AN IMMUNOHISTOCHEMICAL STUDY OF BETA-CATENIN IN HNPCC COLON TUMOURS

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DECLARATION

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ABBREVIATIONS

APC	Adenomatous polyposis coli
CRC	Colorectal cancer
FAP	Familial adenomatous polyposis
GSK	Glycogen synthase kinase
HNPCC	Hereditary non-polyposis colorectal cancer
MDR1	Multi-drug resistance 1
MMP7	Matrix metalloproteinase 7
MMR	Mismatch repair
MSI	Microsatelitte instability
MSI-H	Microsatelitte instability-high
MSI-L	Microsatelitte instability-low
MSS	Microsatellite stable
TCF/LEF	T-cell factor/Lymphoid enhancer factor
VEGF	Vascular endothelial growth factor

ABSTRACT

AN IMMUNOHISTOCHEMICAL STUDY OF BETA-CATENIN IN HEREDITARY NON-POLYPOSIS COLON CANCER (HNPCC) COLON TUMOURS.

Beta-catenin is normally complexed with adenomatous polyposis coli (APC) protein and E-cadherin adhesion molecule, and localized on the cell membrane. If APC/beta-catenin is disrupted, beta-catenin transfers into the nucleus, where it functions as a transcriptional activator, causing unregulated cell proliferation. The localisation of beta-catenin in HNPCC adenomas has not been studied but a shift in beta-catenin to the nucleus has been previously demonstrated in a range of colorectal cancers, including those in HNPCC. The aim of the first part of the study was to determine whether there is a beta-catenin shift occurring as an early event in HNPCC tumours. Coded sections of tumours were immunohistochemically stained with antibody against beta-catenin and counterstained in haematoxylin. 14 HNPCC adenomas, 13 HNPCC carcinomas, 10 FAP adenomas, 10 FAP carcinomas, 10 sporadic adenomas and carcinomas and 10 juvenile polyps –three with dysplasia and seven without- were studied. A score was given for loss of membrane staining (0-1), presence of cytoplasmic staining (0-2) or nuclear staining (0-2) and a total out of five obtained. An shift in beta-catenin was demonstrated at the adenoma phase in HNPCC. HNPCC tumours were compared with sporadic tumours and a statistically significant similarity in prevalence of beta-catenin shift found in adenomas and carcinomas. The early shift in beta-catenin in HNPCC led to the second part of the study evaluating the "down-stream" effects of this shift in HNPCC tumours. The HNPCC sections were immunohistochemically stained with E-cadherin, c-myc and cyclin D1. The results showed a positive correlation between E-cadherin loss, increased cyclin D1 and a shift in beta-catenin. No significant change in c-myc or correlation between c-myc and a shift of beta-catenin was found. In conclusion the study indicates that disruption of the APC/beta-catenin pathway plays a similar role in HNPCC tumours to that in sporadic tumours. A notable exeption is the effect on c-myc and further study is needed in this regard.

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CHAPTER 1: INTRODUCTION

Colon cancer is one of the most common malignancies worldwide, with the incidence in developed countries being about 5% of the general population.[1,2] Approximately 20% of these have a familial risk, of which 5-10% have Mendelian inheritance.[3] This subset can be divided into a number of syndromes, of which hereditary non-polyposis colorectal cancer (HNPCC), familial adenomatous polyposis (FAP) and juvenile polyposis are the most frequent and well-studied.

