherapy with surgery as both neoadjuvant and adjuvant treatments have shown radiotherapy to be beneficial in rectal cancer management (Table 1.13). Neoadjuvant radiotherapy compared to adjuvant (post-operative) radiotherapy has several advantages including a lower local recurrence rate, better compliance and effectiveness due to better tissue oxygenation[67, 68]. Several systematic reviews/ meta-analyses have examined the role of pre-operative radiotherapy in resectable rectal cancer[69, 70]. All studies have shown lower local recurrence with preoperative radiotherapy compared to surgery alone or post-operative radiotherapy however an improved overall mortality was only seen in 2 meta-analyses[69, 70].

The main benefits of neoadjuvant radiotherapy use in rectal cancer are in:-

1) Improving local recurrence rates +/- Improving survival [71, 72]
2) Downstaging and "downsizing" of the primary tumour[73]
3) Increasing the resection rate of T3 and T4 tumours
4) The potential to decrease the abdomino-perineal resection to anterior resection rate[74]

Neoadjuvant radiotherapy versus surgery alone

The Swedish Rectal Cancer Trial (SRCT)[71] was the first randomised trial carried out to evaluate the use of pre-operative radiotherapy in locally advanced rectal cancer. This study was carried out prior to the introduction of total mesorectal excision by RJ Heald. The study group randomised patients with resectable rectal cancer to either receiving preoperative radiotherapy (25Gy in 5 fractions – "short course") followed by immediate surgery or to surgery alone. After a median follow-up of 5 years, the local recurrence rate was 27% in surgery alone group compared to only 11% in those who received radiotherapy ($p<0.001$), highlighting the poor surgical results as well as the advantages of neoadjuvant treatment. The overall 5-year survival was 48% in the surgery alone group compared to 58% in the radiotherapy plus surgery group ($p=0.004$). They concluded that preoperative radiotherapy reduced local recurrence and improved survival. In a recent update of their long-term follow-up results, they found that the benefits of pre-operative

radiotherapy in overall (p=0.008) and cancer-specific survival (p=0.04) and local recurrence (p<0.001) persisted to up to 13 years of median follow-up[75].

The Swedish rectal cancer trial was conducted before the wide-spread adoption of total mesorectal excision (TME). Following a national training programme in the technique of TME and quality pathological reporting, a multicenter randomised controlled trial comparing preoperative radiotherapy (5Gy x 5 days) plus immediate total mesorectal excision with surgery alone was conducted in Netherlands[72]. After 2 years of follow-up, local recurrence was 8.4% in the mesorectal excision group compared to only 2.4% in the radiotherapy plus total mesorectal excision group (p<0.001). Coutrary to the Swedish trial no significant difference in survival was seen in the two groups. Several important observations were drawn from this trial. Radiotherapy reduced local recurrence in only low rectal cancers but not in high rectal cancers; only stage II and III tumours showed significant benefits, the lower recurrence rate of total mesorectal excision after appropriate training and lastly no downstaging effect was observed after the short course radiotherapy used in this trial.

Long versus short course preoperative radiotherapy

Currently, two regimens of radiotherapy administration are being used in rectal cancer, the long and short course. The long course conventional fractionated radiotherapy (upto 50.4Gy in total; 1.8Gy/day x 5 weeks) with concomitant chemotherapy (5-FU) is used followed by total mesorectal excision 4-6 weeks after treatment. In Eastern and Northern

Europe, the short course radiotherapy (5Gy x 5days) regimen is more popular with surgery performed within 7 days of completing treatment. Advantages of the short course approach include a lower rate of early toxicity, better compliance, less expensive and can lead to prompt surgery in places with long waiting lists. However, as a higher dose of radiotherapy is administered, there is a concern regarding late toxicity.

Bujko et al. reported long-term results in terms of survival, local control and late toxicity in the two regimens of RT. Early radiation toxicity was higher in the long course CRT group (18.2% vs 3.2%; $p < 0.001$). No significant differences were observed in terms of overall survival, local control or late toxicity. However, they observed a downstaging effect, with higher rates of complete pathological response (CPR) and negative circumferential resection margin (CRM) after long course chemoradiotherapy compared to those who received short course radiotherapy[76].

In addition to radiation toxicity, pre-operative radiotherapy is also associated with early post-operative complications and long-term side effects. Early complications include perineal wound breakdown following abdomino-perineal resection, diarrhoea, proctitis, urinary tract infection, small bowel obstruction, leucopaenia and venous thrombosis.

Downstaging and downsizing

One of the advantages of the long course radiotherapy +/- chemotherapy is downstaging of the primary tumour. Previously non-resectable large cancers could become resectable after treatment and more importantly, a negative circumferential resection margin could be achieved, and in about 10% to 20% of cases a complete pathological response (CPR), the best surrogate marker of excellent prognosis[77]. In contrast, downstaging does not occur following short course radiotherapy as tumour cells require time to shrink. The Polish study randomised patients to either short course or long course chemoradiotherapy. Marked downstaging was observed in the long course group with 16% complete pathological response compared with only 1% in the short course group ($p<0.001$). In addition, significant "down-sizing' was observed following chemoradiotherapy. The diameter of the tumour was on average 1.9cm smaller ($p<0.001$) after chemoradiotherapy[73].

Sphincter preservation

It is clear that long course radiotherapy can effectively downstage (T-stage and pathological stage) and downsize tumours making them more resectable. However, whether or not radiotherapy leads to a higher rate of sphincter preservation and facilitates an anterior resection (colo-anal anastomosis without permanent stoma) is a debatable topic[78]. A recent systematic review failed to show any advantage of preoperative radiotherapy on anterior resection rates[74].

Non-operative approach

One interesting and potentially important concept that has arisen from the use of preoperative radiotherapy is the non-operative approach and careful follow-up of patients who achieve a complete luminal or clinical response following treatment[79]. Habr-Gama reported their series of 260 patients, of which 28% achieved a complete *clinical* response and were observed. Incomplete responders were treated with surgery. Five-year and disease-free survival rates were in favour of the observation only group[80, 81].

Table 1.13 Representative randomised trials of pre-operative radiotherapy NS: non-significant; RT: radiotherapy; TME: Total mesorectal excision

Surgery alone vs Pre-operative radiotherapy and Surgery				
Study	n	Treatment	Local recurrence	Overall survival
Swedish[71] (1997) *(Follow-up 5 yrs)*	1168	Short course RT No TME	27% (surgery alone) 11% (RT + surgery)) P<0.001	48% (surgery alone) 58% (RT+surgery) P=0.004
Kapiteijn[72] (2001) *(Follow-up 2 yrs)*	1861	Short course RT TME	8.2% (TME alone) 2.4% (RT+TME) P<0.001	81.8% (TME alone) 82% (RT+TME) P = NS
Pre-operative versus post-operative radiotherapy				
Frykholm[82] (1993) *(Follow-up 5yrs)*	471	Short pre-op vs post-op	13% (Pre-op) 22% (Post-op) P=0.02	P=NS
Sauer[4] (2004) *(Follow-up 5yrs)*	823	Long course pre-op chemoradio vs post op	6% (Pre-op) 13% (Post-op) P=0.006	76% (Pre-op) 74% (Post-op) P=0.8
MRC CR07; NCIC-CTG C016[68] (2009) *(Follow-up 4 yrs)*	1350	Short pre-op RT vs selective post-op chemoradiotherapy	4.4% (pre-op) 10.6% (post-op) P<0.0001	P=NS; Disease -free: 77.5% (pre-op) 71.5% (post-op) p=0.013
Long versus short course pre-operative radiotherapy				
Bujko[76] (2006) *(Follow-up 4 yrs)*	312	Short pre-op RT vs Long course pre-op chemoradiotherapy	9% (short) 14.2% (long) P=0.17 (NS)	67.2% (short) 66.2% (long) P=NS

1.12.3 Role of chemotherapy in colorectal carcinoma

Chemotherapy in colorectal cancer is commonly used in 3 settings:-

i) Neoadjuvant treatment in rectal cancer in combination with radiotherapy

ii) Adjuvant treatment in node positive and metastatic disease

iii) Palliative chemotherapy

Neoadjuvant chemotherapy

The addition of 5-fluorouracil (5FU) based chemotherapy to neoadjuvant long course radiotherapy can improve pathological staging and local recurrence rates. Bosset et al.[83] randomly assigned patients with T3 or T4 rectal cancers to preoperative radiotherapy, preoperative chemoradiotherapy, preoperative radiotherapy and post-operative chemotherapy and preoperative chemoradiotherapy and post-operative chemotherapy. The chemotherapy consisted of 5-FU and folinic acid (leucovorin). No effect on survival was observed however, 5-year cumulative local recurrence rates were 8.7%, 9.6%, 7.6% in the 3 groups that received chemotherapy compared to 17.1% in the group that received radiotherapy only. They concluded that chemotherapy use, irrespective of timing of administration, confers a significant benefit with respect to local control.

Adjuvant treatment in node positive and metastatic disease

Adjuvant 5-FU based chemotherapy has become the standard of care in patients with Dukes C (stage III) colon cancer. It is not routinely recommended for patients with stage II disease however it is considered in high - risk cases e.g. T4 tumours, perforation, obstruction, poor differentiation. To assist in decision making with regard to adjuvant CT, Gill and colleagues developed a model to estimate survival benefit based on T-stage, nodal status, grading and patient age. 5-FU may be used singly, or in combination with other effect enhancing agents. Combination with folinic acid (leucovorin) has been shown to improve survival and local recurrence rates and up till recently, has been considered the first-line adjuvant therapy in colorectal cancer[84].

The topoisomerase inhibitor irinotecan and platinum based agent oxaloplatin have been extensively studied for use as part of the 5-FU and leucovorin package. The FOLFOX (5-FU, Leucovorin, Oxaloplatin) regime has been shown to offer an improved disease-free survival in patients with stage III colon cancer compared to 5-FU and leucovorin alone[85]. FOLFOX is becoming the first line treatment of choice in patients with stage III disease. Replacement of oxaloplatin with irinotecan for disease resistance or adverse effects is deemed second line therapy.

Palliative chemotherapy

The aim of palliative chemotherapy is to prolong survival and improve quality of life. Exciting progress has been made in the field of biological agents. Bevacizumab (Avastin), is a monoclonal antibody that targets vascular endothelial growth factor (VEGF). This has been tested in combination with irinotecan, 5-FU and leucovorin, median survival and response rates were shown to be in favour in the Bevacizumab group. This regime is now standard first-line therapy for metastatic colorectal cancer[86]. Cetuximab is a chimeric monoclonal antibody against epidermal growth factor receptor (EGFR). In combination with irinotecan in patients with irinotecan resistant disease, cetuximab has been shown to lead to a response rate of 22.9% compared to 10.8% with cetuximab alone[87].

1.13 Epigenetics

While genetics refer to the study of individual DNA sequences involved in normal development and inheritance, disease and neoplasia; epigenetics refer to the inheritance of information on the basis of gene expression. Conrad Waddington in 1942 first described this concept - a phenotype arises from genotype through programmed change, influenced by the organism's environment[88]. The modern definition of epigenetics is 'heritable changes in gene expression without any alteration in the DNA sequence'[89]. Like genetic changes and inheritance, epigenetics are involved in normal mammalian processes and development. An example of an important epigenetic function is the suppression of one of the X chromosomes in females[90]. However it is now recognised that epigenetic changes are also responsible for a wide variety of diseases including neoplasia, and are at least as common as genetic mutations in giving rise to cancer[91]. A majority of cancers is now known to be the consequence of a combination of genetic and epigenetic alterations[92-95].

Several epigenetic mechanisms (markers) have been identified to date. DNA methylation is the most well known and understood epigenetic modification[96]. Histone protein modifications, modification of chromatin structure and chromatin factors are other types of epigenetic markers that work in conjunction with DNA methylation in regulating gene activity[97].

1.13.1 DNA methylation

DNA methylation plays a crucial role in the control of gene expression in normal human cells. This modification is capable of 'gene silencing'[6]. In normal embryogenesis and development, this process is required for genomic imprinting where by only one of the parental alleles is expressed (mono-allelic expression) and the silencing of genes on the additional X chromosome in females[90, 98].

Through a chemical modification process, a methyl (CH3) group is attached to the 5' carbon position on a cytosine base to form the resultant 5-methylcytosine. The source of methyl group comes from S-adenosyl methionine (SAM), which is converted to S-adenosyl homocysteine (SAH) through the methylation process. SAH is recycled back to SAM using methyl groups supplied by the nutrients folate and cobalamin[99]. (Figure 1.10)

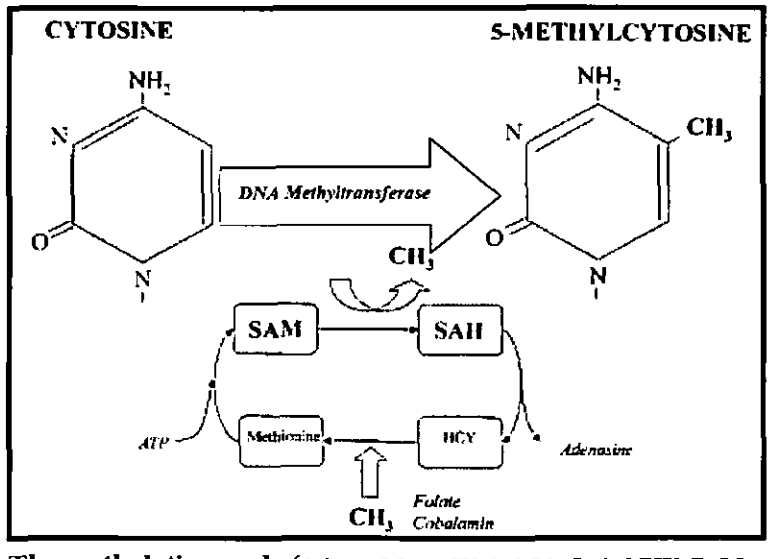


Figure 1.10 The methylation cycle (Adapted from Wajed SA, Laird PW, DeMeester TR. DNA methylation: an alternative pathway to cancer. *Ann Surg* 2001;234(1): 10-20.)

In mammals, DNA methylation only takes place on a cytosine base that is immediately paired up with a guanine base (CpG dinucleotide). 5-methylcytosine bases are observed to be unstable and hypermutable, and prone to undergo spontaneous deamination to thymine. Through evolution this has resulted in a relative paucity of cytosine bases[100]. Approximately 70% of all CpG dinucleotides are methylated in the human genome with the remainder residing in short sequences of 0.5-2 kb in length that are typically located in the promoter regions of genes[101]. These areas are rich in CpG dinucleotides that remain unmethylated and are referred to as CpG islands that are present in 50-60% of all human genes.

The mechanisms involved in initiating methylation of mammalian DNA are not yet clear. However currently, 2 mechanisms have been proposed to explain the mode of gene silencing by DNA methylation[100, 102]. DNA methylation can directly inhibit the binding of transcriptional factors to the targeted sequence, or indirectly by interacting with transcriptional repressor proteins. One such protein is MeCP2 that contains a methyl-CpG binding domain (MBD) which binds to methylated CpG groups. This binding is followed by recruitment and attachment of a group of protein complexes that possess repressor properties including histone deacetylases (HDAC1 and 2). The resultant de-acetylation of histones leads to conformational changes in chromatin structure rendering the gene inaccessible to transcriptional factors, and thereby the corresponding gene is silenced[103].

DNMTS- control of DNA methylation

Methylation of DNA is a highly specific and tightly controlled process and different tissue types express their individual patterns of methylation[102]. The regulation of methylation relies on a group of enzymes called DNA methyltransferases (DNMTs). During development, the methylation pattern across the genome is determined by a series of de novo methylation and demethylation events, once established, the particular pattern is maintained throughout life[104]. Two groups of DNMTs, each with distinctive functions exist to regulate and preserve the methylation pattern across generations. Maintenance DNMTs – DNMT1, is responsible for methylation of newly synthesised DNA sequences based on the parental template. De novo DNMTs – DNMT 3a and 3b, are essential in establishing new methylation patterns in the early stages of life. The exact roles of either of these de novo methyltransferases are unclear however they have been shown to be responsible for methylation of different and specific satellite sequences of DNA[105]. De novo methylation occurs extensively not only in normal the physiological environment but is also responsible in neoplastic transformation of cells[106, 107].

1.13.2 Histone protein modifications

Histones are physiological proteins around which chromosomes wrap around to form chromatin. Modifications to histones play an important role in the regulation of gene expression closely in conjunction with CpG island methylation. Indeed DNA methylation alone may not be sufficient in suppressing gene expression[108]. Histone

proteins may also be methylated but additionally acetylation and ubiquitinylation of lysines and phosphorylation of serine residues have also been observed during chromatin modifications[109]. As mentioned above, one possible mechanism of gene silencing by CpG methylation is through deacetylation of histones by histone deacetylases (HDACs). The histone H3 lysine 9 residue is deacetylated and methylated in silenced genes, whereas acetylation of the same residue is present in gene expression[110]. Histones modifications are accompanied by chromatin conformational changes. Acetylated histones are associated with an open configuration of chromatin, permitting access of transcriptional factors and allowing gene expression to follow. Histone deacetylases on the other hand keep histones deacetylated causing the chromatin to become more condensed, preventing access of transcriptional elements thereby rendering the gene inactive[111].

1.13.3 DNA methylation in carcinogenesis

Aberrant methylation patterns are now widely observed in all types of malignancies[112, 113]. While traditionally mutations in tumour suppressor genes and activations of proto-oncogenes have been seen as the primary driving force for neoplastic initiation and transformation, increasing knowledge of epigenetics leads us to believe that epigenetic markers such as DNA methylation and histone modifications play a substantial role in tumour development and progression. The two most consistent epigenetic findings in cancer are global hypomethylation and focal gene-specific hypermethylation compared to normal tissue[114, 115].

1.13.3.1 Global Hypomethylation

In human cancer, an overall genomic reduction in DNA methylation of 10% - 20% has been observed compared to normal tissue. This reduction in methylation in cancer is found typically at repetitive sequences residing in satellite DNA and peri-centromeric regions of metaphase chromosomes[116]. Satellite global DNA hypomethylation has been observed in ovarian, breast and Wilm's tumours[100]. In a mouse skin multi-stage carcinogenesis model, advancing pre-cancerous lesions have been shown to display increasing levels of hypomethylation as they progress towards the malignant phenotype[117].

Several mechanisms have been proposed to explain the contribution of global hypomethylation to cancer development. Firstly, hypomethylation may increase the likelihood of chromosomal breakage leading to chromosomal instability. An example of this is in the rare inherited ICF (immunodeficiency, centromeric region instability, facial abnormalities) syndrome, a high frequency of chromosomal translocations is observed in association with widespread peri-centromeric hypomethylation[118]. Secondly, hypomethylation can lead to reactivation of previously silenced retro-transposons (additions to chromosomes from reverse transcription) resulting in alterations of normal gene structure and function[119]. Loss of imprinting (LOI) is the process by which the normally silenced inherited allele is abnormally expressed. LOI is responsible for the development of a number of paediatric solid tumours including Wilm's tumour, embryonal rhabdomyosarcomas and hepatoblastomas. Aberrant hypomethylation is

linked to tumour development through the process of LOI[116]. Finally, hypomethylation is frequently observed to be present in proto-oncogenes. Consequently this results in oncogenic activation, promoting neoplastic transformation[115].

1.13.3.2 Focal Hypermethylation

In conjunction with global hypomethylation, individual gene-specific hypermethylation of CpG islands is now regarded as a fundamental event in carcinogenesis[91]. A wide spectrum of genes is hypermethylated particularly in tumour suppressor genes (TSG), which given their normal physiological role in inhibiting cancer formation, gene promoter methylation leads to suppression of their crucial role[115, 120]. The Rb gene in retinoblastoma was one of the first TSGs found to be hypermethylated[121, 122]. Since then a whole panel of genes involved in cell cycle, DNA repair, cell differentiation, signal transduction, metastasis and angiogenesis have been shown to be hypermethylated in cancer[123, 124] (Table 1.14). The p16 tumour suppressor gene located on chromosome 9 is responsible for regulation of cellular proliferation[99]. CpG island methylation of this gene has been shown in oesophageal adenocarcinomas as well as Barrett's epithelium. The VHL (von-Hippel-Lindau) gene in renal cell carcinoma[125] and BRCA1 in breast cancer are also hypermethylated[126]. In haematological malignancies such as myelodysplastic syndrome (MDS) and lymphomas, reversal of methylation has been successful with the use of demethylating agents[127].

CpG island methylation can contribute to carcinogenesis by interacting with genetic mutations. Knudson's widely accepted theory of cancer formation proposed that loss of function of both alleles is necessary for malignant transformation[128]. The first "hit" of the two-hit model is typically a genetic mutational event. Silencing by methylation can act as the second hit in this model, promoting malignancy by suppressing the function of the remaining allele.

1.13.4 Role of DNA methylation in colorectal cancer

The adenoma-carcinoma sequence is well accepted as the principle pathway to colorectal cancer. Beginning from normal mucosa, neoplastic cells transform into mild, moderate, and severely dysplastic adenomatous polyps in a progressive fashion before reaching the final stage of invasive adenocarcinomas. Parallel to these histopathological changes are step-wise accumulation of mutational changes involving the adenomatous polyposis coli (APC), K-Ras and p53 genes[129]. This molecular pathway of progressive genetic alterations is well established and predisposes to increasing chromosomal instability. Microsatellite instability (MSI) refers to expansion or contraction of short nucleotide repeats. Instability across these repeats is generated due to slipping during DNA replication[130]. Defects in DNA mismatch repair proteins result in their inability to repair these errors during replication. The MSI pathway accounts for the second major molecular pathway to colorectal cancer carcinogenesis.

It has been proposed that these two pathways, both leading to genomic instability are not sufficient to explain the vast genetic heterogeneity seen in colorectal cancer and indeed in some tumours, neither chromosomal instability or microsatellite instability could be clearly demonstrated[100]. The epigenetic Methylator pathway is now accepted to play a major role in the pathogenesis of colorectal cancers. Clinically, recognition of DNA methylation changes has opened up new potential diagnostic and therapeutic options in the treatment of colorectal cancer.

1.13.4.1 Global hypomethylation in colorectal cancer

Consistent with other neoplasms, the 5-methylcytosine content in colorectal cancer is reduced at a global level compared to normal non-neoplastic tissue[131]. As mentioned above, loss of imprinting (LOI) is one of the proposed mechanisms by which global methylation can contribute to tumour development. The genes insulin-like growth factor 2 (IGF2) and H19 are both located on chromosome 11. These are normally reciprocally expressed. LOI of IGF2 has been shown to be a risk factor for colorectal cancer[115, 119].

1.13.4.2 Focal hypermethylation + CpG island Methylator Phenotype

Hypermethylation of promoter CpG islands and transcriptional silencing of the gene is now recognised to be a common event in colorectal cancer and hundreds of genes are methylated in a given neoplasm[132]. The list of genes hypermethylated in colorectal cancer is increasing with advancing methods of methylation analysis. This list comprises

tumour suppressor genes, mismatch repair genes and cell-cycle regulatory genes[119]. (Table 1.14)

One major breakthrough in the concept of focal hypermethylation in colorectal cancer was when Toyota et al. allocated the term CpG island Methylator Phenotype (CIMP) to a particular subset of colorectal cancers[133, 134]. They studied a panel of 26 CpG islands in 50 tumours and 15 adenomas. Two distinct categories of methylation were seen when the 26 CpG islands were examined. 19 clones were frequently methylated in the tumours tested but also methylated in normal mucosa to a smaller degree. They named this type of methylation type A for ***aging-specific*** methylation. The remaining 7 clones were exclusively methylated in tumours only and no methylation was identified in normal mucosa. They named this type of methylation type C, for ***cancer-specific***. The frequency of type C methylation was significantly less compared to type A. When these 7 clones were examined further in detail in the 50 tumours, a group of tumours showed a high degree of methylation whereas in another group, methylation of type C clones was extremely rare. Toyota postulated that this distinct group of tumours are more likely to have transcriptional silencing of their tumour suppressor genes by promoter methylation and called these the CpG island methylator phenotype (CIMP). CIMP was also present in seven of the fifteen adenomas studied. Two other interesting findings were noted in their study. Twelve of their colorectal cancers had microsatellite instability, and in all these tumours, hMLH1, the gene known to be responsible for microsatellite instability, was hypermethylated. Most interestingly, all 12 tumours were CIMP positive. An also intriguing finding was that CIMP was more likely to be present in proximal tumours

(caecum and ascending colon) and was significantly less prevalent in distal tumours. A speculative model of how CpG island methylation is involved in colorectal cancer was proposed. Type A methylation occurs as a function of age in normal, non-neoplastic colorectal epithelium upto a certain stage. The methylation may be affecting genes that control growth and proliferation, and could explain the hyperproliferative lesions seen prior to tumour. Type C methylation then takes over for a subset of tumours, with a further group of these becoming CIMP positive.

Following on from this initial proposition of CIMP, other clinical and pathological observations have been made that are associated with this phenotype[135]. CIMP positive tumours are more likely to be present in females and older patients; also more likely to be poorly differentiated with abundance of mucin[136]. A number of further studies have concurred with the observation that CIMP has a close aggregation with microsatellite instability (MSI), and that CIMP MSI +ve tend to have a favourable prognosis, whereas CIMP MSI-ve cases have poorer outcome. Genetically, CIMP is also strongly associated with K-Ras, BRAF and reduced p53 gene mutations highlighting the interplay between epigenetic and genetic mechanisms to carcinogenesis[137, 138]. CIMP is not limited to colorectal cancer, and has also been observed in a wide range of malignancies including oesophageal, leukaemia and ovarian cancers[139].

Table 1.14 Genes hypermethylated in colorectal cancer

Gene	Function
APC	Signal transduction, β-catenin regulation
CDH13	Cell signalling (cell recognition and adhesion)
CDKN2A	Cell-cycle regulation
CHFR	Mitotic stress checkpoint
HIC1	Regulation of DNA damage responses
HPP1	Transmembrane transforming growth factor (TGF)-β antagonist
LKB1	Cell signalling, cell polarity
MGMT	Repair of DNA guanosine methyl adduct
MLH1	Mismatch repair
$P14^{ARF}$	Cell-cycle regulation
RASSF1A	DNA repair, cell-cycle regulation
SOCS1	Cell signalling
THBS1	Angiogenesis
TIMP3	Matrix remodelling, tissue invasion

1.13.4.3 DNA methylation, Aging and Colorectal carcinoma

Increasing age is an important risk factor for the development of many cancers. The incidence of colorectal cancer rises sharply after the age of 60. From a genetic view point, aging together with increasing mutagens exposure may predispose to accumulation of somatic mutations, increasing the likelihood of developing cancer. Since the discovery of promoter CpG island methylation in cancer, it is now clear that this process also plays a role in the aging process in normal colorectal mucosa. Global genomic hypomethylation and non-promoter hypermethylation were the first changes to be observed in aging tissue. Later, promoter CpG island methylation was also shown to play a substantial role in gene silencing in normal tissue.

The oestrogen receptor α (ERα) was the first gene shown to be methylated in normal colonic epithelium[140]. ERα methylation is rarely found in young individuals but increases exponentially with age. It is also found to be present in all adenomas and tumours. Several other genes have since been shown to be hypermethylated in normal colonic tissues as well – IGF2[141], MYOD1[142], N33[142]. Age related hypermethylation appears to be gene-specific, as some genes are methylated in colon cancer only e.g. THBS1 (thrombospondin-1), HIC-1 (hypermethylated in cancer 1)[143], as well as tissue specific e.g. Estrogen receptor α (ERα) is frequently methylated in normal colon[140] and liver[144] but rarely methylated in the normal oesophagus[145].

