Molecular mechanisms in colon cancer
Hardwick, J.C.H.

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Chapter I

Introduction
Introduction

Colorectal cancer is the second commonest cause of cancer deaths in the United States and Europe, second only to lung cancer.¹ Western populations have a 1 in 20 lifetime risk of developing the disease and in many sections of society rates are increasing.² Despite major advances in our understanding of colon cancer, successful treatment remains dependent on early diagnosis and surgical intervention. Present oncological treatments such as radiotherapy and chemotherapy have had relatively little impact on the disease and currently hope is pinned on screening to find cancers and remove them even earlier. However, it is becoming apparent that screening can only reduce colorectal cancer deaths by 20-30% and that new approaches are needed before colorectal cancer can be added to the list of treatable malignancies.³

Understanding of the molecular workings of the cell is at present proceeding at an exponential rate. An understanding of the molecular mechanisms that underlie cancer has been a priority from the outset. Research into the molecular basis of colorectal cancer has been particularly fruitful and colorectal cancer has emerged as a paradigm for how tumours gradually evolve and acquire survival advantages over normal cells.⁴ The existence of inheritable forms of the disease and its slow development has been instrumental in this progress. It is hoped that through advances in the molecular understanding of colorectal cancer new treatments can be designed, treatments targeted at molecules central to the development of the cancer, so called “biological” treatments.⁵

Much of our current knowledge of colorectal cancer stems from genetic analysis of cancer tissue looking for areas of the genome where loss of heterozygocity is common coupled with knowledge gained from studying cancers in other organs. Thus Ras and p53 were among of the first molecules identified as being implicated in colorectal cancer progression.⁶ ¹⁷ However, there are a number of areas in colorectal cancer research where the initial insight was gained through the molecular investigation of clinical and epidemiological findings. For example, the study of families with an inherited predisposition to colorectal cancer has lead to the discovery of the APC gene.⁸ ⁹ There are many large publications providing exhaustive reviews of the current state of our molecular understanding of colorectal cancer.⁴ This is beyond the scope of this introduction. Instead I will concentrate on reviewing the fascinating link between colorectal cancer epidemiology and molecular biology, taking each of the main epidemiological associations in turn and outlining the current molecular explanation and how this has enhanced our understanding of colorectal cancer. This will
provide a background for the work described in the first half of this thesis, which has taken colorectal cancer epidemiology and molecular theories arising from it as a starting point.

**Epidemiology of colorectal cancer**

Risk factors for colorectal cancer include a family history of the disease, obesity, inflammatory bowel disease, meat consumption, smoking, and alcohol consumption. Inverse associations include vegetables, nonsteroidal anti-inflammatory drug (NSAID) use, hormone replacement therapy and physical activity. A subset of patients with a strong family history of the disease can be grouped on clinical grounds into a number of rare inherited syndromes, but well over 90% of all cases of colorectal cancer are so called sporadic cancers where both genes and environment are thought to play a causal role.

I. **Family history**

Despite their rarity the inherited colorectal cancer syndromes have provided many of the key molecular discoveries in colorectal cancer. Their genetic homogeneity has made it relatively easier to identify common underlying mutations and the resulting discoveries have often had remarkable relevance to sporadic colorectal cancers and even to cancers in other tissues.

**Familial Adenomatous Polyposis**

Familial Adenomatous Polyposis (FAP) is a rare dominantly inherited syndrome characterised by the development, sometimes from childhood, of multiple colorectal adenomas numbering from a few polyps to several thousand. The lifetime penetrance of this phenotype approaches 100% and affected patients go on to develop cancer in one or more of the polyps at a mean age of 44 years, approximately twenty years earlier than the mean age for development of colorectal cancer in the general population. There are a number of clinical variants, namely an attenuated form where many fewer polyps are found, Gardener’s syndrome, and Turcot syndrome.