The incidence of colon cancer is reported to be lower in African people in South Africa. The incidence for Africans is reported as 2 per 100 000, whereas the incidence for white males in South Africa is 40 per 100 000.[4-6] This is thought to be partially due to underreporting in Africans, but a real difference in incidence also exists. This low incidence is thought to be related to diet and environment. African Americans have one of the highest incidences in the world, supporting an environmental influence rather than a genetic one.[6] The incidence is thus likely to change with progressive Westernisation.[7]

1.1 FAMILIAL COLON CANCER SYNDROMES

1.1.1 FAMILIAL ADENOMATOUS POLYPOSIS

Patients with this syndrome present with numerous (100's to 1000's) adenomatous colonic polyps and untreated have a lifetime risk of colonic cancer approaching 100%.[3] Inheritance is autosomal dominant with approximately 25% having no family history and

presumably occurring due to new germline mutations.[8] Germline mutations of the *adenomatous polyposis coli* gene are responsible for this syndrome. The *adenomatous polyposis coli* gene was discovered in 1991 and is situated at chromosome 5q21-22.[9] The gene is large, comprising 8535 bp spanning 21 exons. Exon 15 accounts for over 75% of the coding sequence and most mutations occur at the 5' end of exon 15. Over 300 germline mutations have been described. Loss or mutation of both *APC* alleles occurs in FAP adenomas as small as 3mm in size.[10] The *APC* gene product is a large protein with multifunctional domains that mediate both oligomerisation and binding to many intracellular proteins including beta-catenin, gamma-catenin, glycogen synthase kinase-3beta (GSK-3bß), axin, tubulin, EB1 and hDLG.[11] The majority of *APC* mutations lead to loss of the beta-catenin degradation sites and the associated GSK-3ß sites. The mutant APC can thus bind but not down-regulate beta-catenin and levels of free cytoplasmic beta-catenin increase.[10] Studies of sporadic tumours have demonstrated that the *APC* gene is not only important in FAP, but responsible for initiation of the majority (>80%) of sporadic cases of colorectal cancer.[1]

1.1.2 JUVENILE POLYPOSIS

Juvenile polyps are characterized histologically by dilated crypts in a loosely packed oedematous stroma with no glandular dysplasia. The majority of polyps are colorectal. They may be sporadic or occur as part of familial juvenile polyposis. Juvenile polyposis is an autosomal dominantly inherited syndrome characterized by multiple juvenile polyps of the gastrointestine.

The patients also have an increased risk of tumours of the stomach, duodenum, biliary tree and pancreas. Two thirds of patients present in the first two decades of life and untreated about 60% will have colorectal cancer by 60 years of age.[12] Clinically the syndrome is characterized by greater than five juvenile polyps in the colorectum, juvenile polyps throughout the gastrointestinal tract or a family history with any number of juvenile polyps.[13] There are two possibilities for the development of cancer in these patients. These are from an adenoma arising de nova, or via adenomatous change in a juvenile polyp. Pure adenomas have been found to be rare and dysplastic change has been found in about 10% of classic juvenile polyps. Atypical juvenile polyps are multilobated and often adopt a villous or papillary configuration. Dysplasia of varying severity is found in 50% of these lesions. The finding of these mixed juvenile polyp/adenomas favours the later mode of carcinogenesis.[13] Juvenile polyposis appears to be a genetically heterogeneous syndrome, but mutations in the *Smad4* gene on chromosome 18q21.1 have been found in some families. The frequency of this mutation has been reported as anything from 20 to >50% of patients. Smad4 is an important mediator in the transforming growth factor-beta pathway, and thus has a role in regulating the transcription of genes involved in cell cycle and transcriptional regulation. The genes regulated include the cyclin dependent kinase inhibitors *p16* and *p21*, *cyclin D1* and *TGF-* ß itself.[14]

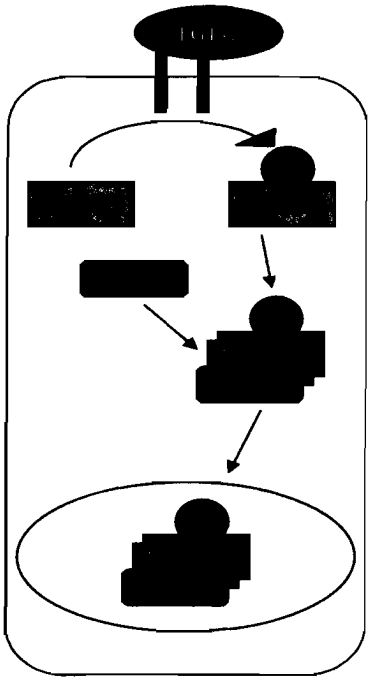

Figure 1.1 :Smad 2 and 3 are phosphorylated by activated TGF-ß. They bind with each other and with Smad4. The complex then transfers into the nucleus were it regulates the transciption of genes.[15]