Based on the observation of ERα methylation in normal colon, a postulated model linking DNA methylation, aging and cancer emerged. A small colony of aging colonic cells with ERα promoter hypermethylation may increase in number and degree of methylation as cells mature with aging. Therefore, the higher the degree of maturity of these cells, the higher the chances of tumour formation[143].

1.13.5 Clinical applications of DNA methylation

CpG island methylation in colorectal cancer offers exciting opportunities of research into its clinical implications. Most research to date has concentrated on the 2 most promising clinical applications. Firstly, it has the potential to act as a molecular disease marker given its high prevalence in colorectal cancer. Secondly, it serves as an ideal therapeutic target for synthetic inhibiting agents.

Currently, there is no reliable biomarker to predict the likelihood of developing colorectal cancer. The fact that CpG island methylation occurs in the aging normal colorectal mucosa, which increases proportionally with age, it serves as potential screening test for colorectal cancer. CpG island methylator phenotype (CIMP) is also observed in pre-cancerous lesions such as aberrant crypt foci (ACF) and adenomas. A close relationship between inflammation and methylation is also present. Ulcerative colitis patients' mucosa contains a high degree of methylation in those who ultimately develop cancer[146]. Other inflammatory based tumours such as Barrett's and oesophageal cancer[145], chronic hepatitis and hepatocellular cancer[144], and lung cancer from smoking[147], have also shown

high methylation patterns in the inflammatory precursors. Since hMLH1 methylation is responsible for 80% of sporadic microsatellite unstable tumours, it is potentially a useful marker for the development of microsatellite instability in colorectal cancer. With more advanced methylation detecting techniques, it is now possible to detect with high sensitivity and specificity promoter methylation using patient's serum[148] and faecal DNA[149]. In a study of 20 patients, hMLH1 methylation was detected in 30% of cases with 100% specificity[148]. However larger studies are now required to evaluate the use of methylation both as a reliable biomarker and prognostic indicator for colorectal cancer.

Epigenetic therapy is a fascinating area of development in the pharmacological prevention or treatment of carcinogenesis. Both genetic and epigenetic modifications are somatically heritable, however, the critical difference between the two molecular processes is that the epigenome is potentially reversible and therefore presents itself as a prime therapeutic target. Reversing DNA methylation has been shown to suppress intestinal neoplasia in a mouse model[150]. DNA methylation and histone deacetylation are currently the two main epigenetic modifications to be targeted by therapeutic agents. Clinical trials on DNA methyltransferase (DNMT) and histone deacetylase inhibitors are ongoing and are showing promising results.

1.13.5.1 DNA methyltransferase (DNMT) inhibitors

This group of agents has shown the most promising results in the treatment of myelodysplastic syndrome (MDS) and leukaemia[92, 127]. Two DNA methyltransferase

(DNMT) inhibitors ***Azacytabine*** and ***Decitabine*** have already been approved by the US Food and Drug Administration (FDA) as therapeutic drugs in the treatment of MDS patients[151]. Inside the cell, both agents covalently bind DNMTs, inhibiting their role in methylation of DNA strands. A 50% to 70% response rate[152] has been shown by both agents in MDS and Azacytabine has been shown to double 2 year survival rates compared to conventional regimens[153, 154]. In colorectal cancer and adenomatous polyps, mouse model studies and in vitro studies have found an encouraging ability of DNMT inhibitors in suppressing tumour development[155]. Clinical trials with these agents in solid tumours including colorectal cancer have shown mixed results so far and further studies are ongoing, focussed on the optimisation of dosage schedule, duration of treatment and combination therapies with chemotherapeutic drugs and histone deacetylase inhibitors[151].

1.13.5.2 Histone deacetylase inhibitors

Histone deacetylases (HDACs) are transcriptional co-repressors which play an important role in the regulation of cell growth and maturation and transformation especially in colorectal tissue. They catalyse the deacetylation of lysine residues on histone proteins which are normally wrapped around by DNA strands (146bp) in the inactive form - heterochromatin. HDACs are antagonistic to histone acetyl-transferases (HAT), which catalyse lysine acetylation[156]. When acetyl groups are removed from lysines, these become positively charged and consequently strongly attract negative DNA. The outcome of this chemical interaction is a condensation of nucleosomes, making it very difficult for transcriptional factors to approach, suppressing transcription[157]. Conversely,

HAT catalysed acetylation confers an open nucleosome structure, allowing transcription to take place freely. Histone deacetylases may also regulate transcription by catalysing the deacetylation of DNA binding transcriptional factors.

There are currently 18 mammalian Histone deacetylases (HDACs) identified, classified into 4 classes (Figure 1.11). Several HDACs are expressed in normal colon and intestinal cells, concentrated in the proliferating crypt compartments. This suggests a role of HDACs in cell proliferation and differentiation[157]. In vivo studies have demonstrated abnormal cell development and differentiation in over-expressed HDACs in foetal mouse explants[158]. Indeed, HDACs are reported to be increased in colon tumours compared to paired normal mucosa[159, 160].

In vitro, histone deacetylase inhibitors (HDACi) are capable of inducing growth arrest, apoptosis and cell differentiation[161]. This has been demonstrated in cell lines of a variety of malignancies including colorectal cancer. The short chain fatty-acid butyrate, a Histone deacetylases inhibitor, is capable of inducing cell maturation and to have anti-tumour properties in colon cancer cell lines[162, 163]. In animal models of intestinal cancer, butyrate and other HDACi have also been found to reduce growth of colon cancer xenografts. Over 30 clinical trials are currently underway to evaluate HDACi's therapeutic potential in a wide range of solid and haemopoetic malignancies[164]. One agent SAHA (Suberoylanilide Hydroxamic Acid), has been approved by the US FDA for the treatment of T-cell cutaneous lymphoma as a monotherapy agent[152].

Combination therapy with different epigenetic agents or, with other chemotherapeutic agents has also shown promising results. DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors acting together have been shown to reactivate gene expression in colon cancer as well as inducing response in leukaemia patients[165, 166]. Moreover, the combination of HDACi with 5-Fluorouracil can synergistically inhibit tumour growth and promote cell killing in xenograft models[167]. HDACi is also capable of enhancing the effect of radiotherapy in multiple cell types, an effect of significant importance in rectal cancer patients receiving neoadjuvant radiotherapy[168].

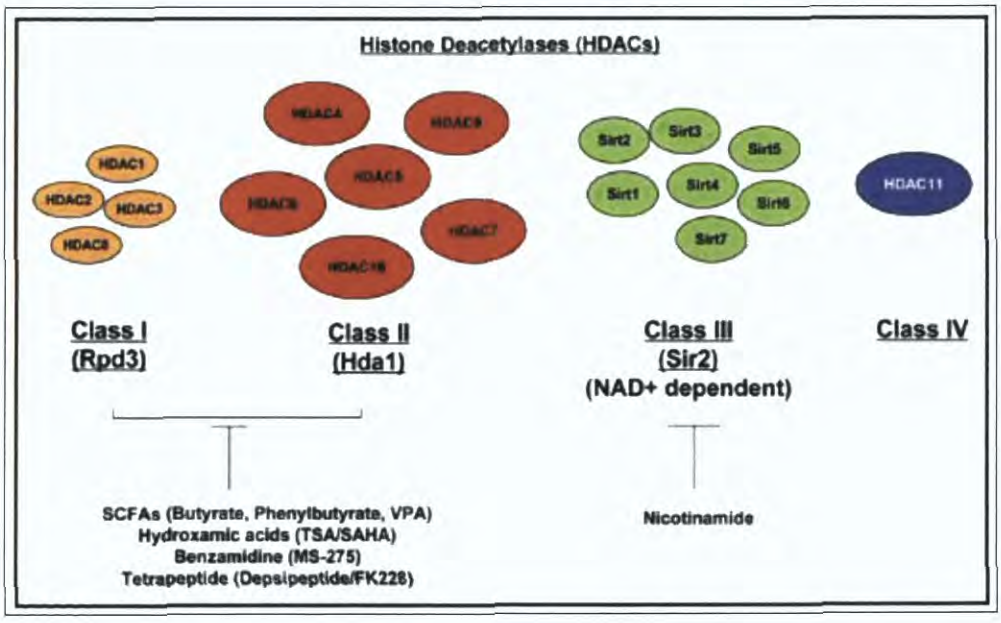


Figure 1.11 Classes of mammalian histone deacetylases (Adapted from Mariadason JM. HDACs and HDAC inhibitors in colon cancer. *Epigenetics* 2008;3(1): 28-37.)

1.14 Summary

Colorectal caner remains one of the commonest cancers in Ireland. Its incidence is likely to increase over the next few decades. The prognosis of colorectal cancer ranges from 10% to 90% 5 year survival rates depending on the stage of diagnosis and treatment given. Currently, histo-pathological staging with the TNM and Dukes' staging systems remain the most important prognostic indictor. Continuing clinical and scientific research aims to further improve the prognosis of colorectal cancer patients. An emphasis is placed on a multidisciplinary approach forming an individualised treatment regime. Colorectal cancer patients now almost always receive treatment from all three treatment modalities including surgery, radiotherapy and chemotherapy. There have been advances in each of these modalities in colorectal cancer management. Total mesorectal excision is now the procedure of choice in rectal cancer resection that leads to significantly reduced local recurrence rates. Minimally invasive surgery is increasingly used for colon and rectal cancer resection. Pre-operative radiotherapy (+/- chemotherapy) is now routinely administered to patients with locally advanced rectal cancers to improve local recurrence. Adjuvant chemotherapy using the FOLFOX (5-fluorouracil, leucovorin and oxaloplatin) is now the regime of choice for patients with advanced disease after surgical resection.

The genetic pathogenesis of colorectal cancer has been well described and understood. The adenoma – carcinoma sequence of mutational events accompany a well defined pathway of pathological changes of normal mucosa, to adenomatous polyps of advancing

dysplasia and ultimately to invasive colorectal cancer. The chromosomal instability pathway contributes to the majority of sporadic colorectal tumours due to underlying mutations in the adenomatous polyposis coli (APC), K-Ras and p53 genes. This pathway underlies the genetic basis for the familial adenomatous coli (FAP) syndrome when patients develop hundreds of adenomatous polyps by their teenage years. Germline mutations of DNA mismatch repair genes lead to microsatellite instability (MSI), which forms the genetic basis for the hereditary non-polyposis colon cancer (HNPCC) syndrome. Although the genetic events leading to colorectal cancer is well understood, the role genetic markers has in diagnosis and predicting prognosis of patients is limited despite ongoing research.

A third molecular pathway leading to colorectal cancer has recently been described. The epigenetic DNA methylator pathway is now recognised as an additional and distinct molecular pathway in colorectal carcinogenesis. Epigenetics describe alterations in gene expression without a change in the DNA sequence. DNA methylation is the most studied epigenetic mechanism. Although underlying predisposing mechanisms are not fully understood, a methyl (CH3) group is observed to be added to the promoter regions of multiple regulatory genes responsible for cancer formation. The resultant effect is silencing the methylated gene leading to gene suppression. In colorectal cancer, multiple genes involved in cellular differentiation and proliferations are now known to be silenced through DNA methylation including the key APC gene known to be mutated in the chromosomal instability pathway. In addition, DNA methylation is responsible for the majority of microsatellite tumours through silencing of the hMLH1 gene. Gene-specific

methylation is thought to promote carcinogenesis primarily through silencing of tumour suppressor genes; global genomic methylation is a separate concomitant phenomenon observed in colorectal cancer, thought to lead to malignancy through predisposing to genomic instability and activation of oncogenes.

Epigenetic mechanisms such as DNA methylation may not only provide more understanding of the molecular heterogeneity of colorectal cancer but may also serve as a potential new marker of diagnosis and prognosis. Traditional genetic indices have not shown great promise in colorectal cancer screening, predicting patient prognosis or response to treatment. Although our understanding of DNA methylation and its clinical implications is still at early stages, through continuing research and clinical studies DNA methylation has the potential to contribute in each of these three areas of colorectal cancer management.

1.15 Aims of thesis

1. To examine the global methylation patterns across the adenoma- carcinoma sequence and any possible association with clinico-pathological details of patients.

2. To examine the global methylation relationship between cancer and paired normal tissue and any possible prognostic role in colorectal cancer patients.

3. To determine the effect of neoadjuvant chemoradiotherapy on the rectal cancer epigenome and any relationship with patient prognosis.

4. To determine whether global methylation patterns in rectal cancer pre-treatment tumour biopsies may predict response to neoadjuvant chemoradiotherapy.

Chapter 2

Materials & Methods

2.1 Patients

Colorectal patients included in this thesis' studies were identified from the 2 hospitals' pathology department (Connolly and Beaumont Hospital, Dublin) computer database system. The system stores histology reports of all patients who have had their specimens processed in the corresponding laboratory. Pathological data were collected by manually reviewing each patient's histology report, and subsequently confirmed by slide review with a consultant pathologist. Clinical and survival (date of diagnosis to death) information were collected by hospital chart review, primary care physician contact and the National Death Registry in Dublin, Ireland. (Table 2.1)

Table 2.1 Clinical and pathological data collected for each study patient

Pathological data	Differentiation Dukes stage T-stage Tumour regression grade (Rectal tumours post chemoradiotherapy) Lymph node status Dysplasia (polyps) Tubular/ villous (polyps)
Clinical data	Sex of patient Age of patient Site of lesion (polyp/ tumour) Patient survival

2.1 Colorectal adenoma and carcinoma specimens

2.2.1 Colorectal adenomatous polyps

Colorectal adenomatous polyps from different patients with mild, moderate and severe dysplasia were retrieved from the Connolly Hospital, Dublin, Ireland, pathology department archive. All polyps were compared to normal mucosa of patents with no disease found at colonoscopy. All polyps were either biopsied or snared with diathermy during routine colonoscopic examinations.

2.2.2 Colorectal carcinoma

Colon and rectal carcinomas were retrieved from the pathology tissue banks of Connolly Hospital and Beaumont Hospital, Dublin, Ireland. All samples were resections with paired normal mucosa from the same patient available for comparison.

2.2.3 Rectal carcinomas – Neoadjuvant chemoradiotherapy

Pre-treatment biopsies and post chemoradiotherapy rectal cancer resections were retrieved from the pathology departments of Connolly and Beaumont Hospital. All subjects underwent the long course chemoradiotherapy (50.4 GY over 5 weeks + concomitant 5-Fluorouracil) regimen and subsequently underwent either anterior resection or abdominal perineal resection with total mesorectal excision (TME) 4-6 weeks after treatment.

2.3 Antibodies for immunohistochemistry

2.3.1 Ki-67 (MIB-1)

Company: DakoCytomation (Denmark)

Host: Mouse

Uses: Immunohistochemistry

Description:

Ki-67 antigen is a nuclear protein which is defined by its reactivity with monoclonal antibody from the Ki-67 clone. Ki-67 antigen is preferentially expressed during all active phases of the cell cycle (G1, S, G2 and M-phases) but is absent in resting cells (G0-phase). During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The antigen is rapidly degraded as the cell enters the non-proliferative state, and so no expression of Ki-67 is detected during DNA repair processes.

Monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1 is used for immunohistochemistry. In diagnostic histopathology and cell biology, the antibody has proven valuable for the demonstration of the Ki-67 antigen in normal and neoplastic cells e.g. prostate adenocarcinomas, soft-tissue sarcoma and breast carcinoma.

2.3.2 5-Methylcytidine

Company: Eurogentec (Seraing, Belgium)

Form: Purified Ascites

Host: Mouse

Uses: ELISA, immunoblotting, cytochemistry, flow cytochemistry, immunohistochemistry and cytogenetics

Description:

5-Methylcytidine is a modified base found in the DNA of plants and vertebrates. DNA methylation is a process involved in gene silencing and therefore plays a role physiologically in the control of gene expression, imprinting and differentiation. It is also implicated in carcinogenesis with gene specific and global changes observed in tumour samples.

The antibody has been developed to discriminate between the modified base and its normal counterpart. It has been used to detect alterations in the urinary excretion of nucleosides in cancer patients, to visualise the distribution of methyl-rich regions along human chromosomes, to quantify *in situ* differences between normal and malignant cells from peripheral blood as well as on tissues sections.

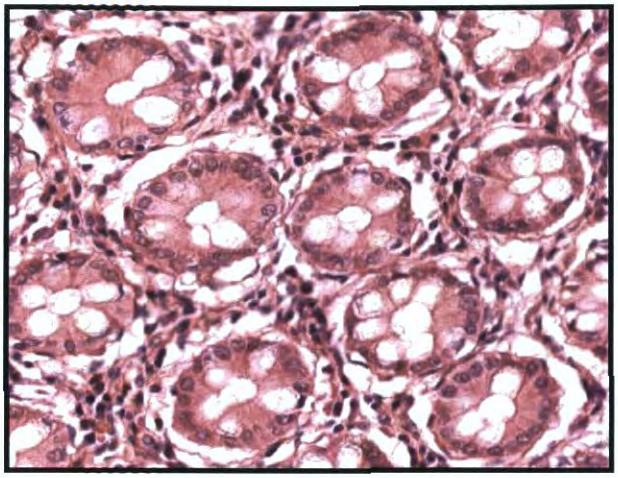
2.4 Histology

Preparation of tissue section

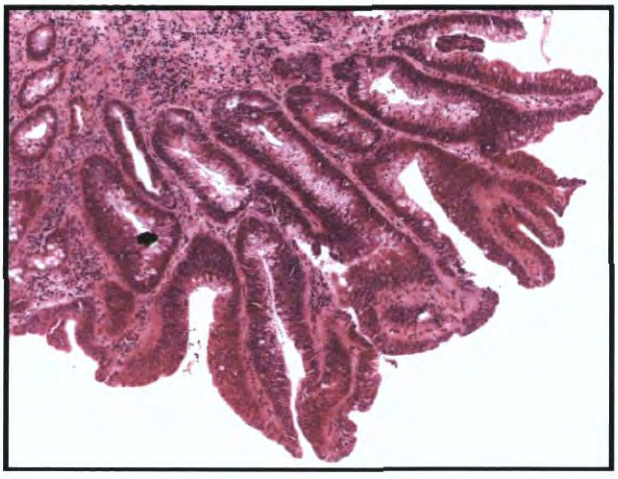
Formalin-fixed colorectal tissue (Adenomas, Cancers, Normal) was removed from the paraffin embedded cassette (Laboratory Instruments and Supply Company, Meath, Ireland). Sections of 4μm thickness were cut from the paraffin embedded tissue using a microtome. These were then mounted on poly-lysine coated (Lahn-Chemical Co.) slides. Sections were air dried at room temperature.

Haematoxylin and Eosin (H&E) Counterstain

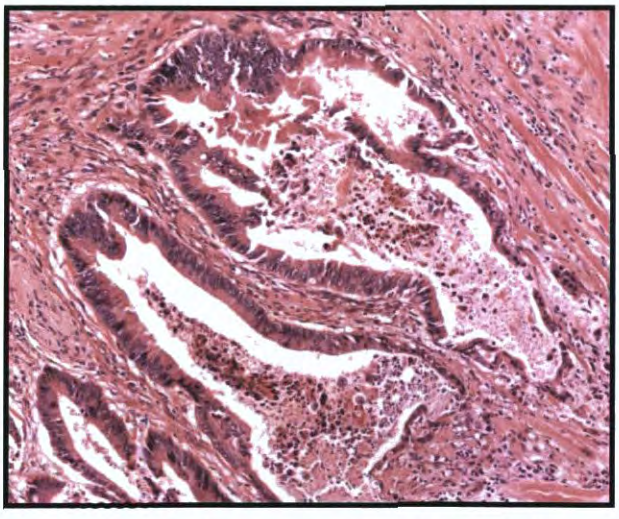
Tissue sections were re-hydrated and then immersed in xylene for 5 minutes followed by immersion in a graduated series of alcohols (100% ethanol for 3 mins and 70% ethanol for 3 mins, water for 3 mins) for wax removal. This stepwise procedure was repeated once. Slides were washed in water and immersed in a Haematoxylin bath (Harris haematoxylin acidified papanicdaou stain, Cellpath PLC, Powys, UK) for 7 minutes. The slides were then "blued" in warm running water for 1-3 mins and then dipped twice into acid alcohol bath. Further washing was then carried out for 6 minutes followed by immersion in 0.05% alcoholic Eosin bath for 3 minutes. Slides were washed again under running water for 1 minute. Finally slides were dehydrated by dipping in 70% ethanol and in 100% ethanol for 10 minutes. Finished slides were mounted with DPX mounting medium (Sigma, Dorset, UK) and coverslipped.



H&E staining of normal colorectal mucosa



H&E staining of adenomatous mucosa



H&E staining of invasive adenocarcinoma

Figure 2.1 Haematoxylin stains nuclei blue and Eosin Stains cytoplasm pink/ red

2.5 Immunohistochemical Staining

Preparation of tissue sections

Formalin-fixed paraffin-embedded colorectal tissue (adenoma, adenocarcinomas, normal) was removed from the cassette (Laboratory Instruments and Supply Company, Meath, Ireland). Sections of 4μm thickness were cut from the paraffin embedded tissue using a microtome. Sections for immunohistochemistry were cut consecutively with sections for H&E staining. These sections were incubated in a 60°C oven for one hour then at 37°C overnight.

Primary antibodies (See section 2.3)

1. 5-Methylcytidine (Eurogentec, Belgium)
2. Ki-67 (DakoCytomation, Denmark)

Controls

Human tonsils for Ki-67, and human normal bowel mucosa for 5-Methylcytidine, were respectively used as positive controls for immunohistochemistry. The primary antibody (Ki-67, 5-Methylcytidine) was omitted and run as negative controls in each batch using the same protocol.

Immunostaining

Immunohistochemistry staining was performed using an automated immunostainer (Leica Microsystems, Bondmax).

Steps:-

1. Deparaffinisation was performed using Bondmax immunostainer automatically using the Bond™ Dewax Solution.
2. Antigen retrieval was performed with the following solutions and incubated for 20 minutes –

a) Ki-67 – Bond™ Epitope Retrieval Solution 1

b) 5-Methylcytidine – Bond™ Epitope Retrieval Solution 2

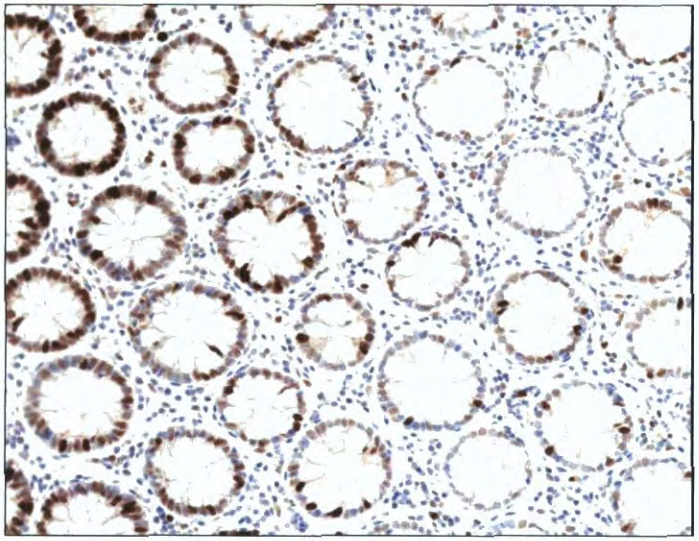
3. Immuno-staining was then commenced using an optimised protocol (Table 2.1)

Dilution for antibodies was prepared using the Bond™ primary antibody diluent:

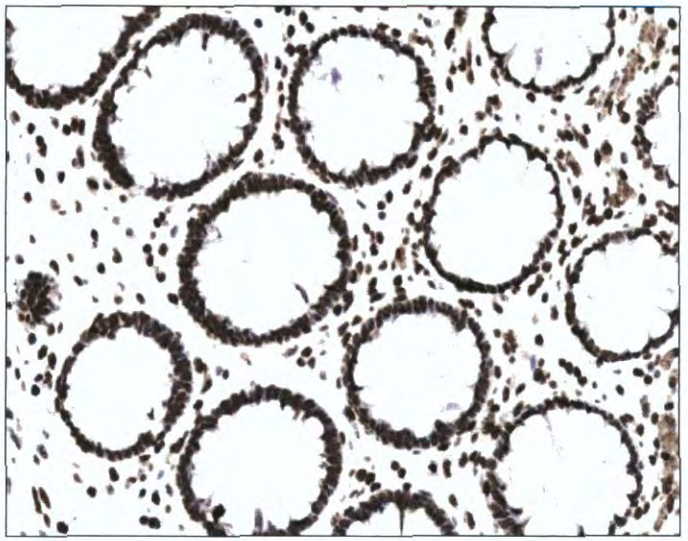
Dilution for Ki-67 -	1:200
Dilution for 5-Methylcytidine –	1:500

Table 2.2 Optimised protocol for automated immunostaining

Reagent	Time (mins)
Primary marker (Ki-67/ 5-Methylcytidine)	15
Post-primary antibody	8
Polymer	8
Peroxide block	5
DAB-refine	10
Bond DAB enhancer	5
Haematoxylin	5



Ki-67 immunostaining



5-Methylcytidine immunostaining

Figure 2.2 Immunohistochemical staining

Figure 2.3 Leica Microsystems, Bondmax - Automated immunostainer

2.6 Image analysis

Automated image analysis and quantification of immuno-staining was performed using the Aperio® (Vista, CA) digital pathology system. Aperio is an integrated digital pathology system that consists of 3 pieces of hardware and software:-

i) ScanScope® - a system that allows digitalisation of pathology glass slides into digital images using a linear-array methodology. It consists of a digital scanner – ScanScope ® XT – a 120-slide capacity high speed scanner for 1 x 3" glass slides. Slides are scanned at either 20X or 40X magnification to give high quality images in colour displayed on a computer monitor.

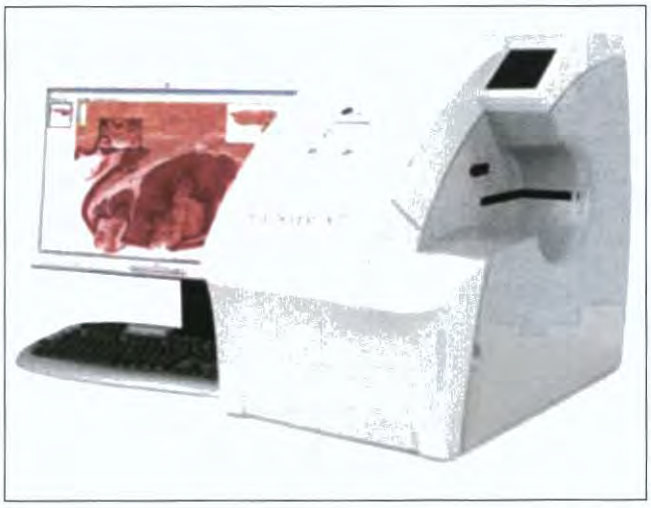


Figure 2.4 ScanScope XT digital scanner

ii) Spectrum® - a web-based digital pathology information management software that allows digital slide viewing and conferencing, workflow management, data retrieval and image analysis. Digital images are stored in a central workstation database but could be accessed remotely via the internet.

iii) ImageScope – a digital slide viewing software that provides viewing of digitalised images from any workstation via the internet. It also incorporates multiple image analysis algorithms (Nuclear, Immuno-histochemistry nuclear, Cytoplamic) for the analysis of pathology staining.