The responsible gene, named the APC (Adenomatous Polyposis Coli) gene, was localised in 1987 to chromosome 5q, and was subsequently cloned and sequenced. Most of the mutations found in families lead to truncation of the APC protein and there seems to be a
correlation between the APC mutation sites and the phenotype e.g. attenuated FAP where the mutations are at the 5' end of the gene\textsuperscript{11} and the profuse Polyposis syndrome where the mutation occurs between 1285 and 1465.\textsuperscript{12} Nevertheless, the same mutation can also lead to considerable variation in phenotype even within the same family.

APC is a large protein whose main function seems to be as part of a signal transduction pathway, the Wnt/β-catenin pathway, that controls the transcription of genes involved in cell adhesion, migration and proliferation.\textsuperscript{13} The central molecule in this pathway is β-catenin. β-catenin plays a dual role in the cell as both a structural protein binding the cytoskeleton to the cell membrane, but also as a monomeric form in the cytoplasm from where it can move into the nucleus and activate transcription of target genes. These two pools of β-catenin are in equilibrium and the size of the monomeric pool determines the activity of the pathway. APC is a vital part of a large complex of proteins that collectively control the size of the free cytoplasmic β-catenin pool.\textsuperscript{14} Cells that lose functional APC exhibit overactivity of the Wnt/β-catenin pathway.\textsuperscript{15} This gives them a selective advantage possibly by reducing apoptosis. This is the likely first step in the formation of a colonic polyp and subsequently colorectal cancer.

Despite the fact that FAP is a rare syndrome accounting for less than 1% of all colorectal cancer cases, the responsible dysfunction of the APC protein and the resulting disruption of the Wnt/β-catenin signal transduction pathway has since been shown to be critical in the development of sporadic colorectal tumours as well. It is estimated that at least 80% of sporadic colorectal tumours have a somatic mutation of the APC gene.\textsuperscript{16} In chapter IV of this thesis we study the effects of nonsteroidal anti-inflammatory drugs on the Wnt/β-catenin pathway.
**Figure 1** A schematic representation of the major elements of the Wnt/β-catenin/APC pathway. β-catenin (β) occurs in two locations within the cell; at the cell membrane as a part of the cell cytoskeleton together with E-cadherin (E) and α-catenin (α) and free in the cytoplasm from where it can move into the nucleus and together with TCF, activate gene transcription. Free cytoplasmic β-catenin levels are tightly controlled by its phosphorylation and breakdown. For this to occur efficiently APC is required in order to bring the kinase GSK into approximation with β-catenin. Loss of full length APC leads to reduced β-catenin phosphorylation and breakdown, thus increasing free cytoplasmic β-catenin levels allowing more β-catenin to enter the nucleus and activate gene transcription.

**Hereditary Nonpolyposis Colorectal Cancer**

A further inherited predisposition to colorectal cancer is hereditary nonpolyposis colorectal cancer (HNPCC), a syndrome first described by A.S. Warthin at the end of the nineteenth century. He noticed a predisposition to colorectal and other cancers in the relatives of his seamstress. Lynch further reported this same family in the 1970’s since when the syndrome has taken his name. Lynch syndrome (now HNPCC) is an autosomal dominant condition, giving rise to early onset colorectal cancers with a predisposition for proximal colonic involvement.

A biological explanation for these observations was found in 1993. The discovery of multiple mutations within short repetitive gene sequences (called ‘microsatellites’) was
correlated to a similar pattern found in mutant yeasts with defective DNA mismatch repair systems. A search for the gene responsible for HNPCC suggested its location to be on chromosome 2p and soon afterwards one of the human DNA mismatch repair genes (hMSH2) was found on chromosome 2p. Germline mutations of this gene were subsequently found in HNPCC families. Thus the underlying reason for the propensity to the development of colorectal cancer in HNPCC families is a defect in DNA repair, and specifically the repair of defectively copied microsatellite sequences. One of the genes that is affected by this is the TGFβ receptor type II. This contains a coding polyadenine tract microsatellite that is often defectively copied in cells with mismatch repair enzyme defects. This results in loss of functional TGFβ receptor II and loss of TGFβ signalling. Since TGFβ inhibits the growth of colonic epithelial cells, this leads to a growth advantage for the TGFβ receptor mutant cells and is the one of the proposed molecular mechanisms whereby the DNA mismatch repair defect found in HNPCC can lead to colorectal cancer.