1.1.3 HEREDITARY NON-POLYPOSIS COLORECTAL CANCER

Hereditary non-polyposis colorectal cancer is the most common hereditary colon cancer syndrome, with the quoted incidence varying between 1 and 10%.[16,17] There is no single pathognomonic feature that characterises this disorder. It can be recognized clinically by a combination of features, described initially by Lynch.[18] These criteria were standardised by the International Collaborative Group on HNPCC in 1990, to provide

uniformity in collaborative studies. The criteria (called Amsterdam Criteria 1) were since modified, to include extra-colonic cancers, and called the Amsterdam Criteria II, namely:

There should be at least three relatives with an HNPCC-associated cancer: colorectal cancer (CRC), or cancer of the endometrium, small bowel, ureter or renal pelvis.

- One patient should be a first degree relative of the other two.
- At least two successive generations should be affected.
- At least one tumour should be diagnosed before age 50 years.
- Familial adenomatous polyposis should be excluded in the CRC case/s, if any.
- Tumours should be verified by histopathological examination .[15]

Diagnosis of HNPCC can be made by fulfillment of these criteria or by genetic testing for germline DNA mismatch repair mutations.[3]

The colonic tumours in HNPCC are frequently of the right colon and may be multiple. Adenomas are less frequently seen and occur in a smaller number compared to those in FAP, hence the name non-polyposis. The relatively small numbers of adenomas is explained by a rapid accumulation of genetic mutations and thus rapid progression from normal epithelium through adenoma to carcinoma.[8]

The genetic defect in HNPCC is an abnormality in DNA repair. The mode of inheritance is autosomal dominant, with a 70-80% penetrance.[16] Germline mutations in *hMLH1, hMSH2, hMSH6, hPMS1 and hPMS2* have been identified, with *hMLH1* and *hMSH2*

accounting for more than 80%.[16,8,3,11] Studies in yeasts show that MSH2 complexes with either MSH6 to recognize single base pair mismatches, or with MSH3 to recognize larger 2 to 4-base pair deletions or insertions. The *MLH1* and *PMS2* gene products then excise the mismatched nucleotides from the newly synthesized strand. Defects in the mismatch repair (MMR) system thus result in many DNA replication errors. These errors are usually detected in simple repetitive sequences, the so-called microsatellites. Microsatellites are present in coding and non-coding DNA throughout the genome. Defects in MMR results in microsatellite instability in which the length of these normally stable repeats varies considerably.[19] This microsatellite instability can be detected in the vast majority of HNPCC cancers. [20]

HNPCC IN THE WESTERN CAPE

294 families composed of 1079 individuals have been identified in the North Western Cape area. 291 of these families have had mutational screening and those who are mutation positive, or have not had genetic testing, undergo regular colonoscopic surveillance.