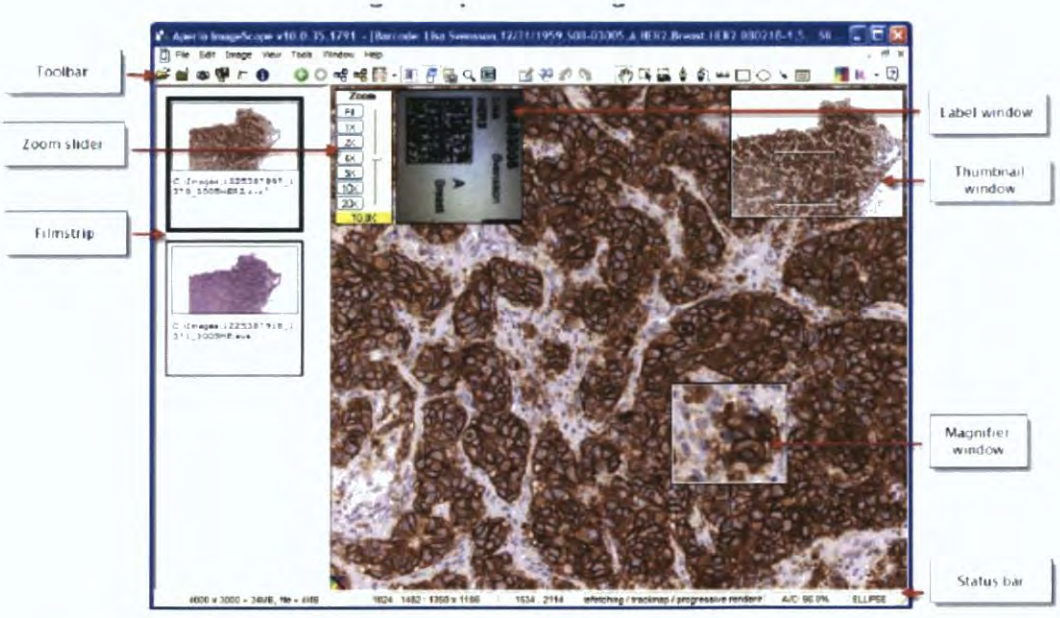


Figure 2.5 Digital slide analysis software

Image analysis steps

Once the H&E and immunohistochemistry slides were ready and available, three main steps were carried out to obtain staining quantification results:

i) Digital scanning – Sets of 3 glass slides belonging to each patient were fed into the ScanScope scanner to produce digital images. Each set of slides contains a H&E, Ki-67 and 5-methylcytidine stained tissue. All slides were scanned at 20X magnification. Slides were coded to protect patient confidentiality, stored and managed using the Spectrum software.

ii) Selecting tumour-only regions – Prior to performing the actual staining quantification, regions of adenoma/ malignant cells within each slide had to be selected so to avoid analysing irrelevant cells to this study (lymphocytes, normal epithelial, stromal and muscle cells). The consultant pathologist used the corresponding H&E slide for each patient as a guide and outlined a representative set of tumour-cell only regions on the Ki-67 and 5-methylcytidine slides. Each region chosen was the same on both immunohistochemistry slide. Ten high powered fields were the target number of regions to be selected on each slide to provide a high number of cells for robust analysis.

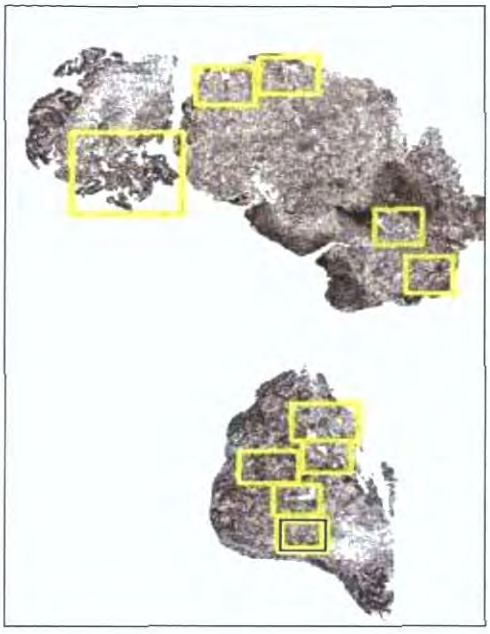


Figure 2.6 Selection of tumour-only regions for analysis

iii) Image analysis using Aperio algorithms

Image analysis of the scanned immunohistochemistry slides were performed using specific algorithms suited to the particular stain used. Several algorithms (Table 2.3) have been developed by the company for different applications, the IHC nuclear algorithm was chosen as the primary algorithm for all our analyses as both Ki-67 and 5-methylcytidine stains are nuclear stains and our interest was in nuclear DNA.

Table 2.3 Image analysis algorithms

Algorithm	Use
Membrane	Membranous staining e.g. HER2
Nuclear	Nuclear staining e.g. Oestrogen and progesterone receptors
Micrometastasis	Detect tumour cells in circulating blood
Colour Deconvolution	Separates staining into 3 channels, calibration of stain colours

Image analysis was performed in 2 steps using 2 separate Imagescope algorithms:

1) Colour Deconvolution Algorithm – Calibration of stain colours
2) (Immunohistochemistry) IHC Nuclear Algorithm – Analysis of immunostaining

The Colour Deconvolution Algorithm

This algorithm was used to accurately calculate the colours of the positive and negative stains, which were then used to calibrate the stain colours in the IHC nuclear algorithm.

a) Deconvolution separates the digital image into 3 colour channels depending on the colour of the stains used:

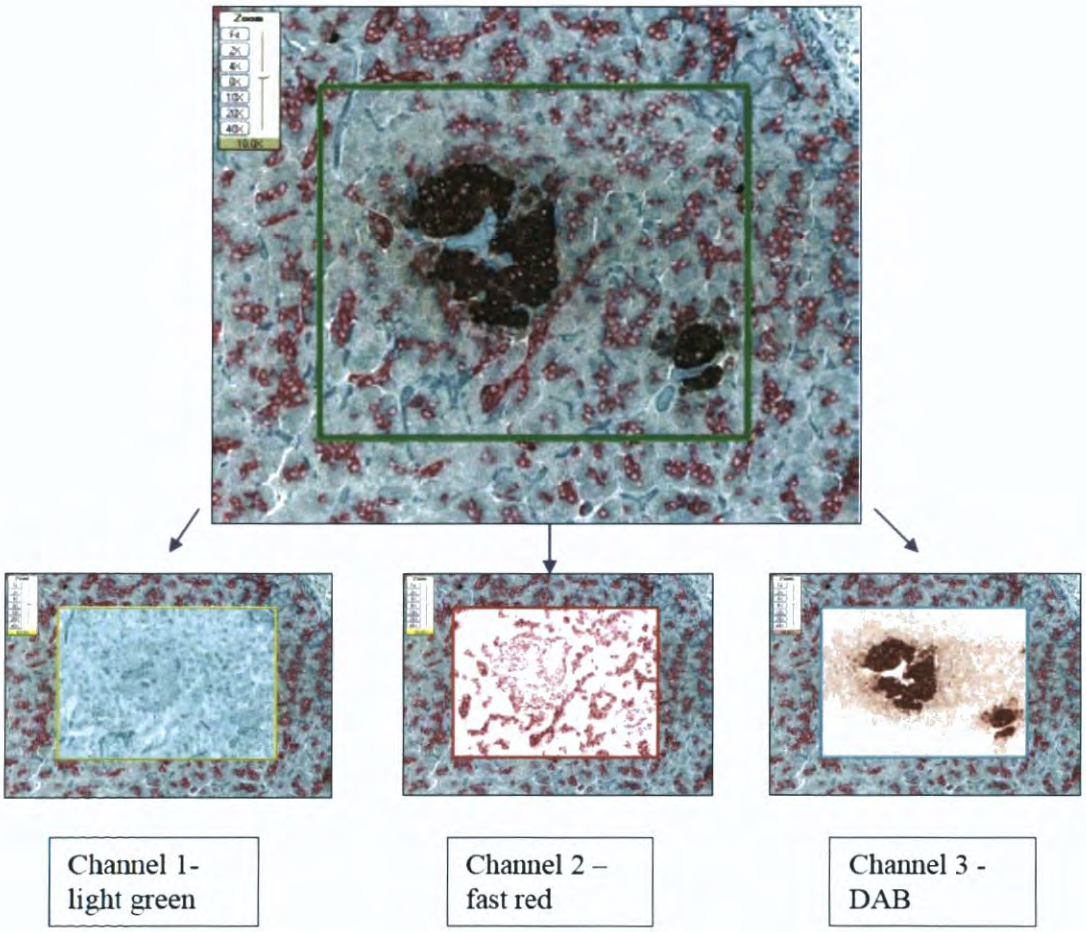


Figure 2.7 Deconvolution of scanned image

b) The algorithm can display either a deconvolved image or as an intensity range:

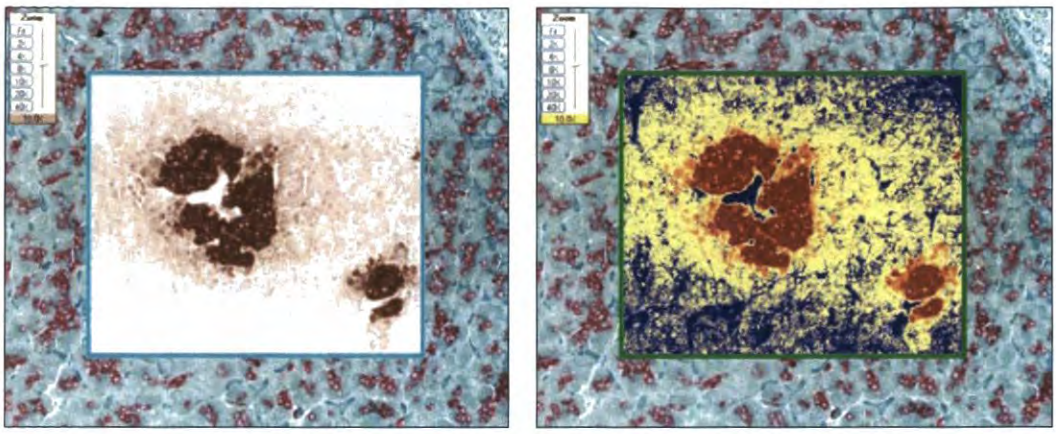


Figure 3.8 Display of deconvolution results – Left: Deconvolved colour channel; Right: Intensity ranges

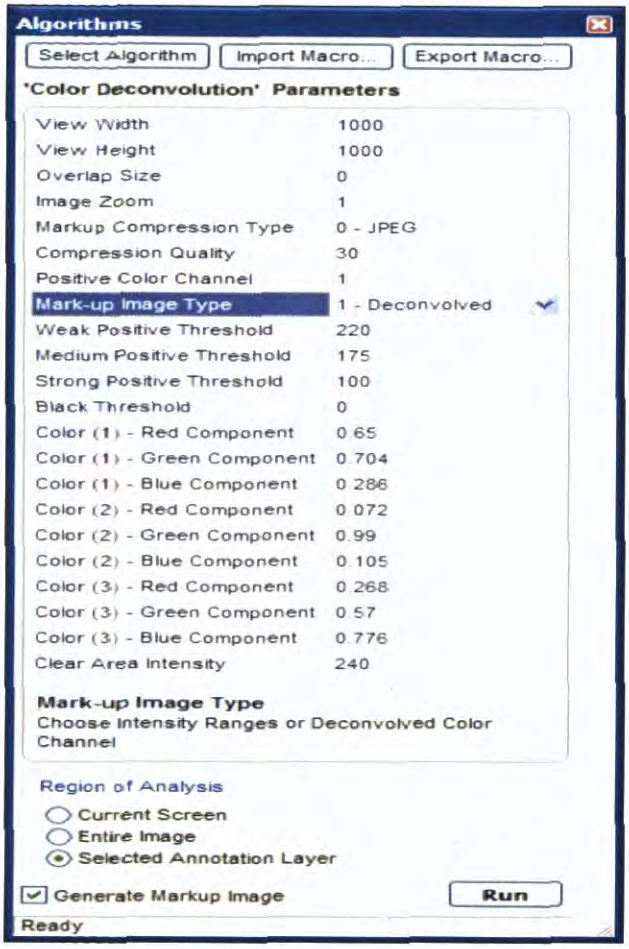
The intensity ranges show 4 colours:

Red	- Strong staining
Orange	- Moderate staining
Yellow	- Weak staining
Blue	- Negative staining

Algorithm	Color Deconvolution
Version	8.001
Average Positive Intensity	177.988
Percent Weak Positive	53.4537
Percent Medium Positive	7.26969
Percent Strong Positive	10.0327
Percent Negative	29.2439
Percent Total Positive	70.7561
Average Weak Positive Intensity	203.063
Average Medium Positive Intensity	142.122
Average Strong Positive Intensity	70.3819
Total Stained Area (mm^2)	0.193882

Figure 2.9 Results of deconvolution can also be shown in numerical values

c) Calibration values for the positive and negative stains are obtained by running the Colour Deconvolution algorithm twice, once for the positive stain and once for the negative stain.



Haematoxylin – Negative stain

An area on slide with negative staining is annotated.

The colour deconvolution algorithm is run and optical density (OD) values for red, green and blue are obtained. These values are substituted into the IHC nuclear algorithm channel 1.

DAB – Positive stain

An area on slide with positive stain only is annotated, corresponding to DAB stain.

The colour deconvolution algorithm is run and OD values for red, green and blue are obtained. These values are substituted into the IHC nuclear algorithm channel 3.

Default Channel 1 = Haematoxylin
Default Channel 2 = Eosin
Default Channel 3 = DAB

Figure 2.10 Calibration displays – Optical density (OD) values are used for the IHC nuclear algorithm

Three different areas of positive and negative stains are chosen with the algorithm run thrice. The average optical density values are substituted into the IHC nuclear algorithm. Average values obtained and used:

Table 2.4 Optical density values used in immunohistochemistry nuclear algorithm

	Haematoxylin (Negative)	DAB (Positive)
Red	0.67	0.50
Green	0.63	0.61
Blue	0.40	0.62

The Immunohistochemistry (IHC) Nuclear Algorithm

The IHC Nuclear Algorithm is designed for the analysis and quantification of immunohistochemistry nuclear staining. The algorithm detects the nuclear staining for a target chromogen for the individual cells in the selected tumour-only regions and quantifies their intensity. In a similar way to pathologists carrying out scoring of, for example HER2 status in breast tumours, the algorithm classifies the nuclear staining as 0, 1+, 2+ and 3+. A nucleus is classified as 0 when it has no nuclear staining; 1+ when it has weak nuclear staining; 2+ when it has moderate nuclear staining and 3+ when it has intense/ strong nuclear staining. Based on the percentages of 0, 1+, 2+ and 3+ nuclei, the percentage of positively stained nuclei as a percentage of 0 to 100% and the average staining intensity of the positive nuclei as a score of 0, 1+, 2+ or 3+ is determined.

In addition to this 3-point intensity scoring system, the algorithm also provides an average staining intensity score for all positive nuclei. This score has a range of 0 to 255. A zero value is assigned to the ***strongest*** staining, while a 255 value is assigned to the ***weakest*** staining. e.g. an intensity score of 230 is weaker than a score of 50.

Nuclear 0

Nuclear 1+

Nuclear 2+

Nuclear 3+

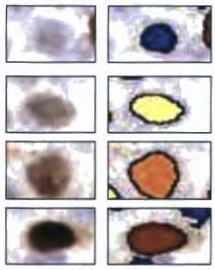
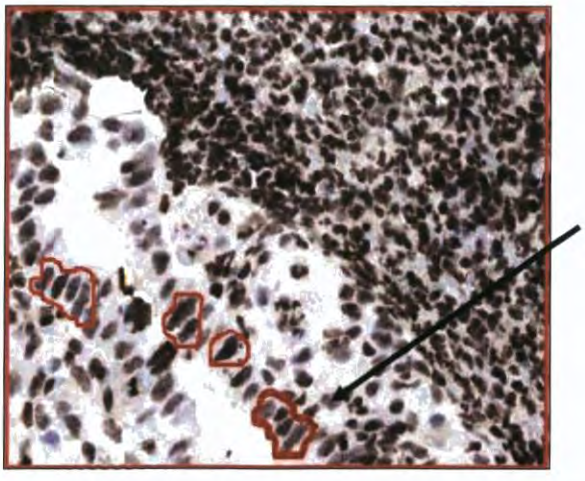


Figure 2.11 **Nuclear staining ranges and display colours**

Application of the IHC Nuclear Algorithm



1. Individual digital images with tumour-only selected regions are retrieved for each patient. Further annotations can be made within these tumour-only regions to minimise analysis of non-tumour cells.

Figure 2.12 **Selection of tumour cells only**

2. Calibrated optical density values of red, green and blue obtained from the Colour Deconvolution algorithm are inserted into the Haematoxylin (Channel 1) and DAB (Channel 3) analysis parameters. Eosin (Channel 2) is set to zero.

3. Intensity threshold values for 1+ (weak), 2+ (moderate) and 3+ (strong) and other cell parameters are adjusted. The 1+ threshold is the detection threshold and separates positive pixels from negative pixels. This value is determined by running the colour deconvolution algorithm on a set of negatively stained regions. The average positive intensity result of these regions is noted down as M0. The algorithm is then run on a set of 1+ regions, the mean positive intensity is noted down as M1. The detection threshold 1+ ie the value that separates negative from positive cells is calculated by (M0 + M1)/2. Threshold values for 2+ and 3+ are calculated similarly by running the algorithm as sets of 2+ and 3+ regions. The 2+ value is calculated by (M1+M2)/2 and 3+ value is calculated by (M2+M3)/2.

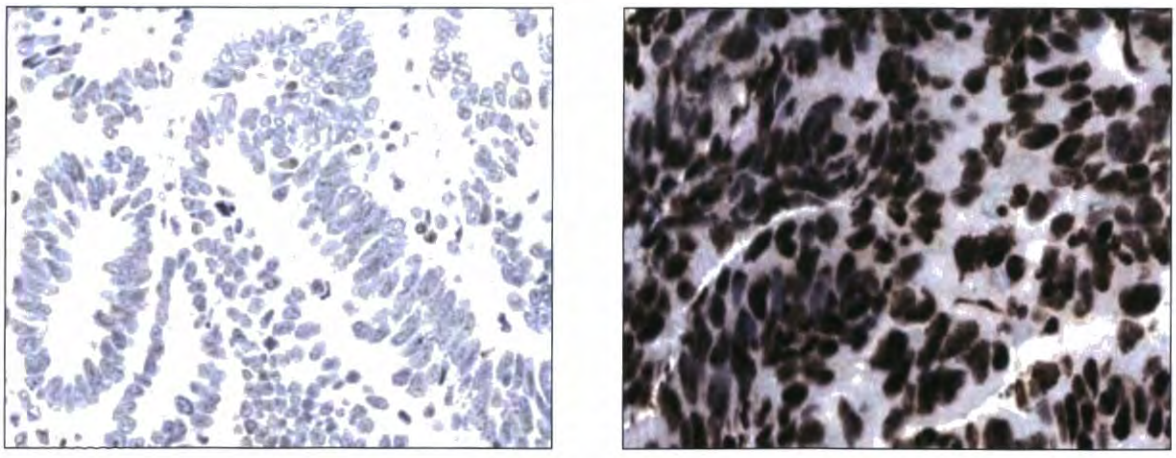


Figure 2.13 Diagram showing negative and strong (3+) 5-Methylcytidine staining

4. The algorithm is run on the displayed annotated regions. Results generated are shown in two formats, a) a mark-up image showing visually the positive nuclei, and the different intensities of the positive nuclei, b) a results table showing numerically values of positivity and intensity.

Figure 2.14 Display of results after as mark-up image and numerical values

5. Results are saved and the process is repeated for each immunohistochemistry (Ki-67 and 5-Methylcytidine) slide of each single patient.

2.7 **Statistical analysis**

All image analysis results were analysed using the Statistical Package for Social Sciences (SPSS®) for Windows version 17 (Chicago, Illinois). Analysis was performed using statistical tests for non-parametric data including the Mann-Whitney and the Wilcoxon Signed Rank tests for both methylation and Ki-67 values. A p-value of <0.05 was considered significant.

Chapter 3

The role of DNA methylation in the adenoma-carcinoma sequence

RCT LIBRARY

3.1 INTRODUCTION

The pathological and molecular events that lead to the development of colorectal cancer have been extensively studied. It is now well established that sporadic colorectal cancer is the result of a predictable pattern of progression of normal colorectal mucosa, to advancing grades of adenomas (benign tumours) and eventually the formation of invasive colorectal adenocarcinoma. Neoplasia begins by widespread hyperproliferation of normal colorectal epithelial cells. A subset of these proliferating cells then undergoes clonal expansion and form a small benign neoplasm termed a tubular adenoma. These adenoma cells then continue to progress through increases in size, dysplasia (atypia) and the acquisition of villous (finger-like) architecture. Increasing dysplasia is accompanied by loss of differentiation and mucin production. Once adenoma cells develop the ability to overgrow adjacent sister cells and invade through the basement membrane, the tumour becomes a malignant invasive carcinoma.

These morphological changes of the adenoma – carcinoma sequence are driven simultaneously by an accumulation of mutational genetic alterations[12]. Of these, adenomatous polyposis coli (APC), K-Ras and p53 have been convincingly shown to initiate and drive the progression of colorectal neoplasia by predisposing to chromosomal instability. These distinctive and well defined stages of colorectal carcinogenesis offer an ideal pathway for interception of progression and also provide multiple targets for screening and early diagnosis.

Epigenetic alterations have recently been confirmed by numerous studies to contribute to colorectal carcinogenesis. Epigenetics refer to heritable mechanisms that control gene expression without altering the individual gene sequences. Aberrant DNA methylation is one epigenetic marker that has received most attention and therefore best understood. Methyl (CH3) groups are attached to cytosine bases in the promoter regions of individual genes. The result of this process is conformational changes in the chromatin that prevent gene expression to take place in the normal manner. In colorectal cancer development, hypermethylation of specific tumour suppressor genes has been observed together with a global (genome-wide) hypomethylation pattern which has been suggested to promote neoplasia through increasing genetic instability[119].

Although research has focussed on using the well described molecular markers in the chromosomal instability pathway such as K-Ras and p53 mutations as screening tools for colorectal cancer, the fact that some of these have low sensitivity and are not detectable until later stages of the adenoma-carcinoma sequence have hindered their usefulness. To date, no accurate or sensitive screening molecular marker is in clinical use. Given that aberrant DNA methylation is detectable early in the pre-cursor lesions of colorectal neoplasia, tumour specific and readily detectable relative to genetic markers, it has the potential to become a promising biomarker in the disease[169].

The aim of this chapter was to examine the global DNA methylation patterns across the adenoma - carcinoma sequence by studying adenomatous polyps with mild, moderate and

severe dysplasia and to determine whether these methylation patterns correlate with any clinico-pathological features.

3.2 PATIENTS AND METHODS

Patients

Forty-five patients with colorectal adenomatous polyps were identified from the pathology database system of Connolly Hospital in Dublin. There were 28 males and 17 females with a median age of 67. Each patient had a single polyp specimen obtained by either biopsy or snared with diathermy during routine colonoscopic examinations following symptomatic presentation. Ten patients with normal colorectal mucosa and invasive adenocarcinoma respectively were also chosen for analysis. A single colorectal surgeon preformed all procedures and all samples were examined by a consultant pathologist in Connolly Hospital. Out of the total 45 adenomatous polyps used for the purpose of this study, 22 (49%) had mild dysplasia, 12 (27%) had moderate dysplasia and 11 (24%) had severe dysplasia. Pathological data of each polyp specimen were collected by manually reviewing each patient's histology.

Tissue processing, immunohistochemistry, image analysis and statistical analysis

Details as described in Materials and Methods – refer to chapter 2. Non-parametric statistical analysis was performed using the Mann-Whitney test.

3.3 RESULTS

3.3.1 Methylation

Clinico-pathological information of all 45 patients with adenomatous polyps is shown in Table 3.1. Mean methylation for normal colorectal mucosa (n=10) and invasive carcinoma (n=10) were 120.7 and 215.9 respectively ($p<0.0001$). Methylation for mildly dysplastic polyps (n=22) was 177.4, moderately dysplastic polyps (n=12) was 173.8 and severely dysplastic polyps (n=11) was 177.3. (Table 3.2) A significant ***decrease*** in methylation as evident by an ***increase*** in methylation staining index (see Methods) was observed between normal mucosa and all dysplasia groups together ($p<0.0001$). No correlation was demonstrated between advancing stages of each polyp dysplasia group (mild vs moderate vs severe) and methylation. A significant reduction in methylation was observed between all polyp dysplasia groups together and cancer ($p<0.0001$). (Figure 3.1; Figures 3.3-3.5) .

No significant correlation was found between methylation and clinico-pathological data of each patient including age, sex, site of polyp and tubular/ villous architecture. No correlation was found between methylation and proliferative index.