Importantly this molecular mechanism is not only applicable to the development of cancers in HNPCC families. The hallmark ‘microsatellite instability’ (MSI) is found in approximately 15% of colorectal cancers whereas HNPCC is responsible for only 3% of colorectal cancers. Cancers with MSI do not display the loss of large chromosomal segments and thus are thought to typify a fundamentally different mechanism of cancer development.

In chapter VI of this thesis we examine the role of a TGFβ family member never previously studied in relation to colorectal cancer, Bone Morphogenetic Protein 2.

**Juvenile Polyposis**

Juvenile polyposis (JPS) is a rare autosomal dominant condition characterised by hamartomatous polyps, usually within the colon but occasionally arising in the stomach and small bowel. Unlike solitary juvenile polyps, which may affect up to 2% of children and adolescents and have little or no malignant potential, JPS patients have an increased risk of gastrointestinal malignancy. Genetic studies of affected families have revealed germline mutations in SMAD4 in approximately 20% of patients. SMAD4 is a central element in the signal transduction pathway of both TGFβ and Bone Morphogenetic proteins (BMPs). However, until the recent findings of BMP receptor 1a mutations in patients with JPS but normal SMAD4, the effects of SMAD4 loss have been attributed exclusively to the
disruption of TGFβ signalling. In chapter VI we investigate the possible consequences of loss of BMP signalling in the colon.

![Diagram of TGFβ and BMP signal transduction pathways](image)

**Figure 2** Diagrammatic representation of the TGFβ and BMP signal transduction pathways showing how the two converge on SMAD4. Sporadic colorectal cancers show frequent mutations of Smad 3 and 4. Mutations of TGFβR2 are found frequently in HNPCC and in Juvenile Polyposis mutations of Smad 4 and BMPR1a have been identified.

**II Non-Steroidal anti-inflammatory drug use**

The observation that the regular use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the size of colorectal polyps in humans was first shown in four patients with FAP. They were following up previous observations in chemically induced colonic tumours in rats where Indomethacin treatment led to reduced tumour formation. Subsequent studies of colorectal cancer death rates in regular NSAID users have confirmed that NSAID use can reduce the likelihood of dying of colorectal cancer by as much as 40%. Randomised controlled trials of the use of NSAIDs in reducing the size of visible polyps have also been encouraging. These findings have led to the prospect of being able to prevent cancers before
they occur and to a large extent have been responsible for the birth of the new field of chemoprevention in which great hopes rest on the potential of NSAIDs and related compounds.

Unfortunately, most NSAIDs are too toxic to be used in the prevention of colorectal cancer. Even in high-risk groups the risk of side effects, including gastric ulcers and renal damage, far outweighs the potential benefits in terms of reducing colorectal cancer. This has led to determined attempts to develop new safer drugs with similar or greater efficacy in preventing colorectal cancer. Central to this is an understanding of how these drugs work in colorectal cancer.

Cyclooxygenase (COX), otherwise known as prostaglandin synthetase, was the first identified target of NSAIDs. It was therefore also the logical starting point for investigation into the molecular mechanism of NSAIDs in the prevention of colorectal cancer. Research highly focused towards establishing the link between COX and colorectal cancer has yielded a strong body of evidence to support the theory that NSAIDs act on colorectal cancer by their ability to block COX enzyme.