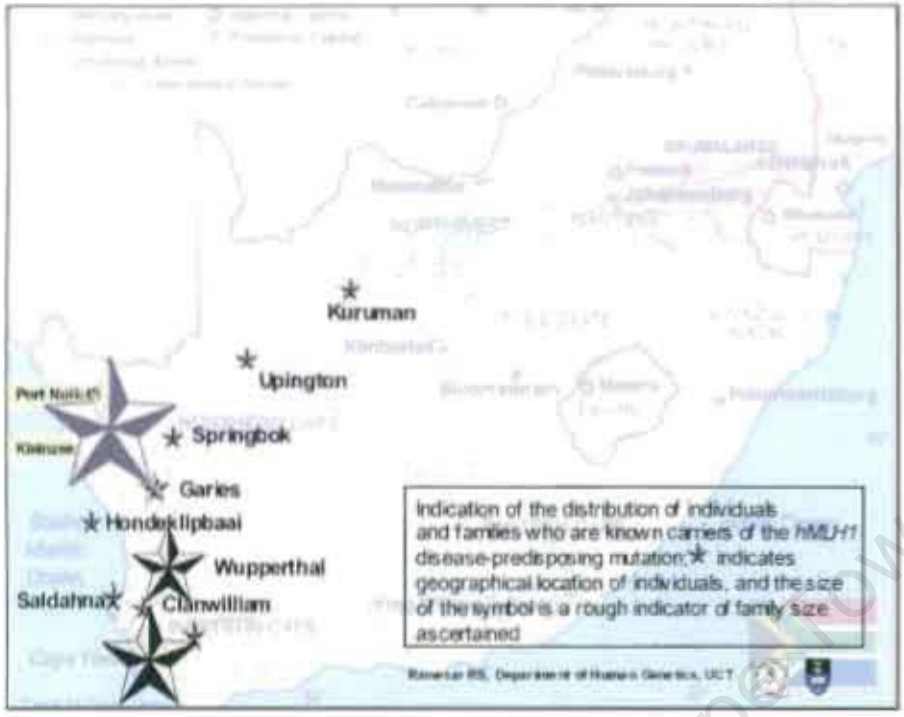

Figure 1.2 Distribution of HNPCC families with *hMLH1* (Map courtesy of Prof Ramesar, Human Genetics, UCT)

1.2 BETA-CATENIN ROLE IN CARCINOGENESIS

Beta-catenin is the 92 kDa protein product of the *beta-catenin* gene localised on chromosome 3p21. Beta-catenin plays an important physiological role in embryonic development.[21] Increased expression of beta-catenin has been demonstrated in a range of cancers, including those occuring in the oesophagus, stomach, colon and endometrium.[22] In normal epithelial and cancer cells beta-catenin is involved in cellular adhesion by interaction with E-cadherin, and with cell cycle regulation via the wnt pathway.[23]

E-cadherin

Beta-catenin was originally discovered as a cadherin-binding protein.[24] The cadherins are mediators of cell-cell adhesion. They are divided into 10 subclasses, of which the E-cadherins are responsible for epithelial cohesiveness. E-cadherin is a 120kd transmembrane glycoprotein that mediates cell adhesion through calcium dependant, homotypic interactions.[21] The cytoplasmic domain of E-cadherin is associated with undercoat proteins, termed catenins (alpha, beta and gamma). Beta and gamma bind to the cytoplasmic domain of E-cadherin and alpha-catenin links beta and gamma-catenin to the actin cytoskeleton. Several studies have shown that the integrity of this complex is necessary for the function of E-cadherin.[25] Loss of E-cadherin function leads to impaired cell adhesion which is responsible for loss of contact inhibition of growth. It is also necessary for an epithelial tumour to acquire metastatic potential.[21]

Decreased E-cadherin has been found in many human tumours.[26] Decreased expression is reported in 40-50% of colorectal carcinomas.[27,28] This decreased expression is associated with loss of differentiation and a worse prognosis.[27,29,26] The study of Takayama et al. of colon, gastric and oesophageal cancer demonstrated immunohistochemically that decreased membrane staining for beta-catenin was associated with decreased E-cadherin staining in a significant number of tumours.[22] Hermiston et al. have suggested, using mouse model experiments, that this loss of E-cadherin can occur at the adenoma stage and that forced re-expression of E-cadherin restores the altered cell cycle.[30] This has been confirmed in sporadic colonic adenomas

where Valizeh et al. and Gagliardi et al. showed decreased E-cadherin expression in 30%, all associated with nuclear beta-catenin expression.[25,31]