3.3.2 Proliferative state (Ki-67)

No significant correlation was observed between proliferative index (Ki-67) expression and site of polyp, sex and tubular/ villous architecture of each patient. Mean Ki-67 positivity was 10.3% for normal mucosa (n=10), 27.8% for polyps with mild dysplasia (n=22), 30.4% for polyps with moderate dysplasia (n=12) and 41.3% for polyps with severe dysplasia (n=11) (Table 3.2). Mean Ki-67 positivity for cancer specimens was 55.6% (n=10). There was a significant increase of Ki-67 expression from normal to advancing stages of dysplasia ($p<0.001$). However no significant difference was observed between each individual dysplasia group. A significant difference was also observed with Ki-67 expression and patients above and below 70 years of age ($p=0.013$). (Figure 3.2)

Table 3.1 Clinico-pathological parameters of adenomatous polyp patients

	Age	Sex	Tubular/ villous	Dysplasia	Site
1	46	M	T	MILD	Left
2	72	M	T	MILD	Left
3	60	M	T	MILD	Left
4	42	M	T	MILD	Right
5	54	M	TV	MILD	Left
6	57	M	T	MILD	Right
7	82	M	T	MILD	Right
8	54	M	TV	MILD	Right
9	54	M	TV	MILD	Right
10	54	M	TV	MILD	Transverse
11	62	M	T	MILD	Rectal
12	64	M	T	MILD	Left
13	63	M	T	MILD	Right
14	28	F	T	MILD	Right
15	46	M	T	MILD	Rectal
16	38	M	TV	MILD	Right
17	71	F	T	MILD	Left
18	71	M	T	MILD	Right
19	77	M	TV	MILD	Left
20	77	F	T	MILD	Left
21	77	F	TV	MILD	Left
22	87	F	T	MILD	Left
23	80	F	TV	SEV	Left
24	81	M	T	SEV	Rectal

25	64	F	TV	SEV	Right
26	82	F	TV	SEV	Left
27	85	F	V	SEV	Right
28	54	M	TV	SEV	Rectal
29	53	M	TV	SEV	Right
30	77	M	T	SEV	Left
31	78	F	TV	SEV	Transverse
32	76	M	T	SEV	Rectal
33	89	M	T	SEV	Right
34	80	F	T	MOD	Transverse
35	80	M	T	MOD	Transverse
36	67	M	TV	MOD	Rectal
37	51	M	TV	MOD	Left
38	78	F	TV	MOD	Right
39	57	M	T	MOD	Right
40	75	F	T	MOD	Right
41	65	F	V	MOD	Rectal
42	46	F	TV	MOD	Rectal
43	52	F	T	MOD	Left
44	77	M	TV	MOD	Right
45	71	M	T	MOD	Rectal

Table 3.2 Mean methylation index and Ki-67 positivity for normal tissues, polyps and cancer tissues used

	Methylation index	Ki-67 (% positivity)
Normal	120.7	10.3
Mild dysplasia	177.4	27.8
Moderate dysplasia	173.8	30.4
Severe dysplasia	177.3	41.3
Cancer	215.9	55.6

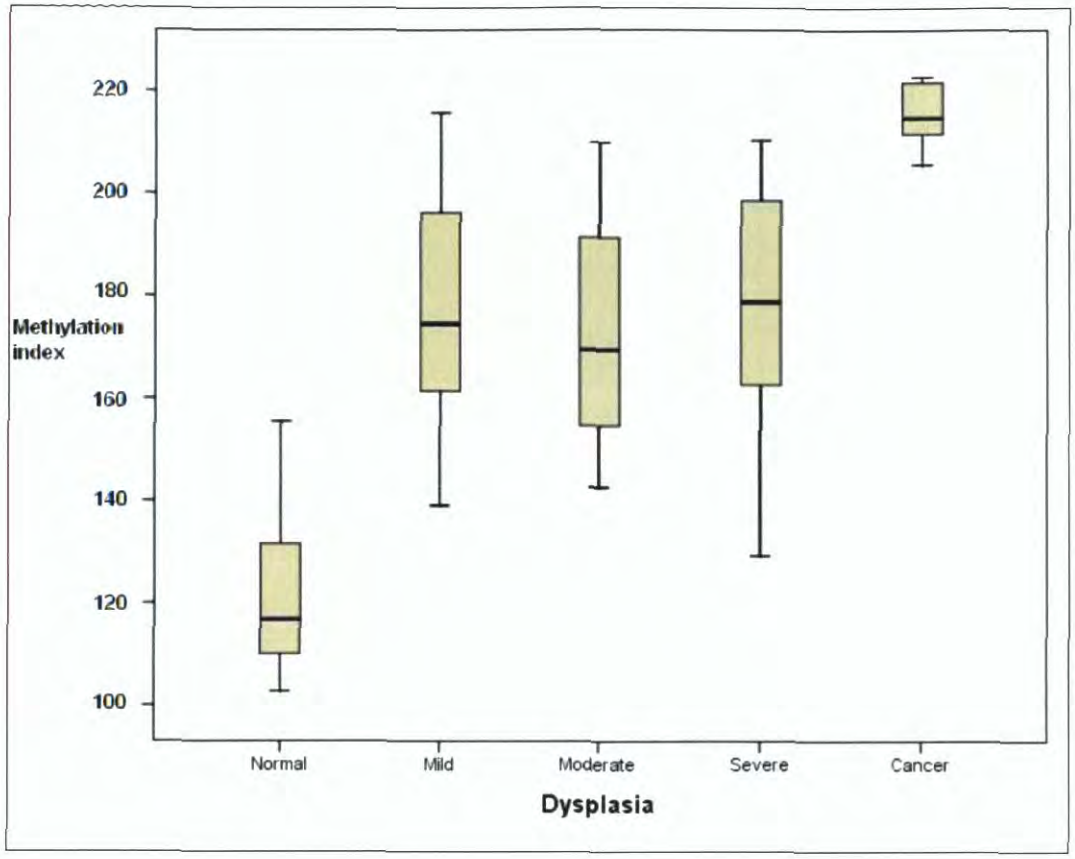


Figure 3.1 **Box plot graph showing methylation differences between normal, *adenomatous* polyps and cancer ($p<0.0001$).**

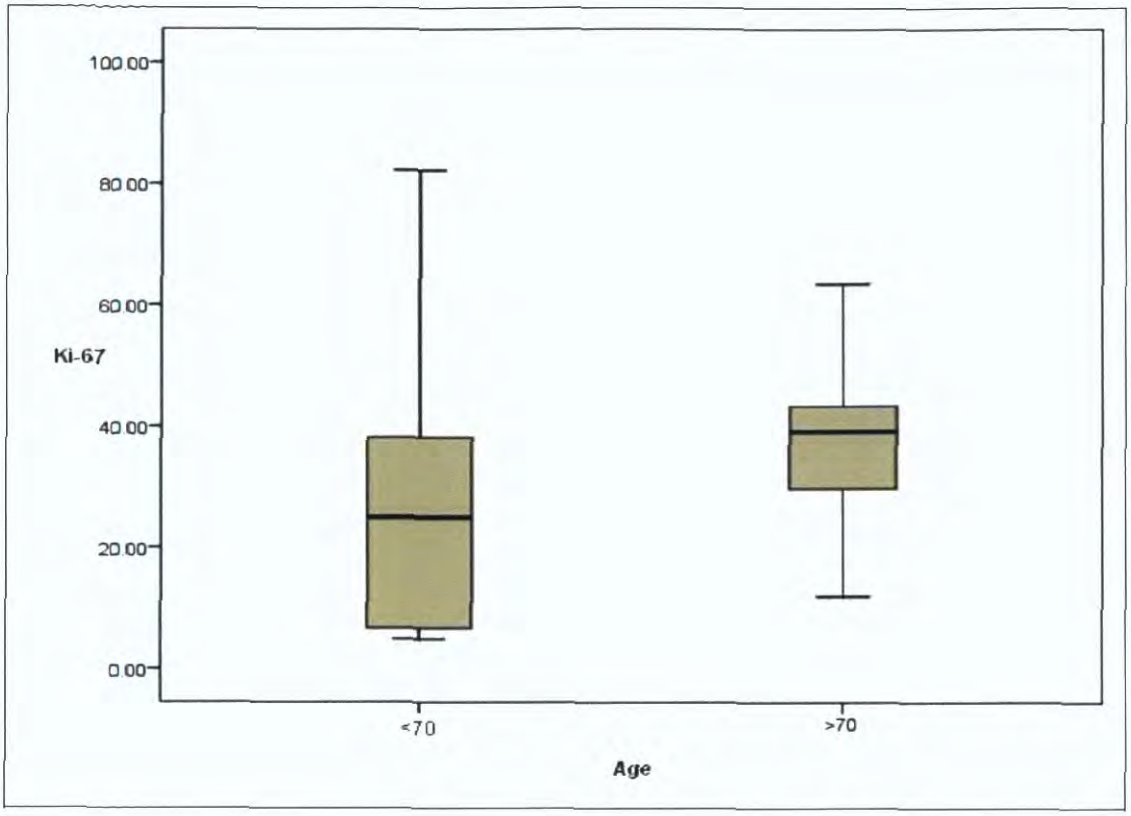
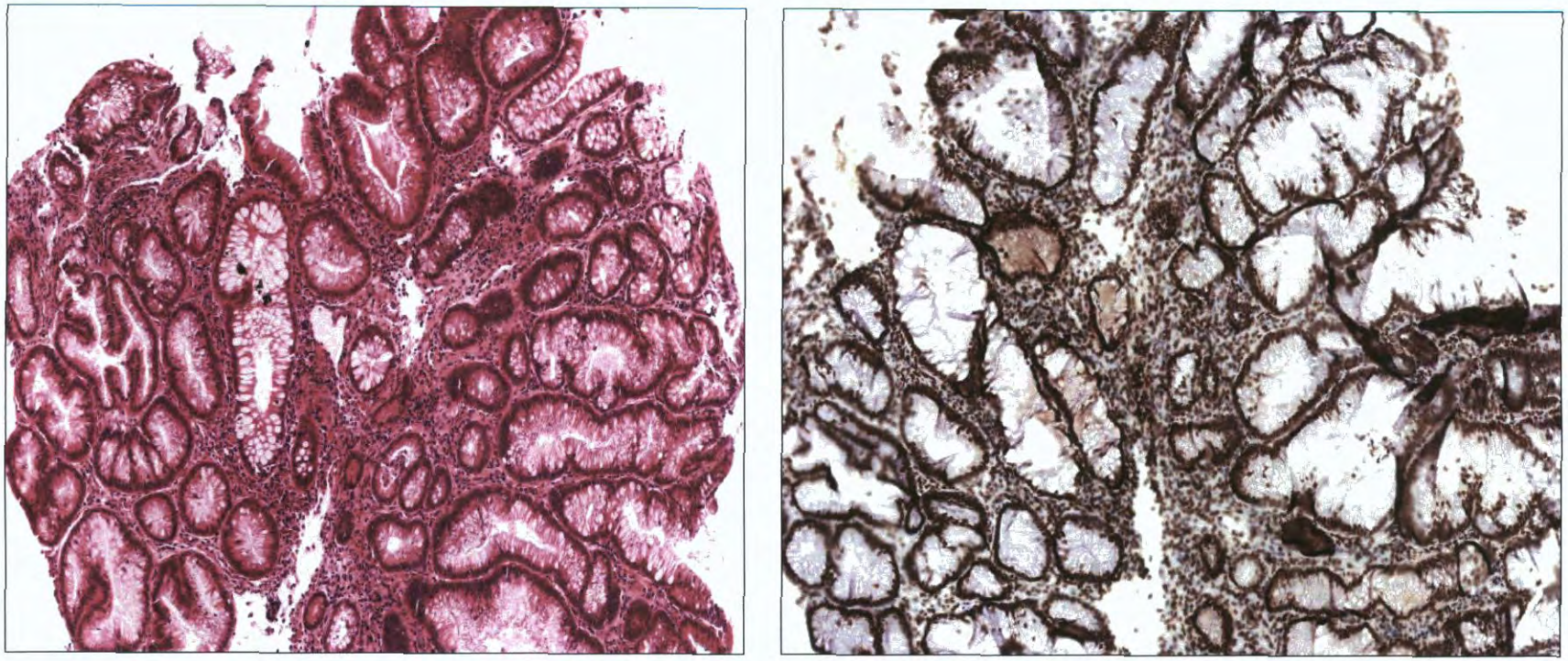


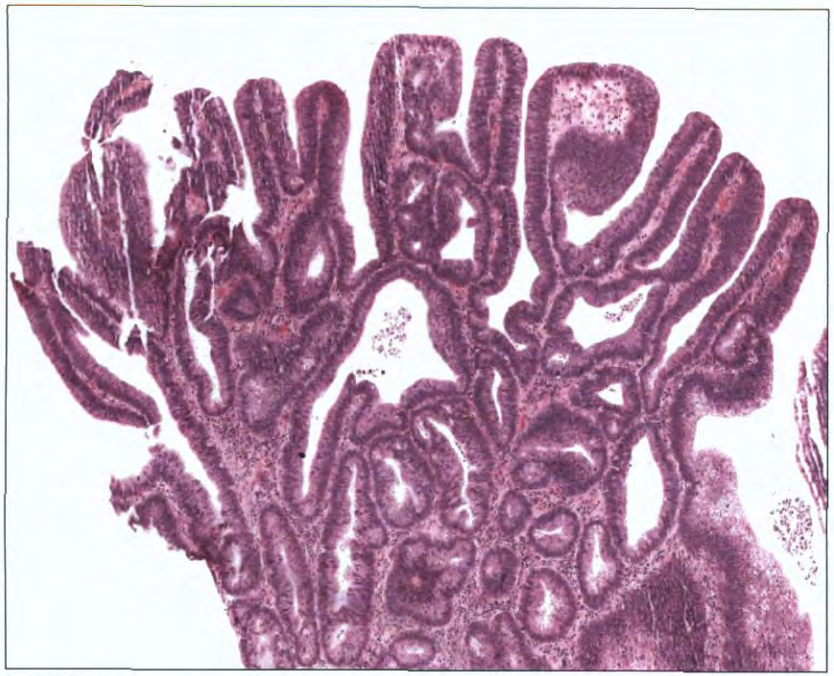
Figure 3.2 **Box plot graph showing significant difference in proliferative index (Ki-67) between patients above and below 70 years of age (p=0.013)**



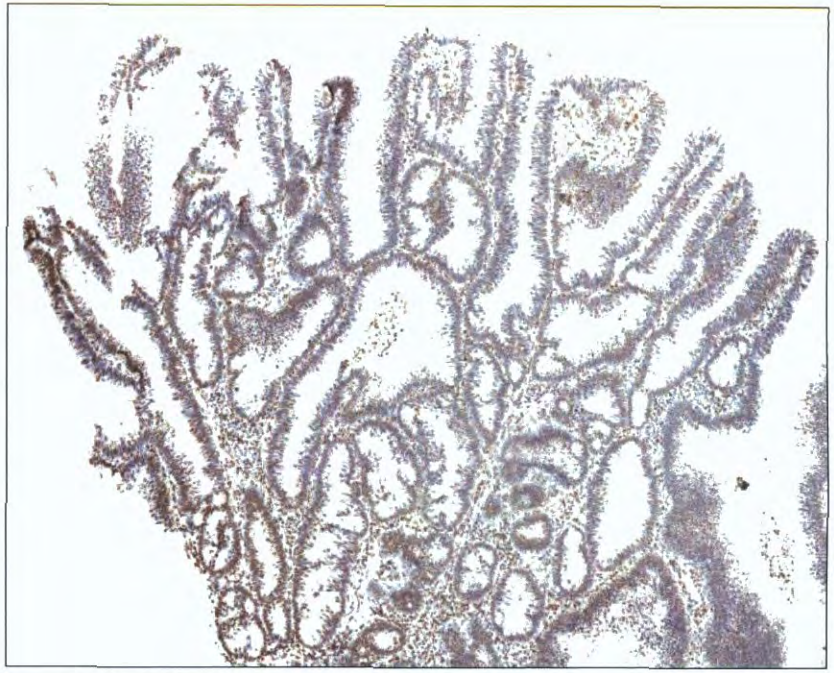
H&E

5-Methylcytidine

Figure 3.3 H&E and methylation slides of adenomatous polyp with mild dysplasia (X20)

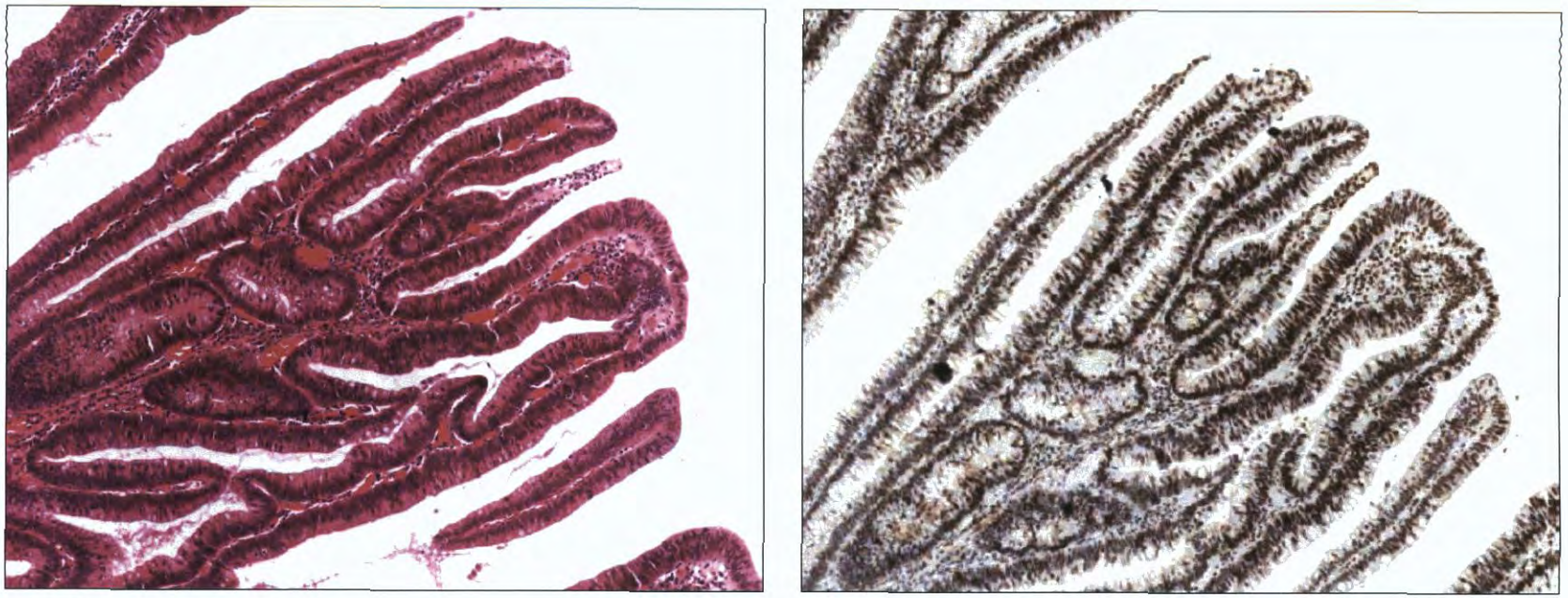


H&E



5-Methylcytidine

Figure 3.4 H&E and methylation staining of adenomatous polyp moderate dysplasia



H&E

5-Methylcytidine

Figure 3.5 H&E and methylation staining of adenomatous polyp with severe dysplasia

3.4 DISCUSSION

Colorectal cancer can be regarded as the genetic prototype for cancer development. The well described and accepted adenoma-carcinoma sequence characterises step-by step progression of normal colorectal mucosa to colorectal cancer. Well defined histo-pathological changes (polyps increasing in size and gaining dysplastic features) are accompanied simultaneously by an accumulation of mutational genetic changes. Gene mutations along this sequence – adenomatous polyposis coli (APC), K-Ras and p53 have been extensively studied[2]. Although potentially these genetic mutations can be used as markers of disease development and progression, study results to date have been disappointing[169].

Epigenetic markers such as DNA methylation are now recognised to play a key role in colorectal carcinogenesis. Promoter methylation of specific tumour suppressor genes e.g. APC, and global hypomethylation have been described in colorectal cancers. Some reports have suggested that aberrant methylation patterns occur early in the adenoma-carcinoma sequence, this makes DNA methylation a potential marker of colorectal cancer development[169]. In this study, we aimed to examine the methylation patterns along the adenoma-carcinoma sequence. This would allow confirmation of whether indeed aberrant methylation changes are found early in colorectal cancer progression; whether such methylation changes progress in a way similar to increasing dysplasia; and whether any correlation can be found with clinico-pathological details of patients and methylation

patterns. The answers to these questions may help determine DNA methylation as a potential new disease marker in colorectal cancer, possible in the era of colorectal cancer screening programs.

A significant difference in global (genome-wide) methylation was observed between normal mucosa and colorectal invasive cancers in this study ($p<0.001$). This finding confirms previous studies showing global hypomethylation in cancers of multiple malignancies including gastric, oesophageal, prostate and breast cancer. Postulated mechanisms of carcinogenesis from hypomethylation include increasing chromosomal breakage leading to chromosomal instability, loss of imprinting and activation of proto-oncogenes from loss of methylation silencing[115].

A significant decrease in methylation was observed between normal mucosa and dysplasia groups however, no correlation was observed between methylation and different individual (mild, moderate and severe) dysplasia groups of the adenomatous polyps in this study. Studies examining methylation patterns across pre-cursor lesions of colorectal carcinoma are limited and most studies have concentrated mainly in gene-specific methylation rather than global hypomethylation changes[170, 171]. Bariol et al [172]studied global hypomethylation in normal mucosa of healthy and colorectal cancer patients (n=45), small adenomas (n=21), large adenomas (n=32) and carcinomas (n=24). Consistent with findings from this study, they did not find any significant difference in hypomethylation levels across all proliferative lesions of the colon, whether they were benign or malignant ($p=0.78$). Not surprisingly, they also observed a significantly higher

5-methylcytosine content in all normal mucosa compared to any type of proliferative lesions (adenomas and cancers) ($p<0.001$), consistent with this study findings. In the same study, they examined hypermethylation of 7 CpG islands. They could not find any association between global hypomethylation and hypermethylation. They suggested that the two processes are independent of each other in causing carcinogenesis. Most studies looking at gene-specific methylation in the adenoma-carcinoma sequence have observed a sequential increase in genes methylated. Ahlquist et al looked at methylation status of eleven gene promoters in 154 tissue samples including normal mucosa and adenomas and carcinomas of colon and rectum. They found a step-wise increase in CpG island promoter methylation towards malignancy with the exception of three genes. They suggested that inactivation of genes through methylation plays a role in progression of the tumour. The three genes (HOXA9, MAL and MGMT) were suggested to be more important in initiation rather than progression of cancer as there was lack of increase between non-malignant adenomas and carcinoma ($p<0.0001$) [173]. In another study, Kim et al looked at frequencies of gene methylation in lesions along the adenoma-carcinoma sequence using a panel of eight genes[174]. Consistent with findings from Ahlquist, they found a significant difference in the number of tumours carrying methylated genes between adenomas and carcinomas ($p=0.01$). The most substantial difference was observed between early and advanced adenomas ($p=0.018$) but no difference was found between advanced adenomas and stage I-II cancers. There was no difference in methylation between adenocarcinomas and metastases. Based on this present study and findings from other available studies on both global hypomethylation and gene-specific hypermethylation, it appears that aberrant methylation tend to occur in the early stages of

the adenoma-carcinoma sequence (Figure 3.1). Global methylation may be more important in the early stages and initiation of cancer development as no hypomethylation changes were observed with different dysplasia groups, while gene-specific hypermethylation is also important in the early stages, it is also involved in driving the adenoma-carcinoma sequence as these changes are seen to increase with advancing stages of neoplasia.

Early methylation changes in colorectal neoplasia makes DNA methylation a potential biomarker of disease. Methylation of genes involved in colorectal neoplasia such as MGMT, and APC have been observed in early (<1cm diameter) adenomas [175-177]. Not only does it have the potential to identify early lesions of the polyp-carcinoma sequence, CpG island methylation also occurs in higher frequencies, and detection is also specific to colorectal cancer only. Traditional genetic markers of detection such as p53 in serum, could also be present in other malignancies such as endometrial carcinomas[169]. Using faecal DNA, Petko et al were able to detect patients with colonic polyps with a sensitivity of as high as 80%[178]. Baek et al in a study using methylation specific polymerase chain reaction (MS-PCR) were able to detect from faecal DNA patients with carcinomas and adenomas, sensitivities of 75% and 59.6% respectively[179]. DNA methylation is therefore a promising biomarker of colorectal cancer, particularly in hypermethylation changes. It can be detected in faeces and serum, which makes it an ideal detection test for screening programs. With further developments in detection methods, sensitivities and specificities of testing should improve and larger population studies should be continued to further define the role of DNA methylation as a screening tool.

In conclusion, this study has shown DNA methylation changes including global hypomethylation and gene-specific hypermethylation occur early in the adenoma-carcinoma sequence. No changes in hypomethylation were observed in different dysplasia groups of adenomatous polyps (Figure 3.1). DNA methylation has the potential to be developed into a useful screening tool in colorectal cancer as it can be detected in faeces and blood in early stages of carcinogenesis[179]. Our study suggests that DNA methylation may have the ability to detect colorectal polyp development leading to significant financial and health benefits in any colorectal screening model. With further studies and developments in detection techniques, sensitivities of detection should improve.

Chapter 4

The prognostic role of DNA methylation in colorectal cancer

4.1 INTRODUCTION

Colorectal cancer is one of the best described cancers in terms of its pathogenesis. The adenoma-carcinoma sequence is well established as the major pathological pathway that characterises the progression from normal epithelium to colorectal cancer. This pathway is accompanied by a step-wise accumulation of genetic mutations that involves gate-keeper genes that initiate and others that drive the progression of colorectal cancer. These genes include the adenomatous polyposis coli (APC), K-Ras and p53 genes first to be associated with colorectal cancer by Vogelstein[2]. This 'traditional' chromosomal instability pathway is responsible for 85% of sporadic colorectal cancers. The other well characterised molecular pathway involves germline mutations in DNA mismatch repair genes that lead to the development of hereditary non-polyposis colon cancers (HNPCC or Lynch syndrome) which account for upto 15% of colorectal cancers[23].

CpG island methylator pathway (CIMP) has recently emerged as a third phenotype of colorectal cancer that displays characteristic clinico-pathological features. The molecular basis of this phenotype involves epigenetic DNA modifications that regulate the expression of oncogenes and tumour suppressor genes[119]. The most understood epigenetic marker, DNA methylation involves an enzyme catalysed addition of a methyl (CH3) group covalently bonded to the 5' position of a cytosine base of a DNA sequence to form 5-methylcytidine. Alterations in gene expression occurs when the promoter regions of regulatory genes known as CpG islands. DNA methylation has been observed to cause complex chromatin conformational changes and histone modifications that

ultimately lead to suppressed expression of the methylated gene[97]. A large panel of key regulatory genes have now been found to be hypermethylated in colorectal cancer. In many human malignancies including colorectal cancer, both gene-specific hypermethylation and global (genome-wide) hypomethylation changes have been observed[119]. DNA methylation is now understood to play a major role in colorectal cancer development.

Despite continuing developments in diagnostic imaging, surgical technique and adjuvant therapies, the survival rates from colorectal cancer have remained from modest depending on clinical stage[180]. Although research continues to explore into new molecular prognostic markers such as p53 and K-Ras, histopatholgical indices including TNM, Dukes' staging and lymphovascular invasion remain the most important indicators of prognosis[181]. The increasing knowledge of the DNA methylation pathway in human carcinogenesis may identify new potential diagnostic and prognostic indicators in this colorectal cancer. Studies on gene-specific methylation have already yielded promising results[182]. However, little is known about the role of global methylation and in particular global hypomethylation as prognostic markers. Hence the aim of this chapter was to evaluate the DNA methylation patterns in colorectal cancer specimens and their relationship with paired-normal mucosa. In addition, we aimed to examine whether DNA methylation in these tumour patients could be correlated with clinico-pathological features as well as prognosis.

4.2 PATIENTS AND METHODS

Patients

A total of 141 colorectal carcinoma patients were identified from the pathology database systems of Connolly Hospital and Beaumont Hospital in Dublin. There were 77 males and 64 females with a median age of 69 years. All patients had confirmed diagnoses of malignancy following surgical resection by one of three colorectal surgeons. Cancers were staged using the Dukes and TNM staging systems according to the American Joint Committee on Cancer (AJCC) staging guidelines. Only resections with availability of both cancer and paired normal mucosa were included for analysis. Rectal cancer patients who received neoadjuvant chemoradiotherapy were excluded from the study.

Tissue processing immunohistochemistry, image analysis and statistical analysis

Details as described in Materials and Methods – refer to chapter 2. Primary outcome measures were the difference in global methylation levels between cancer and paired normal tissue, and patient survival. Secondary outcome measures were the relationship between methylation levels with patient's clinico-pathological data (age, sex, site of lesion, Dukes, TNM staging, lymph node involvement). Analysis was performed using the Wilcoxon Signed Rank test for both methylation and Ki-67 values.

4.3 RESULTS

Clinico-pathological details of all 141 patients are displayed in Table 4.1. Seventy-seven (n=77, 56%) males and 64 (54%) females were identified with a mean age of 69 years.

4.3.1 Methylation of cancer and paired normal tissue

A comparison was made between methylation in cancer tissue and paired normal mucosa. In 124 (88%) patients, there was a significant reduction in global methylation (hypomethylation) in cancer compared to paired normal mucosa (165.3 vs 147.4; $p<0.001$) (A zero methylation index indicates strongest staining; a 255 methylation index indicates the weakest methylation staining – Chapter 2, Materials & Methods). In the remaining 17 (12%) patients, there was an increase in global methylation. (Table and Figure 4.1; Figures 4.7 and 4.8)

4.3.2 Proliferative index of cancer and paired normal tissue

A comparison was made between Ki-67 expression (%) in cancer and normal mucosa. All (n=141, 100%) cancer mucosa had a higher positivity of Ki-67 expression compared to normal mucosa ($p<0.001$). The mean Ki-67 expression was 24.5% in cancer compared to 14.3% in normal mucosa. (Figure 4.2)

4.3.3 Correlation with clinico-pathological details

On univariate and multivariate analysis, no correlation was found between cancer methylation and proliferation (Ki-67) with clinico-pathological details of the patients including sex, age, site of tumour, tumour differentiation, Duke's staging and lymph node status (Table 4.1). Similarly, within the group of patients that showed an increase in methylation (hypermethylation) in cancer tissue, no correlation with patient clinico-pathological details was identified. Comparing the 2 groups of patients, reduced and increased methylation in cancer tissue compared to normal, no significant difference was identified in each of the clinico-pathological parameters statistically.