COX exists in two forms, COX-1 and COX-2. COX-1 is constitutively expressed and is felt to have a 'housekeeper' function, such that its blockade is detrimental to the working of the cell. COX-2 is inducible and found at low levels in normal quiescent cells. Its expression is dramatically increased by a variety of inflammatory stimuli. Studies in normal and neoplastic human colonic tissue have shown that it is the COX-2 isoform that is upregulated in cancer progression. Genetic studies in mice have shown that 'knocking out' the COX-2 gene in mice with a genetic predisposition to intestinal polyposis and cancer, leads to a dramatic reduction in polyp numbers, as does selective pharmacological inhibition of COX-2. The first trials of selective pharmacological COX-2 inhibition in FAP patients show efficacy in reducing polyp size and numbers.

There are, however, anomalies in the COX-2 theory. For example, NSAIDs and NSAID-related compounds with little or no ability to inhibit COX-2 are still able to prevent tumour formation in rodent models of colorectal cancer. Similarly, colorectal cancer cell lines that do not express COX-2 are still killed by NSAIDs and COX-2 inhibitors and in COX producing colorectal cancer cells, restoring the COX-produced prostaglandins fails to reverse NSAID-induced growth arrest. Finally, COX-2 is found by a number of investigators to be most highly expressed not in tumour cells but in macrophages in the stroma of colonic polyps.

Recently a number of new COX-independent mechanisms of action of NSAIDs have been reported that may explain both their anti-inflammatory and antitumour efficacy. NSAIDs
directly inhibit a number of inflammatory signal transduction elements such as nuclear factor-kB, p38 mitogen activated protein kinase and AP-1. \textsuperscript{38} In chapter III we show the expression patterns of a number of molecules central to inflammatory signal transduction in colonic adenomatous polyps in an attempt to shed further light on the mechanism of action of NSAIDs in colorectal cancer using novel methodology. We also show a novel possible mechanism of action of NSAIDs in colorectal cancer in chapter IV and investigate changes in gene expression induced by Aspirin in chapter V.

Figure 3 On overview of the COX-independent molecular targets of NSAIDs and their interactions within the cell. Clearly the action of NSAIDs is more complex than simply inhibition of COX.

Abbreviations: Akt/PKB, protein kinase B; AP-1 activator protein 1; Cdk, cyclin-dependent kinase; Erk, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase 3 beta; HSF, heat shock factor; Hsp, heat shock protein; IKK, I-kappa kinase; JNK, Jun NH\textsubscript{2}-terminal kinase; MAPK, mitogen-activated kinase; MEK/MKK, mitogen-activated protein kinase kinase; NIK, nuclear factor kappaB-inducing kinase; NF-\kappaB, nuclear factor kappa B; PI-3K, phosphatidylinositol-3-kinase; PPAR, peroxisome proliferator-activated receptor; pRb, retinoblastoma protein; p70-S6K, p70S6 kinase; p90RSK, ribosomal S6 kinase;
III Inflammatory bowel disease

Individuals with either Crohn's disease or ulcerative colitis have a dramatically increased risk of developing colorectal cancer. Individuals with ulcerative colitis have about a 20-fold excess risk. In contrast to the development of colorectal cancer in all other cases, these patients seem to bypass the polyp stage. The dysplasia-carcinoma sequence is much more similar to that seen in Barrett's oesophagus or stomach cancer and shares with these gross inflammation as an underlying cause. APC mutations are uncommon, p53 loss can occur early even in histologically normal tissue, as can microsatellite instability, this time in the absence of DNA mismatch repair defects. This pattern may suggest an overwhelming source of mutagenic activity due to exposure of proliferating cells in the crypts to the colonic contents in ulcerated mucosa. Clearly the study of molecular mechanisms in the aetiology of colorectal cancer associated with inflammatory bowel disease has led to very few useful insights into the disease. With so many dissimilarities when compared to sporadic colorectal cancer, it seems a poor model in which to search for generalities relevant to colorectal cancer as a whole.