Signal transduction /wnt signaling

A separate role for beta-catenin has been found in cell signaling by participation in the Wnt/Wingless pathway.[32] The wnt (or its Drosophila homolog wingless) pathway is essential in embryological development and tumourigenesis. The wnt family is composed of more than 15 closely related glycoproteins. Receptors for the wnt proteins are members of the frizzled family of transmembrane proteins. Wnt signalling, which is characterised by nuclear accumulation of beta-catenin, regulates embryological processes. For example it induces epithelial-mesenchymal transitions in human cell culture experiments. Wnt is also involved in development of various organs including intestinal development.[21]

In unstimulated cells free beta-catenin is bound to and destabilised by a multiprotein complex containing axin, glycogen synthase kinase-3beta and APC. Interaction between axin and GSK-3ß facilitates phosphorylation of beta-catenin by GSK-3ß. This phosphorylation targets beta-catenin for degradation by the proteasome pathway.[2]

When cells are stimulated by wnt ligands a signal is transduced to a cytoplasmic protein, Disheveled (Dv10), which upon activation inhibits the activity of GSK-3ß by binding to axin.[33,2] GSK-3ß is usually responsible for the degradation of beta-catenin and the wnt

signaling thus stabilises beta-catenin and leads to increased cytoplasmic levels and transfer of this protein to the nucleus.[23,2] Nuclear beta-catenin associates with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. In the absence of beta-catenin these factors are poor transcriptional activators. Complexed with beta-catenin they are efficient activators and alter the expression of the wnt signaling target genes.[34]