4.3.4 Methylation and proliferative index

No association was identified between methylation levels and proliferative index of the tumours studied.

4.3.5 Survival analysis

The mean follow-up of all 141 patients was 53 months. Sixty-six (n=66; 47%) patients were deceased and the remaining patients (n=67; 48%) were alive at their last follow-up. Eight (n=8, 6%) patients were lost to follow-up. Methylation (Hazard ratio: 0.99; p=0.9) and Ki-67 (Hazard ratio:1; p=0.8) were not found to predict survival of these colorectal cancer patients. Survival was significantly associated with Duke's staging (Hazard ratio

8.9, 15.7, 73.7; p<0.0001) and lymph node status (Hazard ratio 2.6; p<0.0001). (Figures 4.3 - 4.6)

Analysis of survival of patients with hypo and hypermethylation showed a trend towards better prognosis in patients with hypomethylation in tumour tissue compared to those with hypermethylation. However, this did not reach statistically significance (p=0.073).

Table 4.1 *Patient* clinico-pathological characteristics and comparison NS: non-significant

n=141 **Male:77** **Female 64** **Mean age: 69 yrs**	**Reduced methylation** **n= 142**	**Increased methylation** **n=17**	**p-value**
T-stage	T1: 7 (5%) T2: 11 (8%) T3: 90 (64%) T4: 16 (11%)	0 (0%) 2 (1%) 8 (6%) 7 (5%)	NS
Dukes stage	A:14 (10%) B:68 (48%) C:39 (28%) D:3 (2%)	0 (0%) 10 (7%) 4 (3%) 3 (2%)	NS
Differentiation	Well: 3 (2%) Moderate: 105 (74%) Poor: 16 (11%)	0 (0%) 15 (11%) 2 (1%)	NS
Site	Right: 33 (23%) Left: 28 (20%) Transverse: 5 (4%) Rectal: 58 (41%)	3 (2%) 5 (4%) 2 (1%) 7 (5%)	NS
Lymph node	Negative: 84 (60%) Positive: 40 (28%)	11 (8%) 6 (4%)	NS

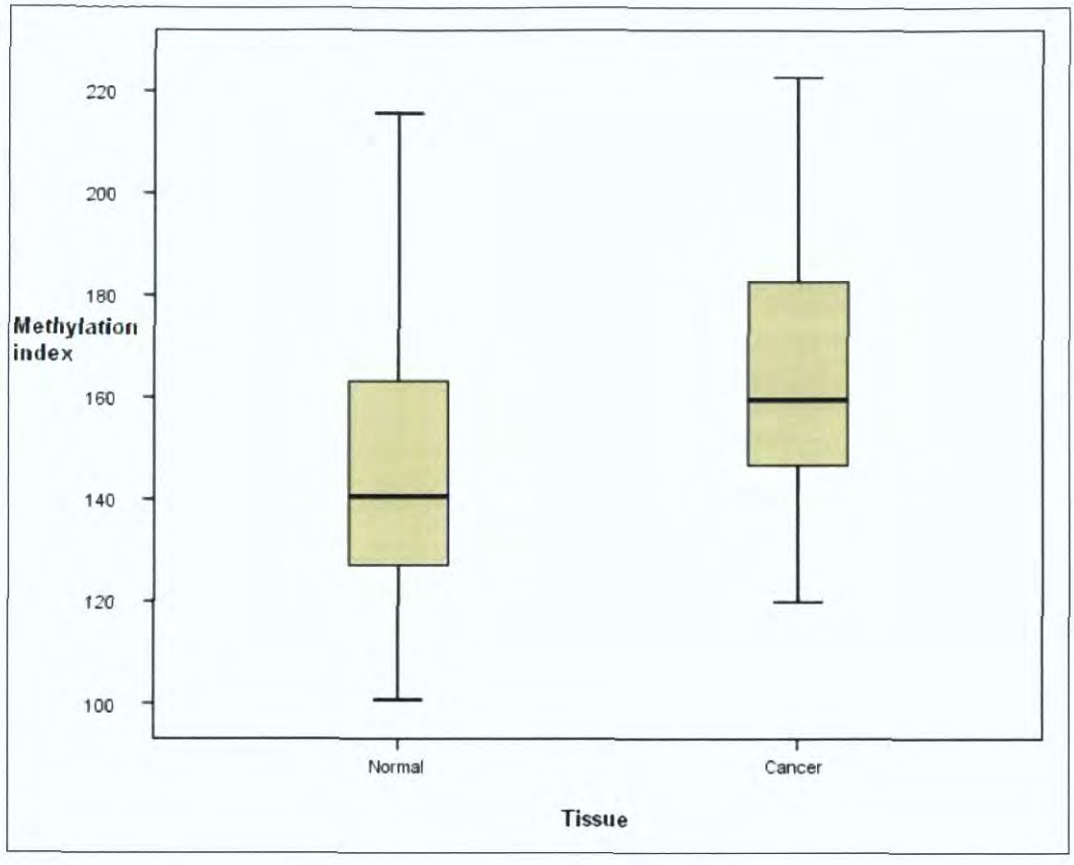


Figure 4.1 **Box plot graph showing significant global hypomethylation in cancer compared to normal tissue ($p<0.001$).**

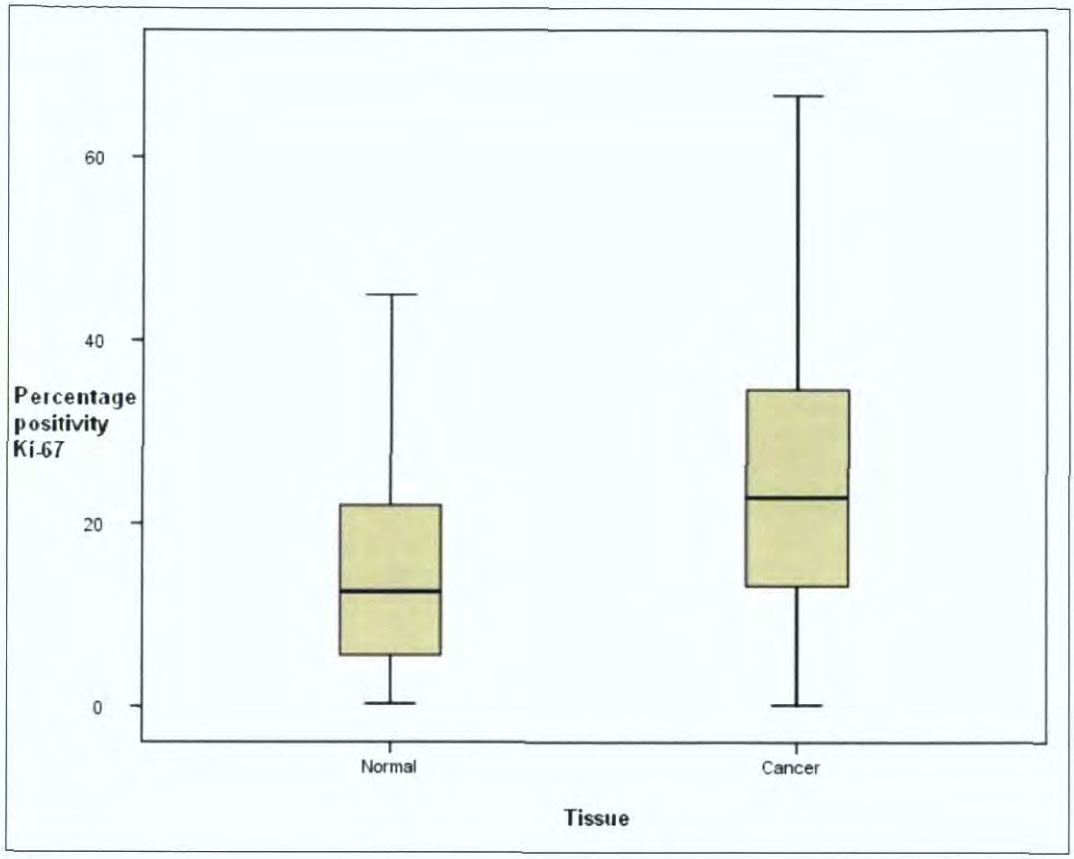


Figure 4.2 **Box plot graph showing significant reduced Ki-67 positivity in normal compared to cancer tissue ($p<0.001$).**

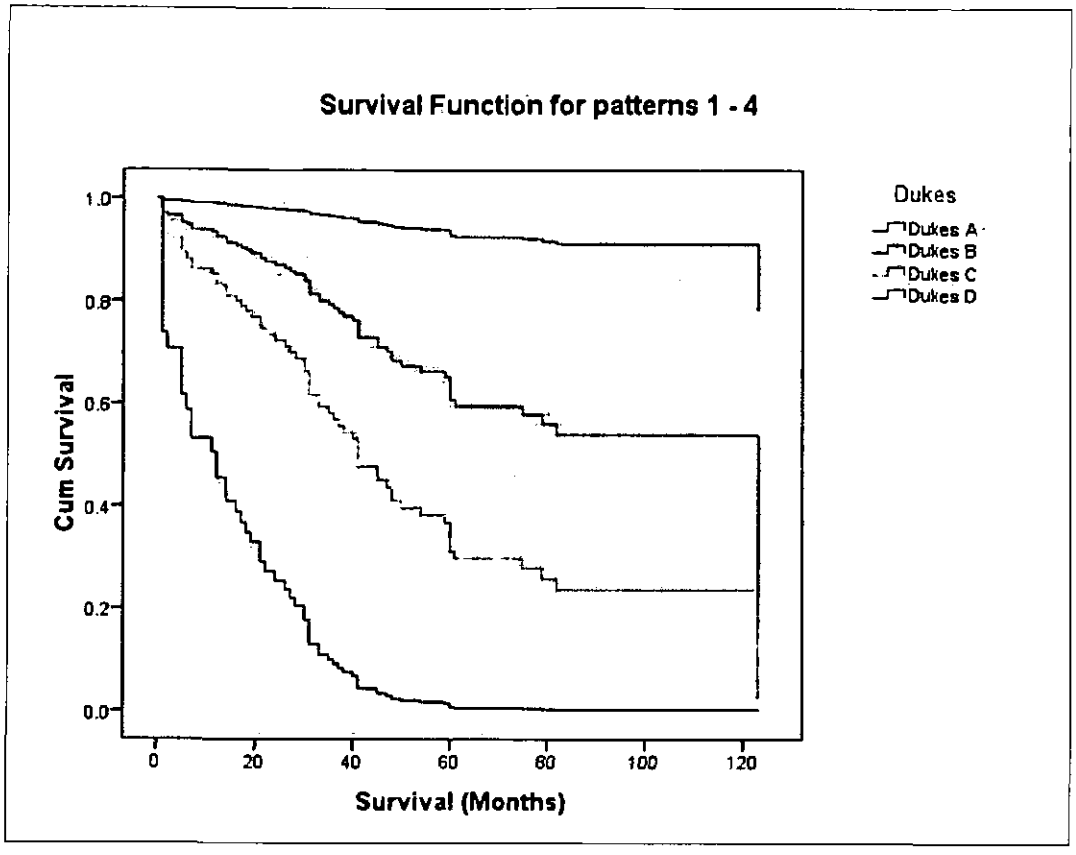


Figure 4.3 **Cox regression model showing association of survival and Dukes' staging ($p<0.0001$).**

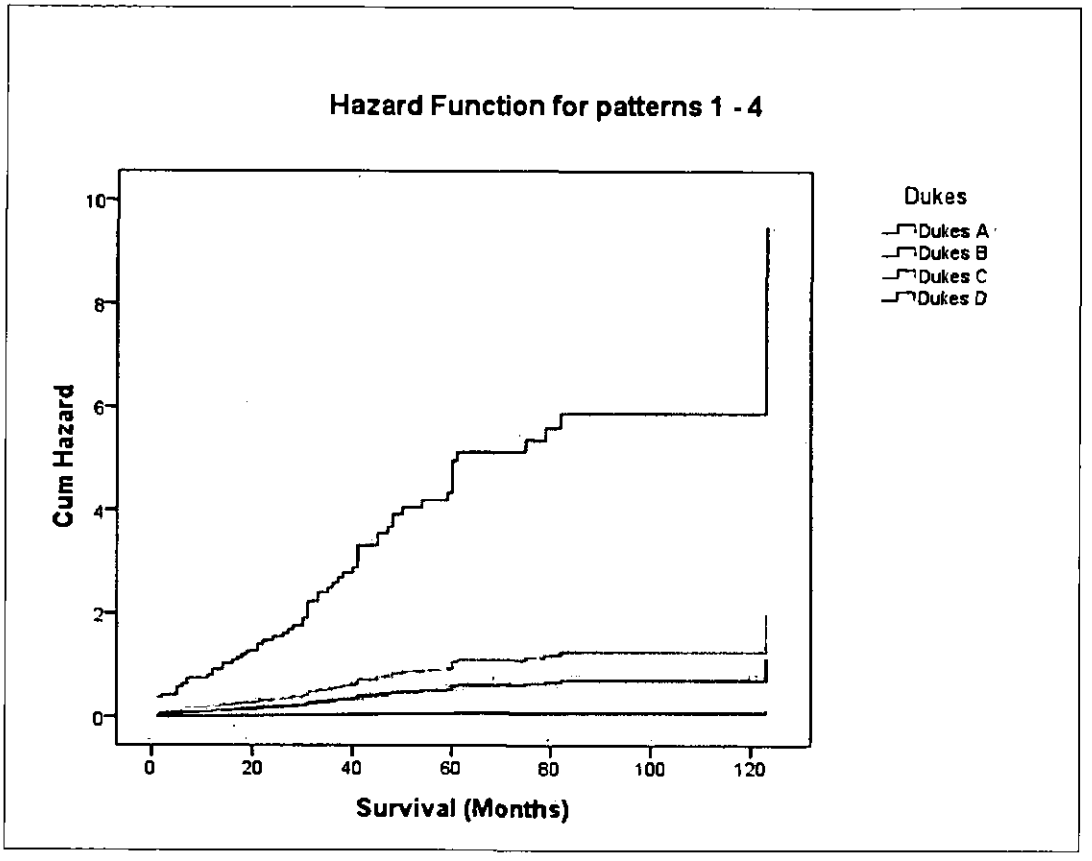


Figure 4.4 **Cox regression model showing increasing hazard ratios with advancing Dukes' staging ($p<0.0001$).**

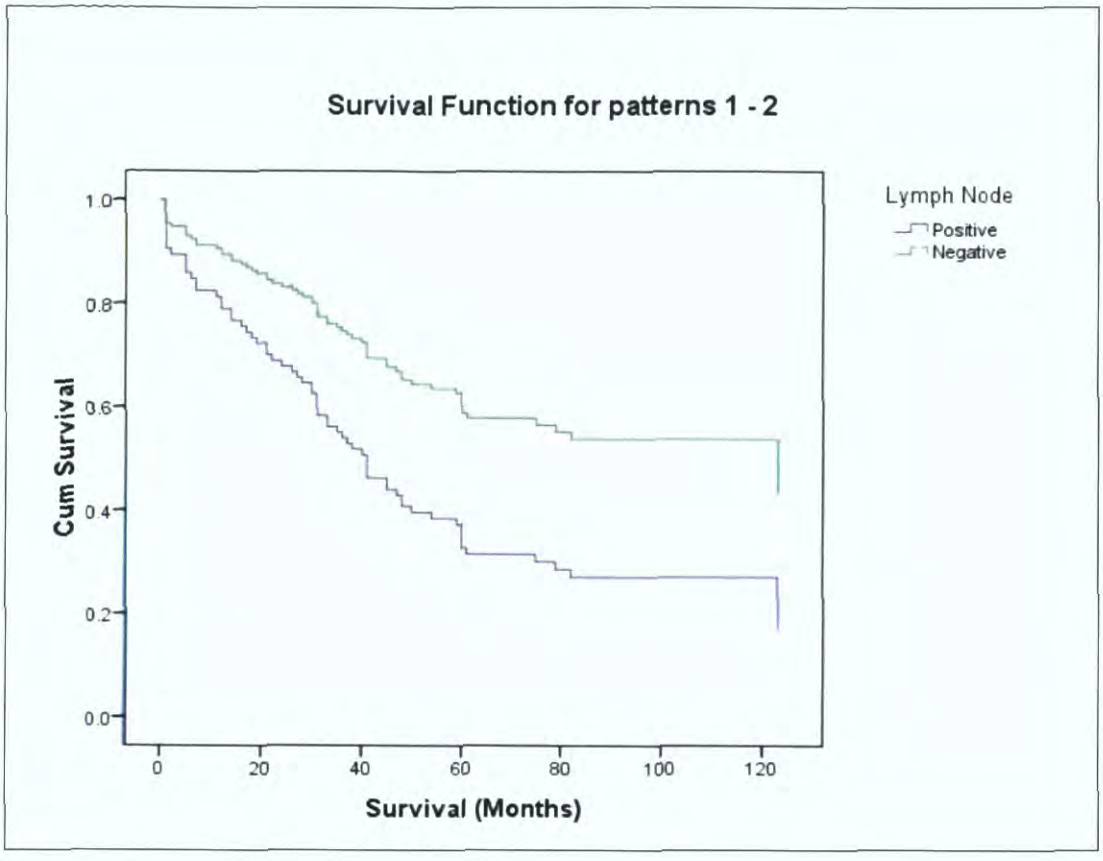


Figure 4.5 **Cox regression model showing significantly better survival with lymph node negativity ($p<0.0001$).**

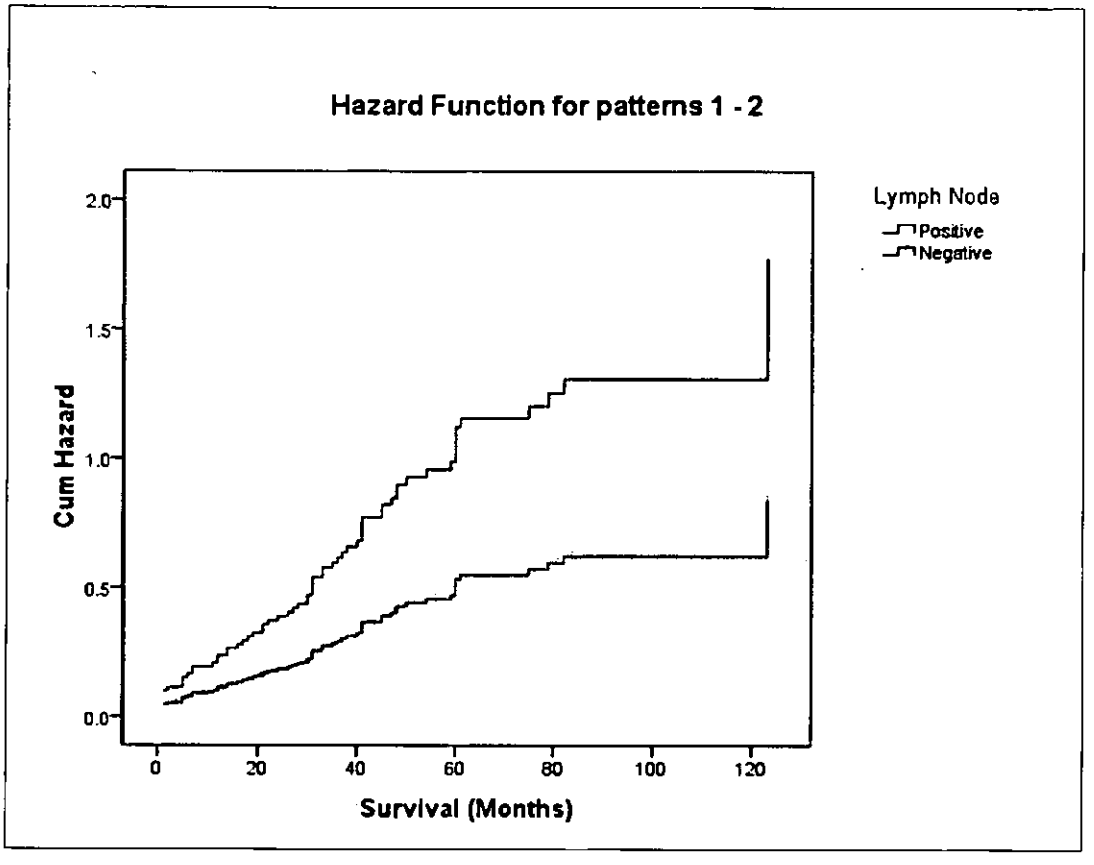
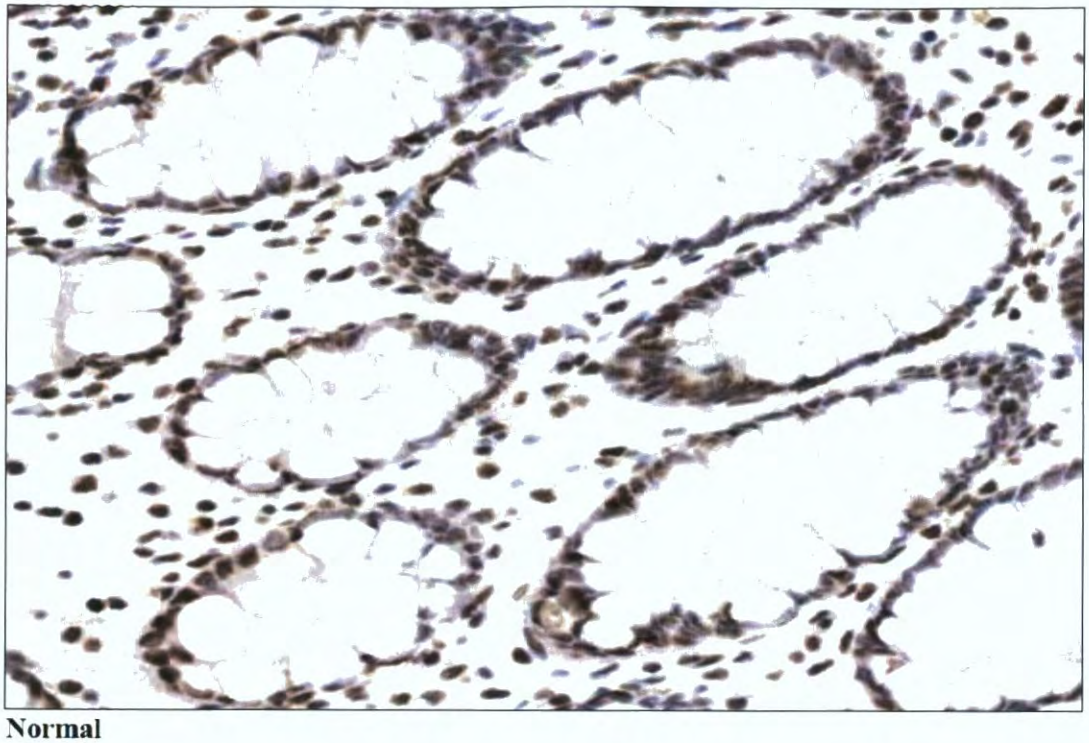
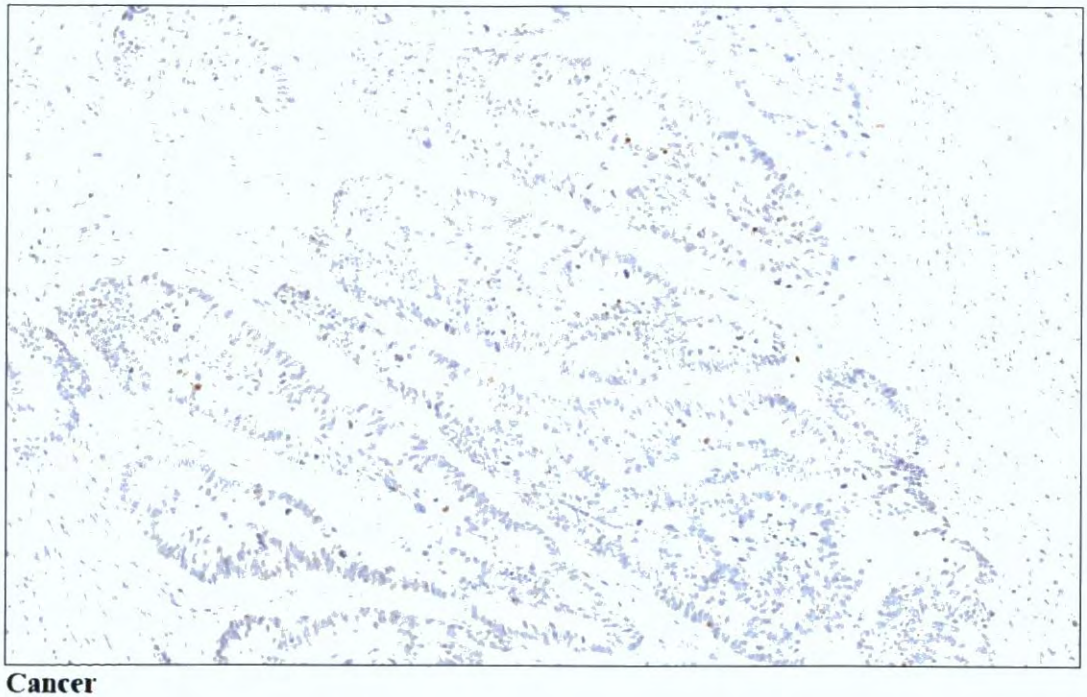


Figure 4.6 **Cox regression model showing increasing hazard ratios with lymph node positivity.**

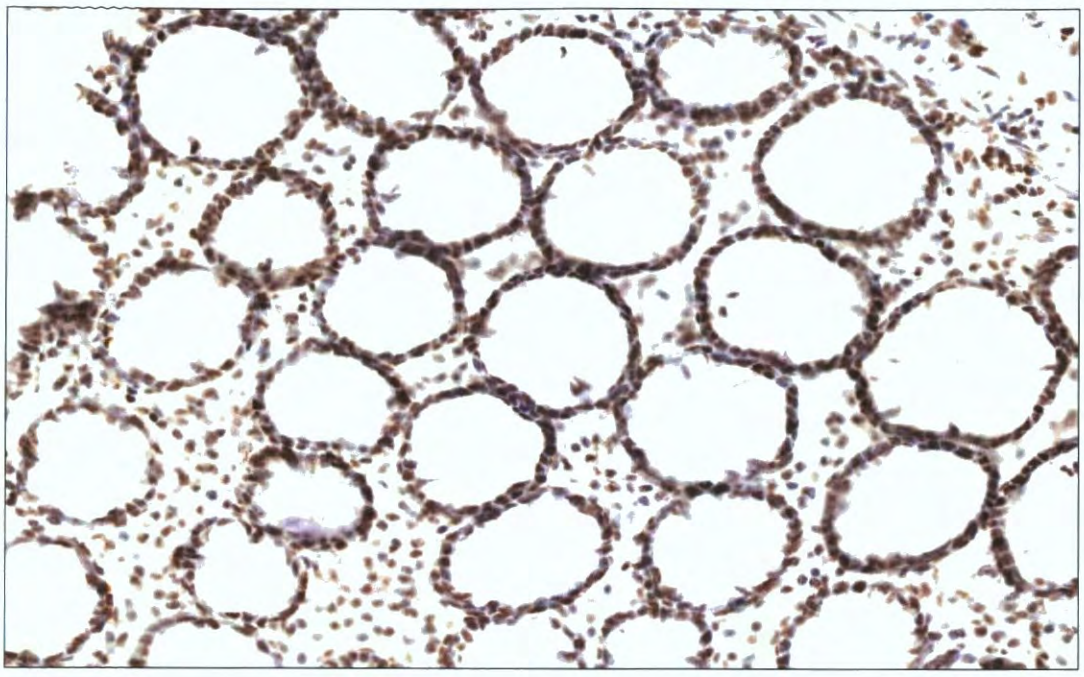


Normal

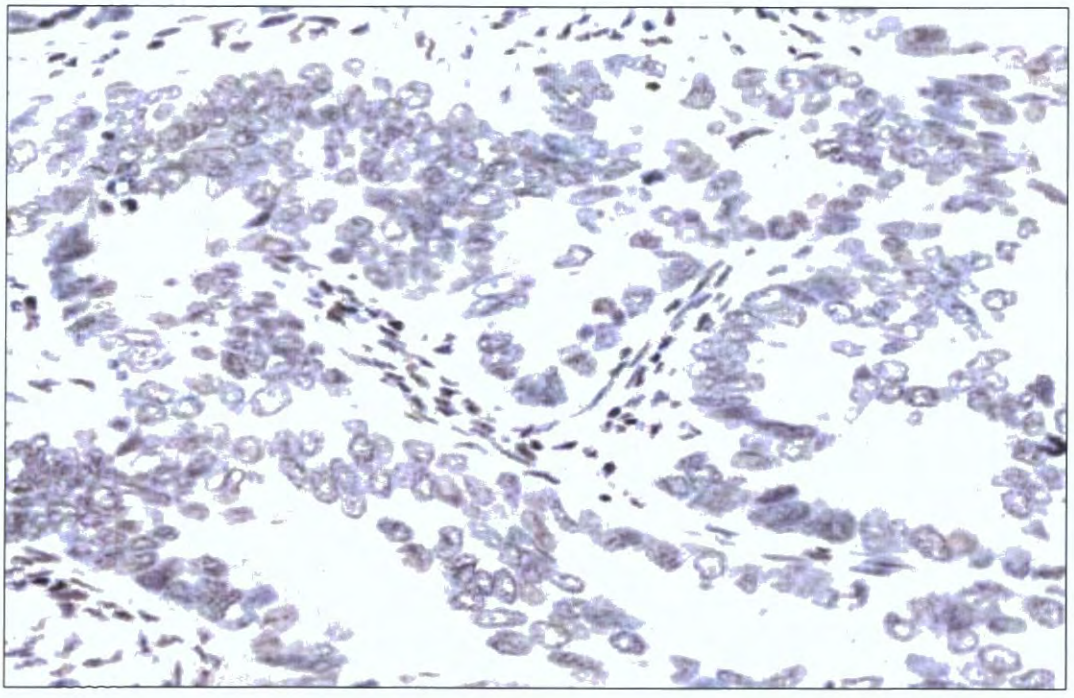


Cancer

Figure 4.7 Hypomethylation in cancer versus normal tissue (Patient 1) (X20)



Normal



Cancer

Figure 4.8 Hypomethylation in cancer compared to normal (patient 2) (X20)

4.4 DISCUSSION

Advances in technology, imaging modalities, pathological understanding and surgical technique in the treatment of colorectal cancer have occurred over the last few decades however the prognosis of colorectal cancer patients has remained more or less the same. The tumour-node-metastasis (TNM) staging system defined by the American Joint Committee on Cancer (AJCC) and Dukes' staging in addition to other pathological indices such as differentiation, lymphovascular invasion and lymph node involvement, are still the most important indicators of prognosis and survival for colorectal cancer patients. As the AJCC stage increases from stage I to stage V, 5 year-survival overall declines from over 90% to less than 10%[1].