IV Diet, Obesity and Physical Activity

One of the most intriguing epidemiological findings in colorectal cancer is the large difference in incidence found between different populations and that these differences are likely to have an environmental basis. This has been shown by rapid changes in incidence in Italy, Japan, urban China and male Polynesians in Hawaii and also by migrant data showing that incidence rates in migrants and their descendants rapidly reach those of the host country, sometimes within the migrating generation. The differences in incidence reach 20-fold with rates among Japanese low even until quite recently and rates in Hawaiian Japanese now the highest in the world. To explain these differences, various components of the diet and of lifestyle in general have been investigated. Essentially the increased risk is associated with the adoption of a 'Western' lifestyle. This involves the consumption of more meat and fat, less fibre and vegetables and an increasingly sedentary lifestyle with a high incidence of obesity.

The role of fibre was first proposed by Burkitt who noted reduced incidence of colorectal carcinoma amongst Africans who eat a diet high in fibre. Recently a huge study of the eating habits of over 88,000 nurses concluded that there was no association between
fibre intake and colorectal polyps and cancer,\textsuperscript{45} making this an unlikely explanation for the increased risk of colorectal cancer in western societies.

A high meat intake, particularly with a heavily browned surface, seems to correlate with an approximately 2 to 3 fold increased risk of colorectal cancer.\textsuperscript{46,47,48} The molecular explanation is proposed to be the carcinogenic properties of heterocyclic amines and nitrosamines found in cooked meat and shown to be carcinogenic in animals. This is complicated by the fact that the metabolism of heterocyclic amines is subject to considerable variability. Investigation as to whether genetic polymorphisms of the genes involved in heterocyclic amine metabolism are themselves a risk factor for colorectal cancer has been inconsistent.\textsuperscript{49}

High dietary fat intake is inconsistently associated with increased risk of colorectal cancer with a relative risk of between 1.3 and 2.2 in case-control studies, but no association in cohort studies or in a combined analysis of 13 case-control studies.\textsuperscript{50} Thus association is weaker than for meat consumption and when corrected for total energy intake is even less apparent.

Diets rich in vegetables seem to be protective against colorectal cancer. The seven cohort studies performed show a modest lowering of risk with increasing consumption of vegetables\textsuperscript{51} but there is a less convincing effect for fruit. On a molecular level several possible explanations have been investigated. Fibre has already been covered. Fruit and vegetables are the main source of folate and folate levels are inversely correlated with colorectal cancer risk.\textsuperscript{52} Similarly folate levels are reduced in those with high alcohol intake and in patients with inflammatory bowel disease treated with Sulfasalazine, both of whom have higher rates of colorectal cancer. Vegetables also contain antioxidants such as carotenoids and ascorbate, which prevent DNA damage.

Overall, despite convincing data that adopting a western diet and lifestyle leads to greatly increased risk of colorectal cancer, this has been difficult to attribute to any single component of the diet. This has led on to the theory that it is the increased calorie intake itself coupled with the reduced expenditure of calories that is the root cause. Studies in humans and animals have shown that reducing calorie intake reduces colonic proliferation and inhibits colorectal cancer formation.\textsuperscript{53,54} Physical activity is consistently associated with reduced risk of colorectal cancer\textsuperscript{55} and obesity that may be seen as the result of a combination of these two factors also shows a convincing association with colorectal cancer.\textsuperscript{56} Despite considerable literature on these associations there is no convincing molecular explanation. In chapter II we
advance a new molecular theory for these findings, namely that Leptin, a hormone associated with obesity and reduced by exercise, acts as a growth factor in colonic epithelial cells.

V  Smoking

Studies of colorectal polyp and cancer incidence in smokers show a convincing association between the two. Blood-borne carcinogens such as heterocyclic amines, polycyclic hydrocarbons and nitrosamines are, as in cooked meat, the implicated molecules and the same questions about their genetically variable metabolism have arisen.