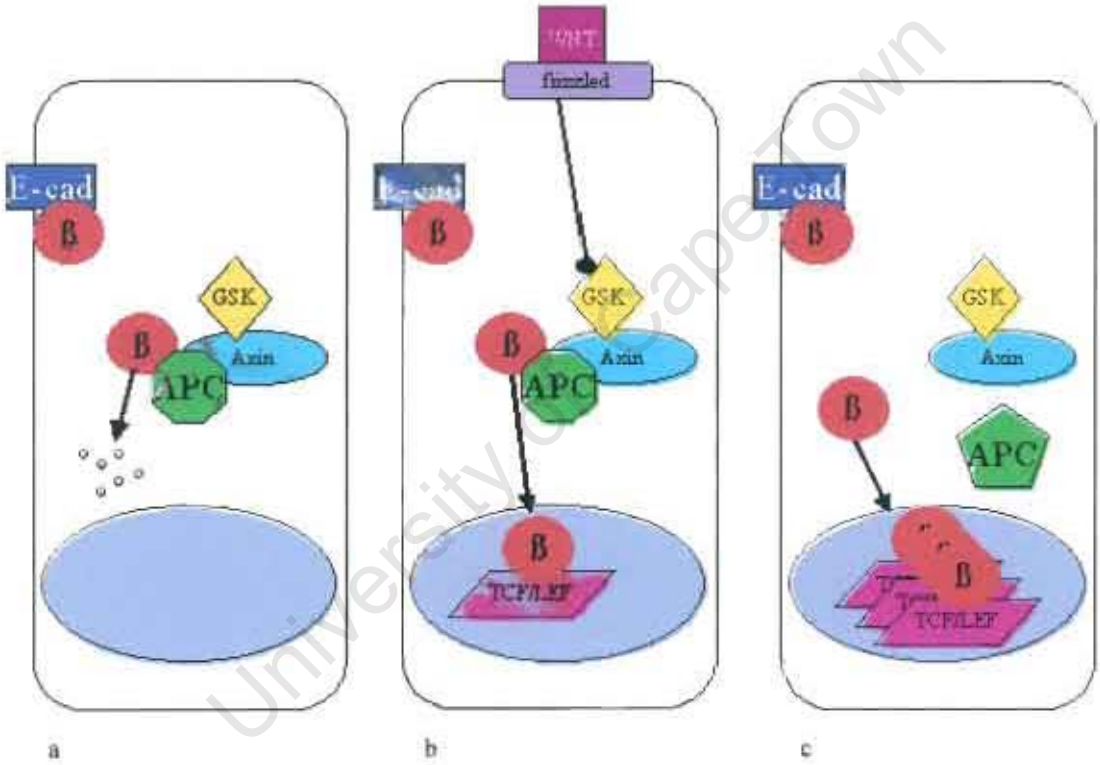

Figure 1.3. Wnt pathway in normal and tumour cells. (a) Normal cells: Beta-catenin bound in a multi-protein complex is phosphorylated by GSK-3ß and degraded. (b) Normal cells: Regulated stabilized beta-catenin translocates to the nucleus and acts as a transcription factor. (c) Tumour cells: disruption of the multiprotein complex due to *APC* mutation leads to beta-catenin upregulation of beta-catenin, transfer to the nucleus and unregulated transcriptional activation.[21]

A number of genes have been identified as targets for wnt signaling. Cyclin D1 is a well known oncogene which stimulates cell proliferation by allowing progression through G1 of the cell cycle. When a cell receives growth promoting signals it synthesizes cyclin D1. This cyclin D1 binds to cyclin dependant kinase (CDK) 4 and then 6 in early G1 of the cell cycle. The CDK 4 and 6 then phosphorylate Retinoblastoma protein (Rb). In the unphosphorylated state Rb is bound to E2F transcription factors and inactivates them. Phosphorylation of Rb releases E2F which is then responsible for the synthesis of factors necessary for progression through the S phase of the cell cycle.[35] *Cyclin D1* gene contains a binding site for TCF/LEF. The binding of TCF/LEF stimulates cyclin D1 synthesis which promotes cell proliferation. Increased beta-catenin has been shown to be associated with an increase in cyclin D1 in colon cancer cell lines.[36] This is thus one mechanism whereby wnt pathway activation and beta-catenin accumulation is thought to lead to uncontrolled cell proliferation. An increase in nuclear cyclin D1 has been demonstrated at the adenoma stage in FAP and in 30% of unselected colorectal adenomas and carcinomas.[37,28]

C-myc is another oncogene identified as a target for wnt signaling. *C-myc* proto-oncogene is expressed in virtually all cells. When a cell receives a growth stimulus the levels of c-myc increase rapidly and then decline to a basal level. After translation c-myc is transported to the nucleus, binds to DNA and acts as a transcriptional activator, promoting cell proliferation.[39] As early as 1989 deregulation of c-myc in colonic cancers was found to be linked to allele loss on chromosome 5q.[40] This is now known to be the site of *APC* gene loss. The link between APC and c-myc has been reported to be beta-

catenin, which regulates transcription of *c-myc* via the TCF transcription factor.[41] Gamma-catenin (plakoglobin), a protein with analogous structure and function to beta-catenin, is also regulated by binding to APC.[42] Kollings et al. showed that gamma-catenin activation of the TCF transcription factor leads to an increase in c-myc expression. This function was independent of beta-catenin, which they found to have no effect on c-myc expression.[43] Gamma-catenin expression is increased in about 85% of sporadic colon cancers.[44]

Numerous studies in the late eighties and early nineties showed an increase in c-myc RNA and protein in colonic carcinomas using Western blotting, Northern blotting and immunohistochemical staining.[45,46,47,48] Elevated levels of c-myc have also been demonstrated at the adenoma stage in FAP.[37] A study comparing c-myc expression with tumour grade showed varied staining results. Increased nuclear staining was seen in well differentiated lesions and decreased nuclear uptake with cytoplasmic staining in poorly differentiated tumours.[49] Predominantly cytoplasmic staining was also found by Imaseki et al., but this was irrespective of tumour grade.[47] It is not known whether cytoplasmic c-myc reflects only inability to reach the nucleus, or whether it has a specific role in the cytoplasm.[49] Wang et al. challenged this elevated c-myc in their recent studies. Using immunohistochemistry and PCR they demonstrated that while c-myc m-RNA was increased in 2/3 of tumours, c-myc nuclear protein expression as detected by immunohistochemistry, was not increased. This was substantiated by Western blotting, which also showed an unaltered or decreased c-myc level.[50]