Researchers have been targeting the molecular biological pathways of colorectal cancer in an attempt to identify a useful diagnostic and prognostic marker. The well recognised genetic mutational pathway described by Vogelstein [2] provides several potential molecular proteins that might be of use including adenomatous polyposis coli (APC), K-Ras, p53 and deleted in colon cancer (DCC). Tumours with microsatellite instability (MSI-H) have also been linked to a better survival compared to microsatellite stable (MSS) colorectal tumours[55] as well as being functioning as a useful predictor to adjuvant chemotherapy.[183]. Although studies of these molecular markers have shown some promising results in predicting prognosis, to date no molecular marker has made it into clinical practice[184]

The epigenetic marker, DNA methylation, is currently undergoing intense evaluation in colorectal cancer. The addition of a CH3 methyl group to the promoter regions of genes is able to cause gene silencing. This phenomenon is now recognised to take part not only in normal physiological processes but also plays a large role in carcinogenesis.[124]. In colorectal cancer, focal gene specific methylation of individual genes and global hypomethylation together with genetic mutations are now understood to initiate and drive cancer formation concomitantly[172]. Some evidence is available in the role of gene-specific methylation as a prognostic marker. De Maat et al investigated the association of multiple genomic methylated-in-tumour (MINT) loci sequences with clinical outcome in a group of patients with rectal cancers. MINT methylation in the sub-group of node-negative patients was associated with disease recurrence, disease specific and overall survival[182].

Global DNA hypomethylation with regards to clinical outcome has been investigated in prostate, liver and leukaemia patients[185-187]. In the previous chapter, a significant reduction in global methylation was observed between normal colorectal mucosa and adenomatous polyps. This change was observed at an early stage in the adenoma-carcinoma sequence (between normal and mild dysplastic polyps) suggesting that hypomethylation takes part in carcinogenesis at the initial stages in colorectal cancer. This points towards a potential role in acting as a biomarker of the disease. In order to further evaluate the role of global methylation in its use as a clinical disease marker (diagnostic and prognostic), this chapter's aim was to investigate global methylation in colorectal cancer and its role in predicting prognosis of patients.

A significant difference in methylation was observed between colorectal cancer and paired normal mucosa in 88% of our study cohort ($p<0.001$). This result confirms the existing knowledge that cancer tissue has a reduced global methylation content compared to normal mucosa[119]. Using a method similar to this current study, Hernandez-Blazquez examined 13 colorectal tumours and found a significantly reduced methylation content in cancer compared to normal tissue ($p<0.05$)[188]. Genomic hypomethylation has been postulated to predispose to malignancy by inducing genetic instability, activating oncogenes and also causing loss of imprinting[119]. However, the influence of global hypomethylation on the survival and outcome of colorectal cancer patients remains unclear.

The second aim of this study was to investigate the prognostic role of global methylation in colorectal cancer. A correlation between methylation status and survival could not be demonstrated in our study. Ogino et al carried out a cohort study of Tumoral LINE-1 (long interspersed nucleotide element-1) hypomethylation and prognosis in colon cancer patients. They observed a significant increase in colon cancer specific ($p<0.001$) and overall mortality with hypomethylation ($p<0.008$)[189]. Using a methylome fingerprinting technique with a sample size of 93 colorectal carcinomas, Frogla et al observed a trend towards a poor prognosis with hypomethylation and good prognosis with hypermethylation[190].

An increase in *global* methylation status in 12% of colorectal cancer specimens was observed in this study. *Gene-specific* hypermethylation has been documented widely to

occur in cancer tissue compared to normal tissue however on a *global* level, hypermethylation seen in our cancers studied was an unexpected event[115]. Sub-group analysis on these patients alone and also with patients that showed hypomethylation did not find any correlation with clinico-pathological data (Table 4.1). Although there was a trend towards poorer prognosis in these patients compared to the 88% of patients showing hypomethylation, this was not a statistically significant finding (p=0.073). Hypermethylation, based on available evidence leads to gene-silencing. Although this finding of global hypermethylation may be due to a technical reason, further studies possibly involving polymerase chain reaction (PCR) on this sub-group of colorectal cancers are needed to evaluate their clinical significance.

Although not demonstrated in this current study, the available evidence on gene-specific and global methylation appears to show some promise in its potential to predict survival in colorectal cancer patients. Since research into traditional molecular indices have been disappointing so far, these preliminary findings in DNA methylation should encourage more studies to be carried out. Indeed, epigenetic modifications such as DNA methylation are now recognised to contribute at least to the same level as genetic events in carcinogenesis and its ability to influence prognosis should not be ignored. Until further and stronger evidence is available however, histo-pathological staging remains the most important indicator of prognosis as indicated by our cohort of patients. Survival was clearly demonstrated to be affected by Dukes' staging and lymph node status (Figures 4.3 to 4.6).

In conclusion, this study has confirmed the previous findings that global genomic methylation is reduced in cancer compared to normal tissue and this reduction appears to be significant. The available evidence suggests a potential in DNA methylation in serving as a disease prognostic indicator. Further studies with more sensitive and sophisticated technology are perhaps required to explore its full potential.

Chapter 5

Effect of neoadjuvant chemoradiotherapy on rectal cancer epigenome

5.1 INTRODUCTION

Neoadjuvant chemoradiotherapy in locally advanced rectal cancer has been shown to significantly reduce local recurrence rates. The Swedish rectal cancer trial showed that neoadjuvant radiotherapy significantly reduced local recurrence rates from 27% to 11% at 5 years follow-up. Additionally, advantages of neoadjuvant treatment include improving the operability of large tumours and some trials have demonstrated an increased survival advantage following treatment[71]. With the recognition of the importance of precise surgery, total mesorectal excision (TME) was introduced and subsequently adopted to further improve outcomes. Randomised controlled trials combining neoadjuvant radiotherapy and TME showed even more impressive results[72]. In many countries, neoadjuvant chemoradiotherapy and total mesorectal excision is now the standard of care in patients with clinical T3/ T4 rectal tumours.

A pathological response, or downstaging from chemoradiotherapy, is more likely to improve the resectability of the primary tumour, and has also been shown to be an indicator of improved survival. Complete pathological response, characterised by absence of residual cancer cells in the resected specimen, has been shown to be an indicator of excellent prognosis. However, this is only seen in approximately 10% of cases. The remaining tumours demonstrate a spectrum of residual disease ranging from microscopic foci of adenocarcinoma to abundant macroscopic tumour cells. It is unclear at present why only some tumours respond to chemoradiotherapy and to varying degrees. Indeed it is impossible to predict accurately which patients do respond. Consequently all

patients are prescribed treatment if deemed physically suitable. An improved understanding of the behaviour of rectal tumours to neoadjuvant chemoradiotherapy would be of clinical significance as neoadjuvant chemoradiotherapy is associated with significant side effects and increases peri-operative morbidity[4]. Moreover, it is costly and could lead to delay in surgery for patients who ultimately do not respond to treatment[191]. Currently, there is no way of predicting which patients would respond to treatment and to what degree. With increasing understanding of the molecular biology of colorectal tumours, research has shifted from the traditional histopathological indicators to molecular predictors of outcome.

The adenoma – carcinoma sequence, genetic alterations in the chromosomal instability pathway and germline mutations in the microsatellite instability pathway have all been extensively studied and described. However, from a genetic view point, limited knowledge has been gained to aid the understanding of rectal cancer behaviour to chemoradiotherapy. The epigenetic pathway is currently undergoing intensive evaluation in all aspects of colorectal carcinogenesis. The most well studied marker, DNA methylation may lead us to some new insights into the behaviour of rectal cancers to treatment. Therefore an investigation into this novel DNA methylation pathway may provide some of the answers and proof to be useful. The aim of this chapter was to evaluate the role neoadjuvant chemoradiotherapy plays in the rectal cancer epigenome in patients with locally advanced rectal tumours.

5.2 PATIENTS AND METHODS

Patients

A total of 53 rectal cancer patients who received neoadjuvant chemoradiotherapy and whose tissues of pre-treatment biopsies and post treatment resections were available, were identified from the pathology database systems of Connolly Hospital and Beaumont Hospital. This group of patients received chemoradiotherapy and surgery from 2004 to 2008. Thirty-seven patients were male and 16 were female with a median age of 67 years. All patients were at least T3 stage on pre-operative clinical examination and MRI scanning with a threatened circumferential resection margin (CRM).

The long course regime of fractionated chemoradiotherapy was employed - 50.4Gy in total; 1.8Gy/day x 5 weeks, accompanied by concomitant 5-fluorouracil continuous 120-hour infusion at a dose of 1000mg/m2/day for 5 days during week 1 and 5. All patients underwent total mesorectal excision (TME) with either anterior resection or abdominoperineal resection 4-6 weeks following completion of treatment.

Tissue processing immunohistochemistry, image analysis and statistical analysis

Details as described in Materials and Methods – refer to chapter 2. Pre-treatment biopsies (PTB) and post-treatment resected specimens were retrieved from the pathology tissue banks of the two hospitals. Primary outcome measures were the difference in

global methylation and proliferative index between pre-treatment biopsy of the rectal tumours and post-treatment resected specimens. Secondary outcome measures were the relationship between difference in methylation and proliferative index (Ki-67) with patient's clinico-pathological data (age, sex, Dukes, TNM staging, lymph node involvement). Statistical analysis was performed using the Wilcoxon Signed Rank test for both methylation and Ki-67 values.

5.3 RESULTS

5.3.1 Patients

Fifty-three (n=53) patients in total with pre and post treatment tissues available were identified over the 4 year study period. All patients had received neoadjuvant chemoradiotherapy prior to surgical resection of their rectal tumours over the 4 year study period from 2004 to 2008. Thirty-seven (70%) patients were male and 16 (30%) were female. The median age of the patients were 67 years (range :41-82 years). Seven (n=7, 13%) patients were complete pathological responders with no residual tumour cells left in the resection specimen. The remaining 46 (87%) patients were partial pathological responders displaying varying degrees of residual tumours cells. The pathological data of the 53 included patients are displayed in Table 5.1.

Table 5.1 Patient clinico-pathological details CPR: Complete pathological response; NS: non-significant

N=53 Median age 67 yrs (41-82 yrs) Male: n= 37 (70%) Female: n=16 (30%)	Reduced methylation post-treatment	Increased methylation post-treatment	p-value
Differentiation: n=53	Well: 1 (2%) Moderate: 33 (63%) Poor: 4 (8%)	1 (2%) 13 (25%) 1 (2%)	NS
Dukes stage: n= 46	A: 3 (6%) B: 16 (30%) C: 12 (23%) ***CPR: 7 (13%)***	1 (2%) 6 (11%) 8 (15%) 0 (0%)	NS
T-staging: n= 46	T1: 0 (0%) T2: 10 (19%) T3: 20 (38%) T4: 1 (2%)	1 (2%) 5 (9%) 6 (11%) 3 (6%)	NS
Lymph node	Positive: 13 (25%) Negative: 25 (47%)	8 (15%) 7 (13%)	NS
Pathological response: n=53	Partial: 31 (58%) Complete: n=7 (13%)	15 (28%) 0 (0%)	NS

5.3.2 Global methylation in pre and post neoadjuvant treatment tumour tissue

A comparison of global methylation intensity was made on the 46 (87%) partial pathological responders. The mean global methylation intensity of pre-treatment biopsies was significantly different compared to the post-treatment resection specimens 152.7 vs 171.5 ($p<0.001$) (A zero methylation index indicates strongest staining; a 255 methylation index indicates the weakest methylation staining – Chapter 2, Materials & Methods). Thirty eight (n=38, 72%) tumours showed a decrease in methylation compared to the pre-treatment biopsy; the remaining fifteen (n=15, 28%) tumours showed an increase in methylation compared to the pre-treatment tumour biopsy. (Figure 5.1; Table 5.1 and 5.2)

5.3.3 Proliferative index in pre and post neoadjuvant treatment tumour tissue

The proliferative index (Ki-67) of the pre-treatment biopsies was significantly different compared to the post-treatment resection specimens, 48.6% vs 27.5% respectively ($p=0.01$). The mean percentage decrease in Ki-67 positivity was 43.5%. (Table 5.2; Figure 5.2)

5.3.4 Correlation with clinico-pathological parameters

The percentage difference in global methylation and proliferative index (Ki-67) between pre-treatment biopsy and post- treatment resected specimens did not correlate with

patients' clinico-pathological parameters including age, sex, Dukes or T-staging, lymph node involvement. Analysis of the percentage difference in methylation within the group of patients with increased methylation in the post-treatment specimen also identified no correlation with patients' clinico-pathological data.

5.3.5 Methylation and proliferative index

No association was identified between methylation levels and proliferative index (Ki-67) in either the pre-treatment or the post-treatment tissues in this cohort of patents with locally advanced rectal tumours receiving neoadjuvant chemoradiotherapy.

Table 5.2 Methylation index and proliferation index (Ki-67) positivity in pre and post treatment tumour tissue. *(methylation intensity: 0= highest; 255=lowest)*

	Pre-treatment	Post-treatment	P value
Methylation index	152.7	171.5	<0.001
Ki-67 positivity (%)	48.6	27.5	0.01

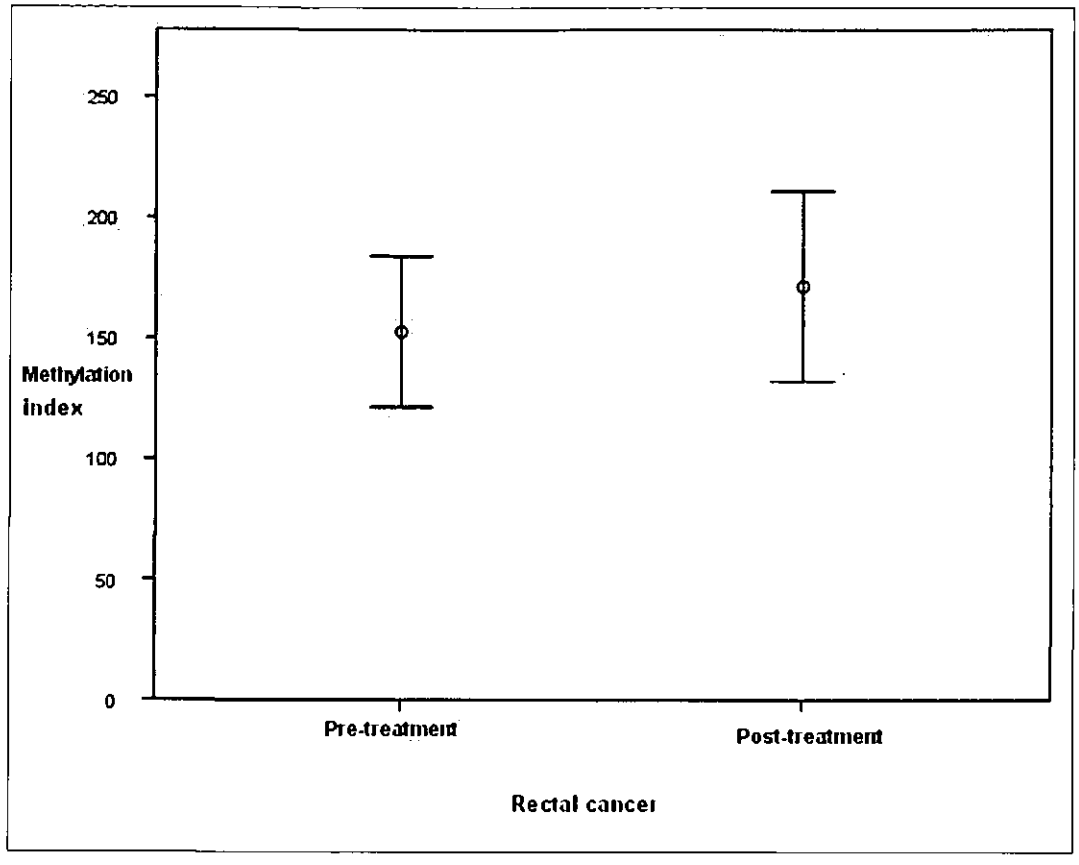


Figure 5.1 Significant reduction in methylation in post–treatment specimens ($p<0.001$, Wilcoxon signed rank test).

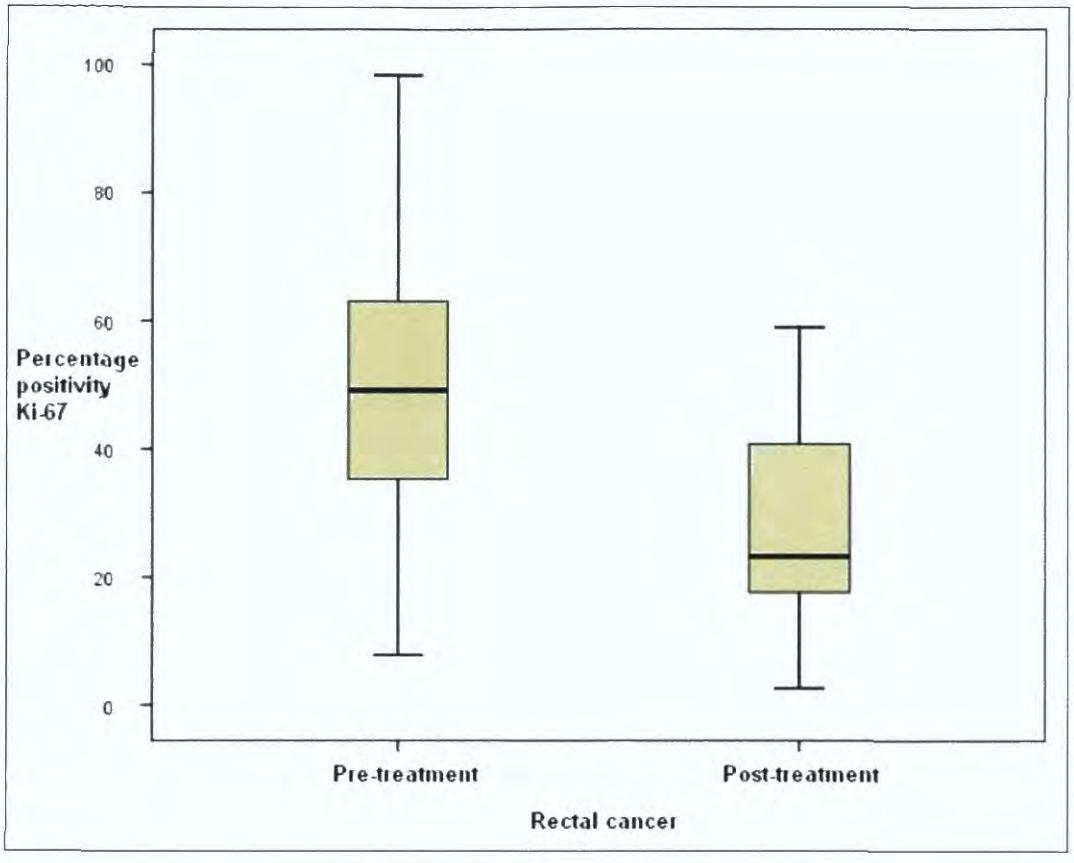


Figure 5.2 **Box plot graph showing significant reduction in proliferative index (Ki-67) positivity post chemoradiotherapy (p=0.01).**

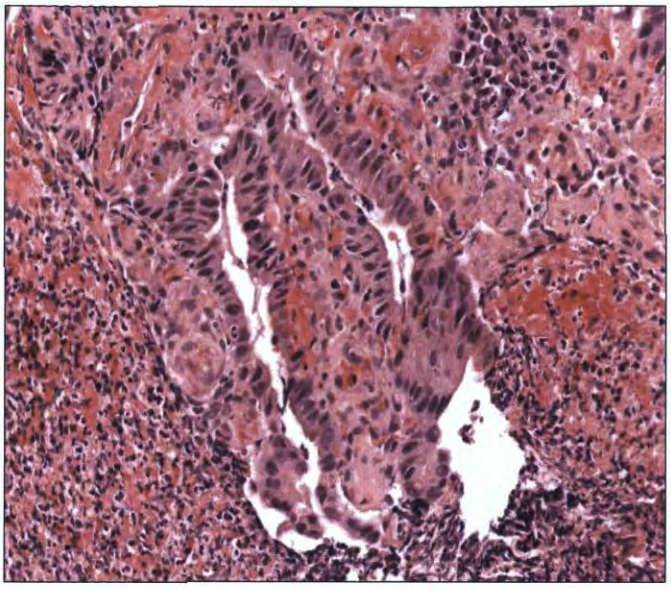
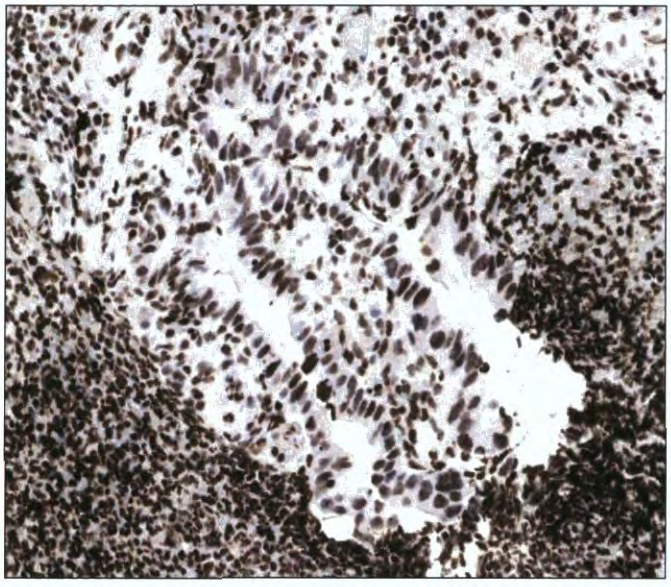
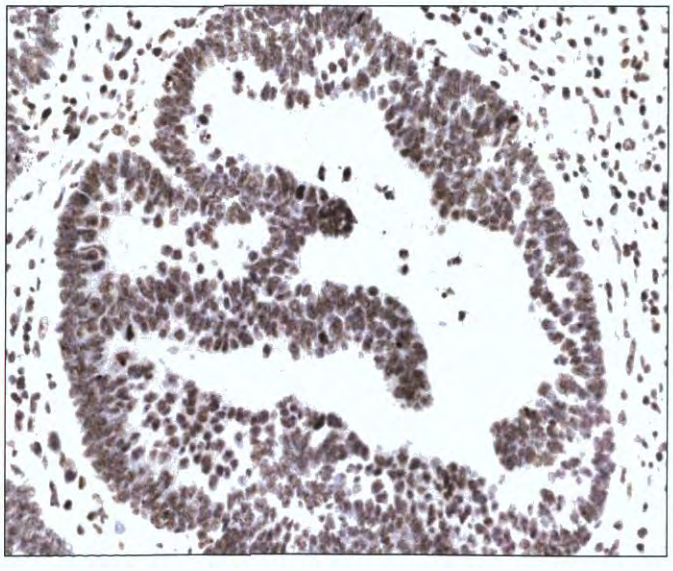


Figure 5.3 Diagrams showing reduction in methylation in post-treatment resection (Patient 1) (x20)