VI  Alcohol

Many studies have shown an elevated risk of colorectal cancer associated with high alcohol consumption. As with meat consumption, studies report either a positive association or no association, with no studies reporting a negative association. Putative molecular mechanisms include the associated folate deficiency found in high alcohol users.

VII  Hormone Replacement Therapy

Since the finding that nuns, who experience an excess of hormone related cancers such as breast cancer, also have an excess of colorectal cancer there have been a number of studies among users of the contraceptive pill and hormone replacement therapy (HRT). The incidence of colorectal cancer and polyps is reduced in HRT users with an approximate halving of the risk. The risk reduction seemed to be maintained for about 10 years after cessation of use. One molecular theory to explain this is that Oestrogen prevents microsatellite instability but that lack of it promotes it. This stems from the observations that women suffering from HNPCC are half as likely as their male counterparts to develop colorectal cancer, but in older women with low oestrogen levels, there is an excess of microsatellite unstable colorectal cancer.

Conclusion

Molecular investigation of associations thrown up in epidemiological studies of colorectal cancer has been an important starting point for molecular biologists. This
fascinating link between clinical findings in patient groups and the intricate workings of the cell has also inspired much of the research in this thesis in which we outline a number of new molecular mechanisms in colon cancer.

**Hedgehogs and the maintenance of the normal gastrointestinal epithelium**

The second half of this thesis originated from drawing parallels between embryonal development and the continuous mini-development program occurring in the gastrointestinal tract throughout adult life. The whole of the lining of the bowel is replaced every 3 to 5 days. Gastrointestinal stem cells continuously divide to produce daughter cells that migrate while gradually maturing into a complex pattern of distinct cell types that make up the mature gastrointestinal tract. This can be compared to cells in an embryo, dividing, migrating and differentiating to adopt the complex patterns that form different organs. Years of detailed genetic analysis of mutant fruitflies and worms have revealed a number of genes central to the patterning of the embryo with names reflecting the characteristics of the mutants like ‘Wingless’ and ‘Hedgehog’. These same genes are conserved from fruitfly to man.

In embryogenesis Hedgehog proteins provide positional information to cells. Cells sense the concentration of Hedgehog that they are exposed to and can infer from this their distance from the Hedgehog producing source. For example at the end of the chick limb bud, the Hedgehog source is on the side of the bud where the chick equivalent of the little finger is due to form. Cells nearby exposed to high Hedgehog levels become chick equivalents of the little finger, and those exposed to low levels the thumb. Moving the source of Hedgehog or having two sources results in disruption of the pattern such as chicks with two little finger equivalents and no thumb. This same patterning control system is conserved in limbs of different species and variations occur to control patterning in a wide variety of other organs.

We hypothesised that the same molecules would perform similar tasks in the adult human gastrointestinal tract. Evidence for the importance of Hedgehogs in the normal development of the gastrointestinal tract is seen in mice deficient in one of the three known vertebrate Hedgehog equivalents, Sonic Hedgehog (shh null mice). We therefore looked at the possible role of human Sonic Hedgehog molecules in controlling patterning in the stomach, in oesophagus that has undergone precancerous changes due to acid exposure that
give it stomach-like features (Barrett’s oesophagus), in stomach-like epithelium occurring in the small intestine (Meckel’s diverticulum), and the role of Indian Hedgehog in the colon.

Normal embryonal development depends on the complex regulation of cellular proliferation, migration and differentiation. The same molecular mechanisms seem to be implicated in the cellular proliferation and differentiation necessary for the maintenance of adult organs. Since cancer results from the malfunction of the normal mechanisms controlling cellular proliferation and differentiation within an organ, it is perhaps unsurprising that the two processes, cancer and development, share many of the same molecular players. We are therefore continuing our research into Hedgehogs by examining the role of Indian Hedgehog in colon cancer.