Aside from disordered cell proliferation, tumour invasion also requires digestion of the basement membrane and extra-cellular matrix. The matrix metalloproteinases are a family of enzymes responsible for extracellular matrix and collagen digestion. Matrix metalloproteinase 7 (MMP7) is, unlike the other metalloproteinases, produced exclusively by tumour cells.[54] MMP7 is overexpressed in 80% of unselected colorectal carcinomas. MMP7 has also been demonstrated to play a role in adenoma development as the inhibition of MMP7 suppresses adenoma formation. The *MMP7* gene has two binding sites for TCF and is activated by beta-catenin/TCF.[55] Beta-catenin increase and shift to the nucleus has been associated with increased MMP7 in immunohistochemical study of human sporadic colon cancers.[21,54] The increased levels were found to be present at the adenoma phase.

Laminin-gamma-2 is an invasion marker which is overexpressed in the invasive front of colorectal carcinomas. Laminin is a component of the basement membrane. It is cleaved by the matrix-metalloproteinases and the gamma-2 chain activates cell migration. Beta-catenin and laminin-gamma-2 have been shown to be overexpressed at the invasive tumour front. Beta-catenin activates laminin-gamma-2 and thus enhances the ability of tumour cells to invade.[56]

Another important factor in tumour progression is angiogenesis. Vascular endothelial growth factor (VEGF) has been found to be important in most tumours. It has also been shown to be increased at the colon adenoma stage. The regulators of VEGF are different in different tissues. In colon cancer cell line experiments VEGF is regulated by K-ras and beta-catenin/wnt signaling.[57]

Beta-catenin is also implicated in colonic cell differentiation. Cell line experiments have shown that down-regulation of beta-catenin/TCF occurs with colonic cell differentiation.[58] This function is thought to be mediated indirectly by down-regulation of transcription factors whose normal role is to repress the expression of genes associated with the onset of differentiation. *Cdx-1*, encoding a homeobox factor, *Id2* (inhibitor of differentiation-2) and *ENC1* (ectodermal neurocortex 1) have been identified. Homeobox genes are genes containing a 180 nucleotide motif, the homeobox, which is conserved between species and in vertebrates is implicated in the patterning of limbs and vertebrae.[21,58,59]

The combination of all these factors makes *beta-catenin* a potent oncogene. The loss of membranous beta-catenin and accumulation of nuclear beta-catenin contributes to the ability of tumour cells to proliferate, survive, dedifferentiate and invade.

1.3 COLORECTAL CANCER CARCINOGENESIS

The multistep process of carcinogenesis, with multiple genetic mutations occurring in sequence, is well recognized and described in well known pathology texts.[35,60] Colon cancer has long been recognised to result from progression of normal epithelium through gradually more dysplastic adenomas to carcinoma. The study of these preneoplastic lesions, particularly in FAP, has done much to contribute to the understanding of carcinogenesis in general.

The well-known classic, or "gatekeeper", pathway of colorectal carcinogenesis was defined mainly through study of FAP adenomas and carcinomas. In FAP a germline mutation in *APC* gene, followed by somatic mutation of the wild type allele, results in the development of numerous adenomas. The somatic mutation is thus the rate limiting step in this process and *APC* is known as a "gatekeeper" gene.[9] "Gatekeeper" genes are responsible for maintaining a constant cell number in renewing cell populations. Mutation of other genes, in the presence of a normal gatekeeper gene, does not result in adenoma development. Evidence supporting this role for *APC* is that *APC* mutations are found in the earliest dysplastic lesions (aberrant crypt foci) and mutation of other genes, such as *p53*, do not result in adenomas in the absence of an *APC* mutation. More than 80% of sporadic adenomas and carcinomas have been demonstrated to have a somatic *APC* mutation.[9] The pathway of carcinogenesis is thus thought to follow roughly the same pattern of mutations in all these lesions.[20] The initial loss of *APC* is followed by loss of other tumour suppressor genes, namely *K-ras* at the adenoma stage, *TP53* on chromosome 17 and deletions on chromosome 18q at transition to malignancy.[11,61] The

inactivation of the second copy of these genes is usually by non-dysjunctional loss leading to loss of heterozygosity. This loss of heterozygosity is correlated with gross chromosomal changes and these tumours are invariably aneuploid.[10]