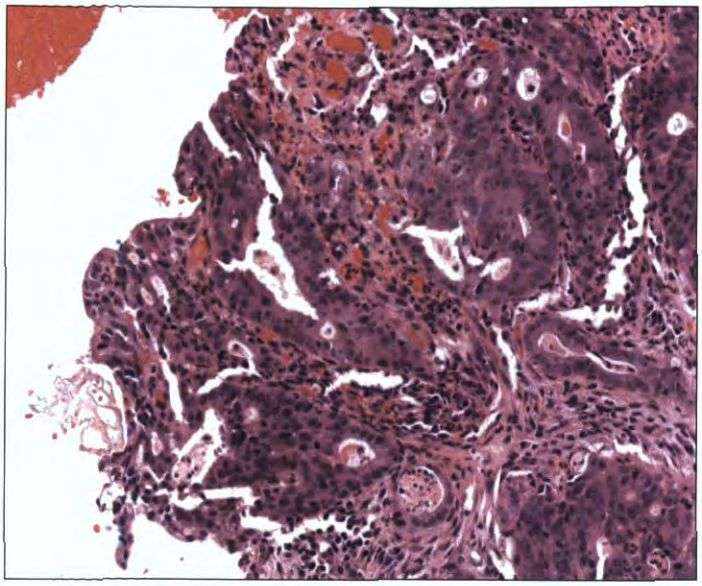
Pre-treatment H&E



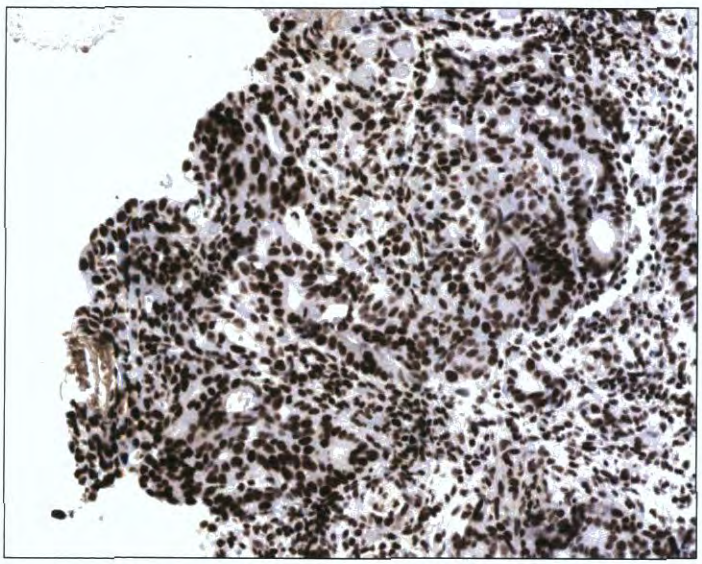
Pre-treatment methylation



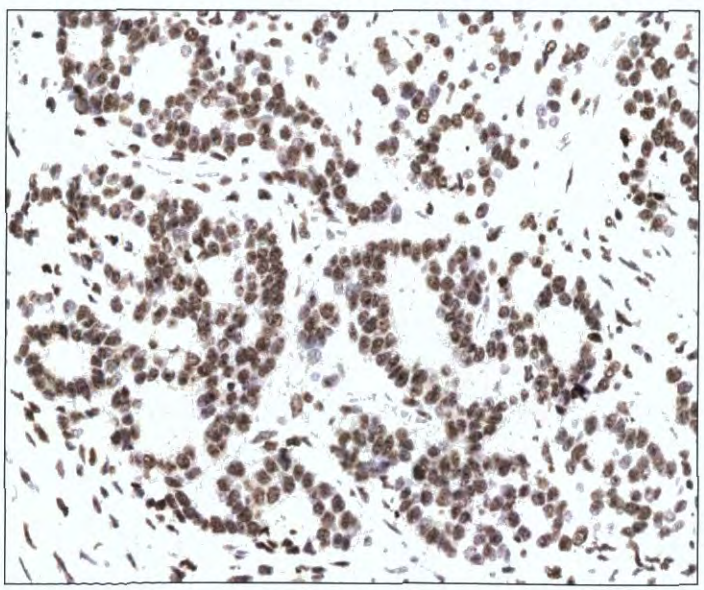
Post-treatment methylation



Pre-treatment H&E



Pre-treatment methylation



Post-treatment methylation

Figure 5.4 Diagrams showing reduction in methylation in post-treatment resection (patient 2) (x20)

5.4 DISCUSSION

The management of locally advanced rectal tumours is particularly challenging. One of most important indicators of treatment success is local recurrence. This is characterised by recurrence of the tumour at the anastomotic site and in the pelvis, associated with intractable pain and morbidity. Five year survival rate is only 5% with recurrent rectal cancer[60]. One crucial determinant of local recurrence is surgical technique. Total mesorectal excision (TME) is now widely accepted as the surgical technique of choice for resection of locally advanced rectal tumours. Local recurrence rates of <10% can be achieved with precise surgery alone[66]. Neoadjuvant radiotherapy targeted at the primary tumour, with or without chemotherapy can further reduce local recurrence in combination with TME. Neoadjuvant chemoradiotherapy followed by rectal resection involving total mesorectal excision is now the standard of care in patients with locally advanced rectal tumours.

Although neoadjuvant chemoradiotherapy can significantly reduce local recurrence rates, not all patients benefit from treatment. Only about 10% of patients achieve a complete pathological response characterised by a complete sterilisation of tumour cells in the resection specimen. The remaining patients display varying degrees of residual tumour with some showing very little response. Treatment is also costly, associated with morbidity and can delay definitive surgical resection[191]. It is not surprising therefore that research in this area has focussed on the molecular basis of tumour behaviour to chemoradiotherapy. An improved understanding could spare patients from unnecessary

pre-operative treatment and can help direct a more patient tailored treatment regime. To date, research into the molecular aspects of rectal tumour behaviour has been relatively unsuccessful and currently the decision for chemoradiotherapy is still based on pre-operative imaging[45].

Recently, the epigenetic pathway involving DNA methylation is being recognised to play a major role in colorectal carcinogenesis. This heritable modification of gene expression has been shown to lead to gene suppression. Gene-specific hypermethylation and global hypomethylation have been observed in a wide variety of tumours including rectal tumours[172]. Genomic instability and tumour suppressor gene silencing as a result of DNA methylation is now thought to contribute to cancer initiation and progression at an early stage in colorectal tumours[192].

In this study, we evaluated the effect of neoadjuvant chemoradiotherapy on global methylation content in rectal tumours using quantitative image analysis. We observed a definite change in the methylation content following chemoradiotherapy, showing a statistically significant reduction in DNA methylation in the post-treatment resection specimens in 72% of patients and increase in DNA methylation in 28% of patients ($p<0.001$).

This is the first study carried out to show the effect of chemoradiotherapy on DNA methylation in any human malignancy, exemplified in this study by examining rectal cancer. Previous studies on the radiation effect of DNA methylation were based on the

deleterious effect on normal animal tissue. Tawa et al examined the effect of ionising radiation on genomic DNA methylation of normal organs in mice. Forty percent reduction in methylation content was observed within 8 hours of irradiation in mice liver[193]. Pogribny et al also observed a reduction in methylation following irradiation in mice. They also noticed that this effect was both sex and tissue specific[194].

Several mechanisms have been proposed to explain the reduced methylation content based on these studies on normal animal tissue. Radiation is a potent DNA damaging agent and this property is used as the basis for targeting tumour cells. It leads to DNA strand breaks, deletions, insertions and crosslinks. These lesions may potentially give rise to changes in DNA methylation directly or indirectly by interfering with processes and enzymes involved. One possible mechanism of hypomethylation after treatment is the shift in DNA methyltransferases from the nucleus of tumour cells into the cytoplasm, disrupting further methylation[195]. Another possibility is the activation of demethylating enzymes by ionising radiation, leading to the active removal of methyl groups from promoter regions[196]. Pogribny postulated that DNA repair mechanisms may play a role in causing hypomethylation[194]. They observed a strong negative correlation between DNA strand breaks and DNA hypomethylation 6 hours following DNA hypomethylation. Since DNA strand breaks are associated with DNA repair processes, which consist of the polymerase catalysed synthesis of DNA with cytosine and not methylcytosine, DNA hypomethylation observed may be DNA repair-related. Radiation exposure has also been noted to cause reduced expression of DNA methyltransferases (DNMT) including DNMT1, DNMT 3a and 3b as well as methyl-binding proteins such as MeCP2. Both

DNA methyltransferases and methyl-binding proteins are important machinery and transcriptional repressor proteins involved in epigenetic silencing through DNA methylation[197]. Diminished levels of these proteins may explain the hypomethylation observed in tumours treated with chemoradiotherapy in this present study.

We also observed a significant decrease in Ki-67 positivity compared to the pre-treatment biopsies. Ki-67 is a nuclear protein responsible for cell cycle regulation. It is present in all phases of the cell cycle except in the resting G0 phase. Therefore it is a good indicator of cellular proliferation. Our observation is consistent with previous studies. Using tissue micro-arrays, Debucquoy et al showed a significant reduction in Ki-67 expression after pre-operative therapy in 99 patients ($p<0.0001$) [198]. Bertolini et al also observed a significant reduction in Ki-67 expression after chemoradiation ($p<0.0001$) and a high Ki-67 value after treatment was an independent poor prognostic factor for overall survival [199]. These observations on Ki-67 indicate that the proliferating rectal tumours cells are most sensitive to the effect of neoadjuvant chemoradiotherapy.

Although no previous studies have evaluated or identified an increase in methylation post irradiation in animal or human tissue, from the available evidence discussed above on animal tissue, this finding in 28% of this study's patients is perhaps an unexpected event. Sub-group analysis of these patients did not find any correlation with clinico-pathological data and difference was also not identified comparing with patients showing reduced methylation post chemoradiotherapy.

In the majority of this study's patients (72%), it seems contradictory that a global hypomethylation pattern is observed after neoadjuvant chemoradiotherapy, a strategy which is aimed at improving prognosis of rectal cancer patients. Hypomethylation is thought to predispose to genomic instability in tumours and increasing hypomethylation is observed in advancing stages of colorectal neoplasia. Further studies will have to be carried out to determine the exact significance of the methylation change, in particular the role this hypomethylation plays in affecting patient prognosis. Results of this initial study suggest that the epigenetic DNA methylation pathway may play a role in the behaviour of rectal tumours to chemoradiotherapy. The next sensible step would be to evaluate whether DNA methylation could be a predictor or prognostic factor of response to chemoradiotherapy. This forms the basis of the next experiment chapter.

In conclusion, neoadjuvant chemoradiotherapy does appear to alter the rectal cancer epigenome. Overall, a decrease in global methylation level is observed in the post-treatment resection tumour cells compared to pre-treatment tumour cells. Radiation induced DNA damage and subsequent DNA repair mechanisms as well as DNA methylation control processes may be responsible for the changes observed. Further studies will be required to determine the exact significance of the methylation changes observed in this study, in both decrease and increase in methylation, paying particular attention to their role in determining patient prognosis.

Chapter 6

Role of DNA methylation in predicting response to neoadjuvant chemoradiotherapy in rectal cancer

6.1 INTRODUCTION

Local recurrence is a major determinant of prognosis in rectal cancer patients. Once diagnosed, the median survival is 18 months. Neoadjuvant chemoradiotherapy followed by high quality precise surgery involving total mesorectal excision is now employed to prevent local recurrence. The benefit of this is clearly illustrated by the Dutch rectal cancer trial which showed a reduction of local recurrence rates from 8.4% to 2.4% at 2 years in patients receiving preoperative radiotherapy and total mesorectal excision as opposed to total mesorectal excision alone[72]. In addition, neoadjuvant treatment has also been shown to increase sphincter-saving procedures, patient survival[75] and downstage rectal tumours.

The significance of downstaging is emphasised by the 10% of cases which show a complete pathological response to neoadjuvant chemoradiotherapy, where no remaining tumours cells are identified in the post-treatment resection specimen. These complete responders have been shown to have an improved local recurrence and survival compared to those with partial response[77].

It would be clinically beneficial to predict accurately which patients respond to therapy since neoadjuvant treatment is associated with increased peri-operative morbidity, cost and could lead to delay in definite surgery in those who ultimately show no response[191]. Recent research has focussed on identifying molecular biomarkers in pre-treatment rectal cancer biopsies to predict response and prognosis. Although Bax[200], epidermal growth

factor receptor (EGFR),vascular endothelial growth factor (VEGF) and p21[199] have shown promising results, currently there is still no accurate predictor of response in clinical use.

Recently a novel molecular pathway involving epigenetic mechanisms has been recognised as an additional, distinctive pathway to colorectal carcinogenesis. Unlike the traditional chromosomal instability and microsatellite pathways which consist of direct mutational changes of the key regulatory genes, APC, K-Ras and p53, epigenetics involve regulatory changes that do not alter the DNA sequence. In normal mammalian development, this mechanism is responsible for suppressing one of the X chromosomes in females[90]. DNA methylation is the key epigenetic marker responsible for a large variety of tumours including colorectal cancers. The process involves an enzyme catalysed addition of a methyl (CH3) group to the promoter sequence of cancer regulatory genes such as APC, leading to "silencing" of the gene, gene expression is inhibited.

Since little has been gained in searching for a useful predictive marker from a genetic perspective, a shift towards evaluating epigenetics in rectal cancer may yield potential markers. The aim of this chapter was to determine the value of DNA methylation as a predictive marker of rectal tumour response to neoadjuvant chemoradiotherapy.

6.2 PATIENTS & METHODS

Patients

A total of 53 patients with locally advanced rectal tumours treated with neoadjuvant chemoradiotherapy were identified from the pathology database systems of Connolly Hospital and Beaumont Hospital. This study cohort was the same as the patient group used in Chapter 5, treatment regime received by these patients was as described in Chapter 5. Clinico-pathological details of the patients are shown in Table 5.1.

Tissue processing immunohistochemistry, image analysis and statistical analysis

Details as described in Materials and Methods – refer to chapter 2. The primary outcome measure was the relationship between pre-treatment methylation and proliferative index (Ki-67) and tumour regression grading. Secondary outcome measures were pre-treatment methylation and Ki-67 with Dukes' and T-staging, and patient's clinico-pathological data (age, sex, lymph node involvement). Analysis was performed using the Wilcoxon Signed Rank test for both methylation and Ki-67 values.

Tumour regression grading

H&E slides of all post-treatment resected specimens were reviewed by a single consultant pathologist for determination of regression grade. Mandard et al originally proposed a tumour response grading system (5-point) for oesophageal cancers after pre-operative chemoradiotherapy[201] (Figure 6.1). A similar grading system has since been applied to locally advanced rectal cancers. A modification to this original grading system was proposed to minimise subjectivity and to improve reproducibility[202]. The modified 3-point Rectal Cancer Regression Grade (RCRG) was devised by combining tumour regression grade 1 and 2 to form one single category – RCRG 1, and combining tumour regression grade 4 and 5 to form another single category = RCRG 3 (Table 6.1). This modified 3-point grading system was used in this study.

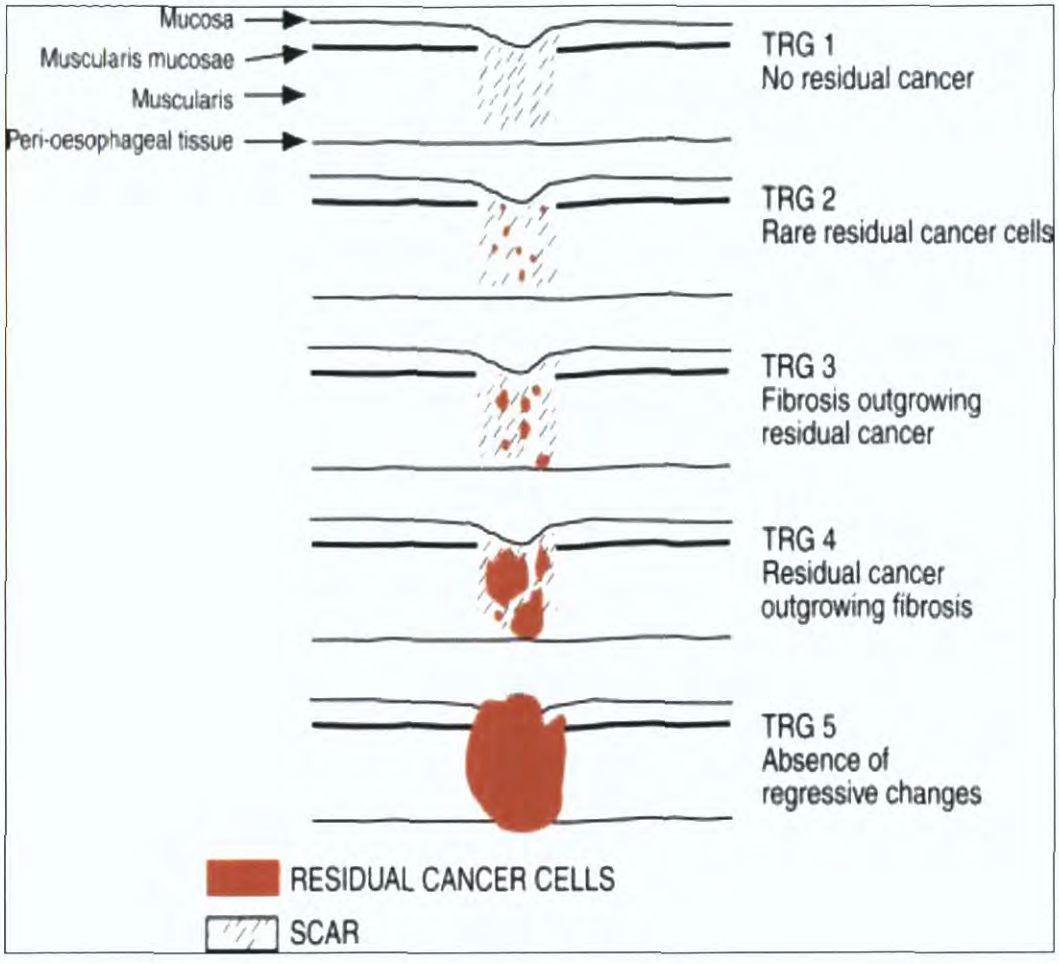


Figure 6.1 Mandard's tumour regression grading for oesophageal cancer

Table 6.1 Modified tumour regression grading system

Regression grade	Histological features
1	Sterilisation or only microscopic foci of adenocarcinoma remaining with marked fibrosis
2	Marked fibrosis but macroscopic disease present
3	Little or no fibrosis with abundant macroscopic disease

6.3 RESULTS

The clinico-pathological data of the 53 included patients are shown in the previous chapter in Table 5.1. Forty-six (n=46, 87%) patients had partial pathological response displaying varying degrees of residual tumours cells in the resected tumour. Seven (n=7, 13%) patients had complete pathological response (ypT0 N0 M0) to neoadjuvant chemoradiotherapy with no evidence of residual tumour cells left in the resection specimen.

6.3.1 Tumour regression grading

Using the modified 3-point Rectal Tumour Regression Grade (TRG), post-treatment residual tumours were graded by a consultant pathologist. Seven (n=7, 13%) tumours had a complete pathological response (ypT0 N0 M0) (TRG1). Forty-six patients (n=46, 87%) were partial pathological responders and of these 15 (28%) patients were Grade 1, characterised by only microscopic foci of tumour cells remaining (TRG1). Fifteen (28%) patients' response was Grade 2 and 16 (30%) patients were Grade 3. (Table 6.1 and 6.2)

6.3.2 Correlation of pre-treatment biopsy with tumour regression grade

Pre-treatment methylation was correlated with tumour regression grading of the cohort of rectal tumours studies. The average pre-treatment tumour biopsy methylation of all 53 patients was 152.7 (range: 104.4-220.7). A significant correlation was found between

pre-treatment tumour global methylation and tumour regression grading of the rectal cancers ($p<0.001$). No correlation was found between Ki-67 expression and tumour regression grading. (Table and Figure 6.2)

Table 6.2 Tumour regression grades (TRG) and pre-treatment methylation index.

CPR: complete pathological response; Methylation index: 0= highest methylation; 255=lowest methylation

	Number (n=53)	Methylation (mean)	P value (Wilcoxon signed rank test)
CPR (TRG1)	7 (13%)	190.5	<0.001
TRG1	15 (28%)	176.9	
TRG2	15 (28%)	156	
TRG3	16 (30%)	127	

6.3.3 Pre-treatment biopsy in complete and partial pathological responders

A comparison was made between the pre-treatment biopsy methylation and proliferative index of partial and complete responders to neoadjuvant treatment. There was a significant difference between pre-treatment methylation of complete pathological responders and partial responders, 190.5 vs 152.7 ($p=0.01$). There was no association identified with proliferative index (Ki-67) and pathological response. (Figure 6.3)

6.3.4 Pre-treatment biopsy and patient clinico-pathological parameters

Pre-treatment methylation did not correlate with patient's age, sex, lymph node involvement, differentiation or survival. There was a significant correlation between higher levels of PTB methylation and advancing T-staging ($p=0.005$). (*Figure 6.4*) Proliferative index (Ki-67) was found to have no correlation with patient clinico-pathological parameters.

6.3.5 Pre-treatment biopsy and patient survival

The mean follow-up of all 53 patients was 25 months. Two (4%) patients were lost to follow-up. Eleven (n=11; 21%) patients were deceased and the remaining 40 (75%) patients were alive at their last follow-up. Pre-treatment methylation and proliferative index (Ki-67) were correlated with patient survival, no association was identified with pre-treatment biopsies and individual patient survival.

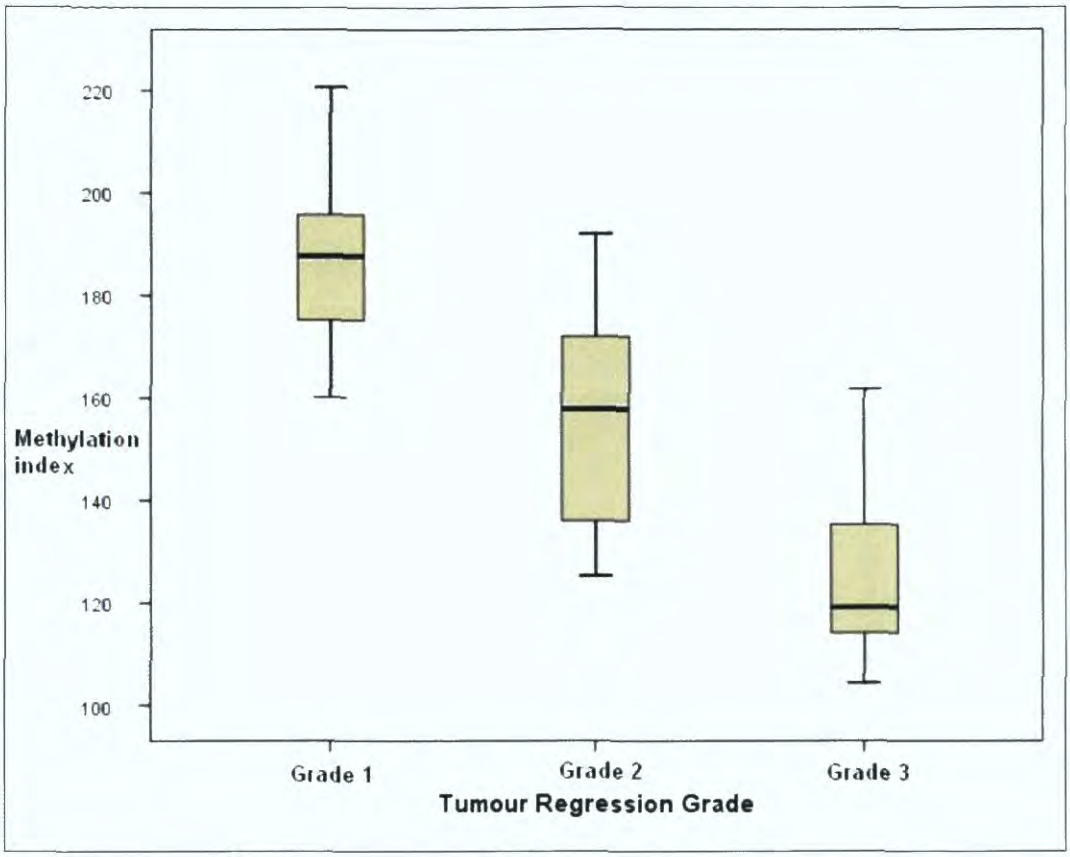


Figure 6.2 Box plot showing correlation of pre-treatment methylation with tumour regression grading ($p<0.001$)

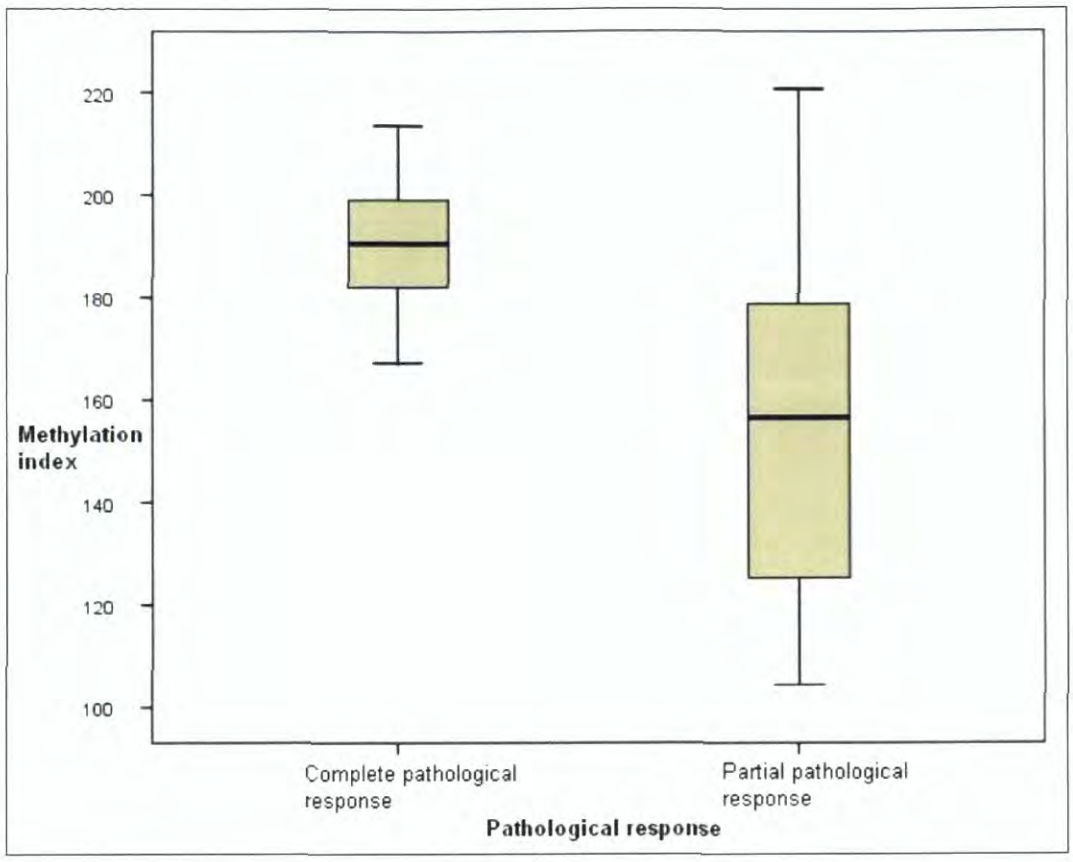


Figure 6.3 **Box plot graph showing significant difference in pre-treatment methylation between partial and complete pathological responders (p=0.01)**

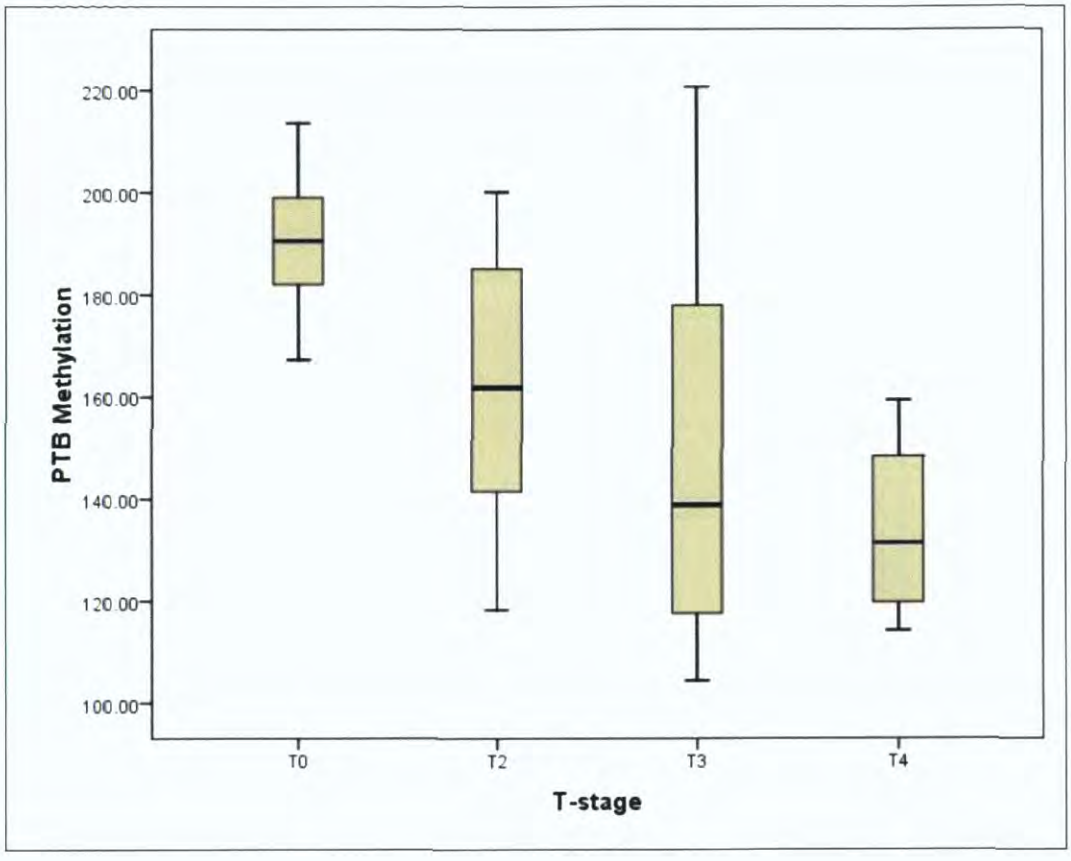


Figure 6.4 **Box plot graph showing significant correlation with high pre-treatment methylation and advanced T-stages (p=0.03)**

6.4 DISCUSSION

Rectal cancer treatment remains one of the most challenging aspects of colorectal cancer management. Local recurrence leads to significant morbidity and is associated with a very poor survival. Surgical resection continues to be the mainstay of treatment and this is highlighted by the widespread adoption of total mesorectal excision (TME) which emphasises the importance of precise surgical dissection along the mesorectum aimed at removing all lymphatic channels around the tumour[62]. This technique has been shown to significantly reduce local recurrence rates in rectal cancer[63]. The addition into the treatment regime of chemoradiotherapy prior to TME has been shown to further reduce disease recurrence and improve survival. Neoadjuvant treatment provides improved local recurrence rates, less toxicity compared to post-operative treatment[4, 71, 72]. Neoadjuvant chemoradiotherapy combined with total mesorectal excision is now considered to standard of care in patients with locally advanced rectal cancer.

About 40%-60% of patients with locally advanced rectal cancer respond to varying degrees to neoadjuvant chemoradiotherapy[203]. A complete pathological response, characterised by a complete absence of rectal tumour cells in the post-treatment specimen is observed in approximately 10% of cases. The significance of response lies in the fact that responders have been found to benefit from an improved survival, lower recurrence rates and more likely to have sphincter saving procedures[204-206]. The current challenge for colorectal surgeons and radiation oncologists is to identify those patients who will respond and benefit from neoadjuvant treatment. Not only can the course of treatment be

time-consuming and may lead to delay in definitive surgery in eventual non-responders, treatment is also associated with significant adverse effects and tremendous cost[207]. Traditional histopathological markers have been disappointing in predicting response. Research into genetic biomarkers is the appropriate next step focussing on potential proteins involved into main genetic pathways likely to be affected by chemoradiotherapy. Numerous studies have evaluated different molecular markers to date, however currently there is still no effective and accurate predictor of response in clinical use.

Epigenetics is an area that is receiving intense attention recently for its contribution to multiple malignancies. Epigenetic mechanisms are described as heritable changes in gene expression without any alteration to the DNA sequence. The most studied and well understood epigenetic marker is DNA methylation. This process takes place at promoter regions of key regulatory genes in cancer formation, most notable tumour suppressor genes, leading to silencing of these genes. In colorectal cancer, both gene-specific hypermethylation and global hypomethylation have been observed and both events are now thought to play a major role in colorectal cancer initiation and progression[192].

Interesting results were observed from the last chapter, when a significant difference was observed in global methylation levels of rectal tumours post chemoradiotherapy. This indicates that DNA methylation may be involved in rectal tumours' behaviour to chemoradiotherapy. This present study was carried out to further investigate the role DNA methylation plays in the chemoradio-responsiveness of rectal tumours. We

evaluated whether DNA methylation in pre-treatment biopsies of locally advanced rectal tumours were able to predict their response to neoadjuvant treatment.

The most interesting finding from this study was that there was a significant correlation between pre-treatment DNA methylation and the degree of remaining post-treatment residual tumour cells indicated by both tumour regression grading ($p<0.001$) and T-staging ($P=0.005$). The lower the methylation, the more likely it was to achieve a complete pathological response in the patients. In addition, pre-treatment methylation level was able to predict the likelihood of complete pathological response and partial pathological response ($p=0.01$) A low methylation was associated with complete pathological responders.

No previous studies have evaluated the role of global DNA methylation in predicting response to neoadjuvant chemoradiotherapy in rectal cancer. In one study involving oesophageal cancer, promoter methylation in a 9-gene panel was examined using methylation specific polymerase chain reaction (MS-PCR) in 35 pre-treatment biopsies. The number of methylation genes per patient was found to be significantly lower in responders compared to non-responders ($p=0.026$)[208]. This is in accordance with this study's findings.

No correlation was found between pre-treatment Ki-67 expression and tumour response in this study. Several previous studies on Ki-67 predictive value have yielded similar results. The two studies with positive findings were also contradictory. Kim et al

[209]showed a high Ki-67 expression was associated with better response in a group of 23 patients (p=0.029) while Jakob et al found a low expression was associated with responders in a study with 22 patients ($p<0.01$)[210]. From current available evidence, it appears that Ki-67 has little value in predicting response to neoadjuvant treatment in rectal cancer. Multiple studies have evaluated potential molecular biomarkers in predicting response and outcome. Although still no biomarker at present has shown definitive usefulness to be successfully incorporated into treatment protocols, recent studies on p21 (tumour suppressor gene activated in response to DNA damage) [211], epidermal growth factor receptor (regulation of cell proliferation, apoptosis and differentiation) [212], thymidylate synthase (DNA synthesis) [213]and vascular endothelial growth factor (pro-angiogenic protein) [214]have shown promising results which warrant further investigation.

In conclusion, neoadjuvant chemoradiotherapy in locally advanced rectal cancer can help improve patient prognosis. Moreover, responders to treatment have better outcomes than those who do not respond. Identification of biomarkers for response prediction can greatly improve patient selection and tailor treatment individually. No genetic marker at present can accurately predict response. This study shows a significant correlation between pre-treatment DNA methylation with tumour response and T-staging. This suggests that pre-treatment DNA methylation may have the poteutial to become a useful biomarker of response. Future larger prospective studies using perhaps more sensitive detective methods of gene methylation such as methylation specific polymerase chain reaction should be carried to further assess this potential response predictor.

CHAPTER 7

DISCUSSION

7.1 DISCUSSION

Colorectal cancer is one of the commonest cancers world-wide. It is the second most common cancer in Ireland behind lung cancer and the incidence is set to increase over the next few decades as a result of an increase in the aging population. Over the last decade, we have seen developments and advances in all aspects of colorectal cancer management including imaging, screening, increased understanding of cancer spread in rectal cancer, neoadjuvant and adjuvant treatments. However, surgical resection remains the treatment modality of choice.

The pathogenesis of colorectal cancer is well described. The adenoma-carcinoma sequence has been well studied and documented to be the characteristic pathway of colorectal cancer formation. Normal mucosa undergoes epithelial proliferation initially. Few proliferating cells then undergo clonal expansion to form a benign adenoma. Increases in dysplasia, size and aggressiveness then follows upto the stage when the adenoma acquires all the characteristics of a carcinoma (malignant tumour) termed a carcinoma- in-situ. When this tumour invades through the basement membrane, it then has the freedom to spread through the submucosa, muscle layers, lymph nodes and eventually to distant organs – invasive adenocarcinoma. This well-defined pathway of histo-pathological stages of progression is accompanied by a step-wise accumulation of mutational events that involve several key regulatory genes in colorectal cancer. The adenomatous polyposis coli (APC), K-Ras and p53 genes[2] have been shown to be involved in the initiation and progression of colorectal carcinogenesis. Seventy to eighty

percent of all colorectal cancers develop via this chromosomal instability pathway. The remaining 10% - 15% of colorectal cancers develop via the microsatellite instability pathway characterised by germ-line mutations of DNA mismatch repair genes leading to impaired DNA repair. In the autosomal dominant condition hereditary non- polyposis colon cancers (HNPCC), patients develop cancers at an earlier age, more right-sided tumours and have better prognosis, is a result of this germ-line mutation.

From a molecular view-point, these well-defined stages and identifiable genetic mutations along the adenoma-carcinoma sequence represent ideal targets to be used for diagnosing colorectal cancer at its earliest stages. The ability to identify these genetic mutations in asymptomatic patients with high sensitivity and specificity should in theory be a feasible method for screening. However, research into molecular events as diagnostic tools have yielded little promising results and currently clinical tests such as faecal occult blood and colonoscopy remain the commonest screening tools in some countries, while in other countries, still no screening program is in place. Moreover, there is also a limited role for genetic mutations as prognostic indicators in colorectal cancer. Pathological indices in the resected cancer specimen including Dukes' and TNM staging, lymphovascular invasion and lymph node status remain the most important indicators of prognosis[181]. Research therefore continues to search for new and more accurate predictors of response to treatment as well as prognosis.

Recently, a novel molecular pathway that involves epigenetic mechanisms has been described in cancer formation and it is receiving intense attention as oncogenic changes

in this pathway appear to occur as frequent as genetic events [91]. Epigenetics refer to heritable changes in gene expression that do not involve a modification in the DNA sequence [89]. Multiple epigenetic mechanisms have been described including histone modifications, modification to the chromatin structure and chromatin factors as well as the best understood DNA methylation [98]. Epigenetic mechanisms are physiologically active, responsible for regulation of gene expression such as the suppression of the X chromosome in females[90].

DNA methylation is an epigenetic mechanism that leads to individual gene silencing[6]. The resultant effect is inhibition of gene expression. The process involves an enzyme catalysed addition of a CH3 methyl group to the promoter regions of genes. This addition involves a covalent bond to a cytosine base that is immediately adjacent to a guanine base only – termed CpG islands[114]. Following methylation, gene silencing is thought to occur as a result of direct inhibition of attachment of transcriptional factors; and also due to the recruitment of repressor proteins such as histone deacetylases[102]. Deacetylation of histones lead to conformational changes in chromatin structure which prevent access for transcriptional enzymes[103].

In carcinogenesis, aberrant methylation changes are now widely observed in all types of malignancies[112]. In colorectal cancer particularly, aberrant DNA methylation is now thought to play a substantial role in neoplastic transformation and progression[192]. Discovery of DNA methylation and the role it plays in colorectal cancer as well as other malignancies has sparked off a cascade of research studies. These have focussed on a

wide spectrum of areas including a search for individual genes that are methylated in a given tumour; evaluation of gene methylation at a global level; potential of DNA methylation as a screening tool in colorectal cancer; prognostic value of DNA methylation as well as therapeutic roles in preventing or treating colorectal cancer. Although numerous studies have been carried out on all these aspects, understanding of DNA methylation in colorectal cancer is still in its early stages. Much larger and more investigations are needed to explore fully the significance and potential of DNA methylation, especially in its prognostic role in colorectal cancer formation.

This thesis was carried out to further evaluate the role DNA methylation plays in colorectal carcinogenesis on top of current available evidence. Particular attention was paid to the clinical significance of this epigenetic mechanism in colorectal cancer including its potential as a biomarker and prognostic indicator of the disease and also as a predictor of response to treatment.

In chapter 3, global DNA methylation was examined in a group of normal colorectal mucosa, adenomatous polyps of different dysplastic stages and invasive adenocarcinomas. These tissues represent lesions along the adenoma-carcinoma sequence and the aim of the study was to evaluate the correlation between methylation and Ki-67, and dysplasia of adenomatous polyps as well as clinico-pathological features of the polyps. No correlation was observed between adenomatous polyps' methylation and dysplasia. There was a significant reduction in 5-methylcytosine content between normal mucosa and mildly dysplastic adenomas ($p<0.001$). In a previous similar study of

pre-cursor lesions in colorectal cancer, no difference in hypomethylation was observed across the adenoma-carcinoma. Consistent with findings from chapter 3, a significant hypomethylation was found between normal tissue and all proliferative lesions. Most other studies have examined methylation at a gene-specific level[170, 173, 174]. Overall, a step-wise increase in methylated genes was found as normal mucosa progress through advancing stages of dysplasia to cancer. From these findings, it appears that methylation changes do occur at early stages of colorectal cancer development with hypomethylation possibly involved with initiation and hypermethylation of individual genes involved in driving tumour progression. Importantly, the presence of identifiable methylation changes at early stages of neoplasia, especially gene-specific methylation, makes DNA methylation an attractive marker as a screening tool.

Following the evaluation of DNA methylation as a diagnostic and screening tool in chapter 3, the next step was to investigate its role as a prognostic marker in colorectal cancer. The aim of chapter 4 was firstly, to examine the methylation pattern in cancer tissue compared to paired normal tissue in resected specimens and secondly, to determine whether cancer methylation is associated with survival of these patients, and therefore has the potential to act as a prognostic indicator.

Consistent with findings from chapter 3, cancer methylation was significantly lower compared to normal mucosa ($p<0.001$). This is consistent with previous studies on multiple malignancies[115, 215]. A small sub-group of patients showed hypermethylation compared to their paired normal tissue. This was an unexpected finding given the

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available evidence widely documenting hypomethylation in cancer. Further analysis is warranted to evaluate the clinical significance of these patients. Global hypomethylation appears to act independently of geue-specific hypermethylation in driving neoplasia however both processes certainly contribute to the neoplastic process from very early stages[172, 190]. In addition, both methylation processes are now thought to accompany, at least equally, genetic mutational changes in caner formation. Cancer methylation levels and change in methylation levels were then correlated with clinico-pathological parameters and survival of the patient cohort examined. No correlation was observed between methylation in cancer and survival of patients.

The evidence on global hypomethylation and survival is limited. No definite association has been seen although two studies have shown a trend towards poorer prognosis with hypomethylation[189, 190]. While current ongoing research still has to identify a useful and feasible prognostic marker using genetic mutations such as p53 and microsatellite instability, preliminary results on DNA methylation studies offer a new and exciting pathway to be targeted for future studies. The contribution of epigenetics in carcinogenesis can now be accepted as significant and the clinical implication of this pathway is potentially substantial and may lead to improved survival. Histopathological staging of tumours remain the most important indicator of outcome at present however, continuing studies into the role of epigenetics, especially DNA methylation must be carried out.

Rectal cancer can be regarded as a separate disease entity to colon cancer. Although pathological spread of rectal tumours within the bowel wall is still similar to all other colon cancers its distinct anatomical position within the pelvis, makes rectal tumours invasion into surrounding viscera and lymphatics much more readily than colon cancers. Surgery remains the gold standard of treatment however surgical resection is also more technically demanding compared to routine colon cancer resection especially in a narrow, male pelvis. As a result, local recurrence is accordingly more common in rectal cancers. Local rectal cancer recurrence is characterised by recurrence of tumour at the site of anastomosis, the perineum and the pelvis. It results in severe morbidity with debilitating pelvic pain, ureteric obstruction, fistulae formation and poor bowel and urinary function[60]. Once diagnosed, survival is poor and treatment is mostly palliative[60].

Another determinant of prognosis is rectal cancer patients is the importance of the circumferential resection margin. A positive circumferential resection margin, defined as tumour within 1mm of the edge of resected specimen, is associated with a 12 fold increase in local recurrence rates compared to a negative margin[57]. Two major developments in rectal cancer treatment have occurred in the last decade and have had huge impact on both reducing the involvement of the circumferential resection margin as well as local recurrence rates.

Total mesorectal excision described by RJ Heald, emphasises the importance of sharp dissection to completely excise the mesorectum which contains all potential lymphatic spread of the rectal tumour[216]. Local recurrence rates reduced from over 20%-30% to

<10% with the introduction of total mesorectal excision[63]. Neoadjuvant chemoradiotherapy was introduced into the treatment regime of locally advanced rectal tumours to further reduce circumferential resection margin involvement and local recurrence rates. The combination of neoadjuvant chemoradiotherapy and total mesorectal excision is now the first line treatment in advanced stage T3/ T4 rectal tumours. Local recurrence rates are reduced from over 30% to around 10% with the use of pre-operative radiotherapy[71]; following introduction of total mesorectal excisiou, local recurrence rates are further reduced to around 2% at 2 years[72]. In addition to improving local recurrence rates, added benefits of neoadjuvant treatment include down-staging of the primary tumour and improved survival[217].

Not only can the down-staging effect of neoadjuvant chemoradiotherapy make a previously non-resectable tumour resectable, pathological response to treatment has been shown to be a predictor of prognosis[205]. Complete pathological response, is the best surrogate marker of excellent prognosis[218]. Response rates range from 40%-60% in patients with locally advanced rectal cancer showing varying degrees to neoadjuvant chemoradiotherapy[203]. However, a complete pathological response in only seen in about 10%-20% of cases. Current research focuses on identifying predictive markers that could predict rectal tumour response to treatment. Such markers would enable us to draw out a patient-tailored treatment plan, sparing patients who are unlikely to respond to chemoradiotherapy and be prescribed alternative adjunctive treatment instead or to undergo surgery directly[203].

In chapters 5 and 6, the role of DNA methylation in locally advanced rectal tumours requiring neoadjuvant chemoradiotherapy was examined. A significant change in global DNA methylation was observed in post-treatment resected specimens compared to pre-treatment biopsies. The majority, 72% of patients showed a reduction in global methylation, and 28% showed an increase in methylation post neoadjuvant treatment ($p<0.001$). This is the first piece of evidence available on human neoplasms and in rectal tumours specifically. Published studies on animal normal tissue suggest mechanisms such as radiation induced transfer of DNA methyltransferases into the cytoplasm, activation of demethylating enzymes and processes involved in DNA repair post irradiation may be responsible in an overall decrease in methylation. This observation suggests that DNA methylation may be involved in the behaviour of rectal tumours to chemoradiotherapy and could potentially prove to be a useful biomarker of response. More sensitive and in-depth analysis of the sub-group of patients showing increased methylation post- treatment is required to assess this change and its clinical significance. A significant decrease in Ki-67 positivity compared to the pre-treatment biopsies was also observed, a finding that is in accordance to previous studies[198]. Ki-67 is a reliable indicator of cellular proliferation and this observation indicates that the most proliferating cells in rectal tumours are most susceptible to the effect of chemoradiotherapy.

The next step in increasing our knowledge in DNA methylation with chemoradiotherapy was to evaluate the usefulness of DNA methylation in pre-treatment biopsies of this group of rectal tumours. We aimed to investigate whether methylation in pre-treatment biopsies could predict tumour response. Interestingly, there was a significant correlation

between pre-treatment DNA methylation and the degree of response to treatment indicated by both tumour regression grading ($p<0.001$) and T-staging ($P=0.005$). Low pre-treatment methylation was associated with better response to treatment. Furthermore pre-treatment methylation was able to predict the likelihood of complete pathological response and partial pathological response ($p=0.01$). Pre-treatment methylation was not able to predict survival in this group of rectal cancer patients.

This is also the first study to evaluate and show the ability of DNA methylation in predicting response to chemoradiotherapy. Promoter methylation in 9 genes was evaluated in oesophageal cancer previously, a low number of methylated genes was associated with better response to treatment[208]. Although a significant decrease in Ki-67 positivity was observed after chemoradiotherapy in chapter 5, it was not able to predict response to treatment. This finding is consistent with others and from current available evidence, Ki-67 has little value in predicting response to neoadjuvant treatment in rectal cancer[209,210].

No molecular marker at present is suitable to be used as a response predictor although p21[211], epidermal growth factor receptor[212], thymidylate synthase[213]and vascular endothelial growth factor[214] show some evidence that requires further investigation. Based on the findings we observed with the effect of chemoradiotherapy in the rectal cancer epigenome, DNA methylation appears to play a role in the behaviour of rectal cancers to treatment and may have the potential to act as a response predictor. This would allow identification of those patients likely to benefit from treatment and the

possibility of a complete pathological response with no residual tumour. This scenario is of particular interest at present since recent studies have found a favourable prognosis in patients receiving conservative, non-surgical management after chemoradiotherapy[80]. Patients unlikely to respond to neoadjuvant treatment may then be offered alternative treatment options and could also be spared from the potential adverse effects of radiotherapy[4].

In conclusion, DNA methylation is now clearly established as a distinctive and alternative molecular pathway contributing to colorectal carcinogenesis. The process is observed early on in the adenoma – carcinoma sequence and may be responsible for both initiation and progression of neoplasia. A genomic reduction in methylation is observed in colorectal cancer tissue compared to normal mucosa. In rectal cancer, neoadjuvant chemoradiotherapy appears to induce an overall hypomethylation status in the epigenome. Methylation status in pre-treatment biopsies appears to have the potential to act as a predictor of tumour response to chemoradiation. In particular, it may be able to predict the likelihood of complete pathological responders to treatment. Future studies need to focus on the prognostic ability of DNA methylation in colon and rectal cancers as well as its sensitivity as a predictor of response to neoadjuvant treatment in locally advanced rectal cancer.

7.2 Future directions

Encouraging results have been identified from the work carried out in this thesis. Two limitations of the studies performed are the retrospective nature and relatively low patient numbers available. Prospective studies involving larger patient numbers, ideally as multi-centre trials should be carried out to confirm the findings observed.

In this thesis, global methylation levels were studied throughout using immunohistochemistry. More sensitive techniques of methylation detection such as polymerase chain reaction (PCR) should be the next logical step in confirming the findings of methylation changes. As mentioned, global methylation is only one part of the DNA methylation phenomenon. Gene-specific promoter methylation constitutes the other half of the DNA methylation process and studies involving this, and possibly using PCR should be carried out to allow a more in-depth exploration into the screening role, specifically using patient faeces and serum in identifying patients with a high likelihood of colorectal cancer. In addition, the prognostic role of DNA methylation along the adenoma – carcinoma sequence as well as in rectal cancers to predict response to neoadjuvant treatment should also be examined using polymerase chain reaction.

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Appendix I - Figures

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Appendix II- Tables

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Appendix III - Abbreviations

ACF	Aberrant Crypt Foci
AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposis Coli
APR	Abdomino-perineal Resection
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CPR	Complete Pathological Response
CRC	Colorectal Cancer
CRM	Circumferential Resection Margin
DCBE	Double Contrast Barium Enema
DCC	Deleted in Colon Cancer
DNA	Deoxy-ribonucleic Acid
DNMT	DNA Methyl Transferase
EGFR	Endothelial Growth Factor Receptor
ERUS	Endo-Rectal Ultrasound
FAP	Familial Adenomatous Polyposis
5-FU	5-Fluorouracil
FOLFOX	5-FU, Leucovorin, Oxaloplatin
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase Inhibitor
hMLH1	Human MutL Homolog 1

hMSH2	Human MutS Homolog 2
HNPCC	Hereditary Non-Polyposis Colon Cancer
IHC	Immunohistochemistry
LOI	Loss Of Imprinting
MBD	Methyl- CpG Binding Domain
MDS	Myelodysplastic Syndrome
MINT	Methylated-In Tumour
MIS	Minimally Invasive Surgery
MMR	Mismatch Repair
MRI	Magnetic Resonance Imaging
MSI	Microsatellite Instability
MSS	Microsatellite Stable
PET	Positron Emission Tomography
SAHA	Suberoylanilide Hydroxamic Acid
SAM	S-Adenosyl Methionine
SAH	S-Adenosyl Homocysteine
TSG	Tumour Suppressor Gene
VEGF	Vascular Endothelial Growth Factor
UICC	Union Internationale Contra la Cancer

Appendix IV- Ethics approval

Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

Connolly Hospital
Blanchardstown
Dublin 15

Tel: (01) 821 3844
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CONNOLLY HOSPITAL BLANCHARDSTOWN

Certificate of Research Ethics Committee Approval

Date: 8th February 2008

To: Mr Eadhbhard Mulligan
Dr Julian Tsang

From: Dr Eamon Leen, Chairperson

Protocol title: Epigenetics in colorectal carcinogenesis

The Research Ethics Committee approved human subject involvement in your research project on 8th February 2008.
There is no expiration date for this approval, but the protocol must be reviewed by the Ethics Committee before December 31st 2011. If this project is to continue beyond that date, please submit an updated proposal one month prior to the expiration date. If this proposal is used in conjunction with any other human experimentation or if it is modified in any way, it must be re-approved for these special circumstances.
Note that the following should be reported to the Ethics Committee: 1) all serious adverse events, occurring at this institution, regardless of whether or not they are thought to be study related should be reported within 2 business days to one of the members of the Research Ethics Committee, 2) any unanticipated problems, and/or 3) and injuries to subjects enrolled.
Please remember that all data including all consent form documents must be returned for a minimum of three years past the completion of the research. Additional requirements may be imposed by your funding source, your department, or other entities. This institution protects personal health information of human subjects.

Dr Eamon Leen, Chairperson

Approval Period: 8th February 2008 – 31st December 2011.

Beaumont Hospital
Ethics (Medical Research) Committee

Chairperson: Professor Gerry McElvaney
Convenor: Professor Alice Stanton
Administrator: Gillian Vale

11th November 2008

REC reference: 08/93

Prof Elaine Kay
Consultant Histopathologist
Beaumont Hospital

RE: 08/93 – Prof. Elaine Kay - The DNA methylation in colorectal carcinogenesis

The above protocol was reviewed at a recent meeting of the Ethics (Medical Research) Committee, held on the 31st October 2008.

The Committee noted that approval for this archived tissue study is in place from James Connolly Memorial Hospital Research Ethics Committee. Beaumont Hospital Ethics (Medical Research) Committee confirmed that this study may proceed at Beaumont Hospital.

A formal approval document is attached.

With best regards

Yours sincerely

Professor Alice Stanton
Convenor
Ethics (Medical Research) Committee

c.c. Mr. Eadhbhard Mulligan, Consultant Surgeon, Connolly Hospital, Blanchardstown, Dublin 15
c.c. Mr. Julian Tsang, Research Registrar, Connolly Hospital, Blanchardstown, Dublin 15

Appendix V- **IP Declaration**