Not all adenomas have a polypoid configuration. Flat adenomas are described and may be early (not yet polypoid) lesions or represent horizontal spread of proliferating tubules. The flat adenomas have been noted to often lack k-ras mutations.[62]

A second "mutator" pathway has more recently been elucidated through study of HNPCC cancers.[20] In HNPCC an inherited mutation of a mismatch repair gene, with somatic loss of the second copy, leads to failure to repair errors that occur during DNA replication. This results in mutations of single nucleotides and alterations in simple repetitive microsatellite sequences. Microsatellites are tandem repeats of simple sequences that occur abundantly and randomly throughout the genetic sequence. They consist of 10-50 copies of 1-6bp motifs that can occur as perfect tandem repeats, imperfect (interrupted) repeats or as a combination of the two types. The repeats are prone to replication errors during DNA replication, but these are normally repaired by DNA mismatch repair proteins. The length of a repeat is thus normally transmitted unaltered through mitosis and meiosis. Novel alterations of these sequences have been demonstrated in tumours.[63] These alterations in the microsatellites, termed microsatellite instability (MSI), are the characteristic phenotype of HNPCC and are detected in the majority of these tumours.[11,20] Thus there is DNA instability rather than the chromosomal instability seen in the classic

pathway. Once the initial mismatch repair defect is present the lesions progress rapidly to carcinomas, because of the increased mutation rate.

The presence of MSI, in sporadic and HNPCC tumours, has been noted by various groups since 1993. A National Cancer Group Workshop in America met in 1997 to establish acceptable criteria for defining a MSI tumour. Five markers, including two mononucleotide and three dinucleotide markers, were selected. MSI-high (MSI-H) tumours were defined as having instability in two or more markers, while MSI-low (MSI-L) have instability in one marker. Microsatellite stable (MSS) tumours are those showing no instability using these five markers.[64] Subsequent studies have usually included these markers, although additional markers have often been added.[65] Using these criteria 10-15% of all colorectal cancers are MSI-H.[64]

The vast majority of sporadic MSI-H tumours have been shown to have hypermethylation of the MLH1 promoter, suggesting that this is the primary mechanism underlying MSI-H in sporadic tumours.[11,66] Both hypermethylation and hypomethylation of DNA have been linked to colonic cancer. Methylation occurs at CpG sites. CGIs are short sequences rich in CpG dinucleotide found in the 5' region of about half of all human genes. Methylation of cystine within 5' CGIs is associated with loss of gene expression.[67,68] Physiological methylation is seen in conditions such as X chromosome inactivation. Aberrant methylation of CGIs is associated with transcriptional inactivation of tumour-suppressor genes in neoplasms, including colorectal cancer.[69] Two distinct types of hypermethylation are described in cancer. Type A (A for aging-specific) and Type C (C

for cancer-specific). Type A methylation of genes occurs in an aging-related manner in normal colonic epithelium, and hypermethylation of these genes occurs at a high frequency in colorectal cancer. In contrast type C methylation is never seen in normal epithelium and is relatively infrequent in cancer epithelium. A minority of tumours display high levels of type C methylation. MLH1 is methylated in about 50 % of these tumours.[67] These account for the majority of MSI-H sporadic colorectal cancers.[11]

Morphologic features noted in MSI-H cancers are predominance of right sided lesions, poor differentiation, mucinous differentiation, intra-epithelial and Crohn's-like lymphocytic infiltration, reduced invasiveness, improved prognosis, female preponderance and multiplicity.[70] With the exception of lymphocytic infiltration these features have been found to be more pronounced in sporadic MSI-H cancers than in HNPCC.[71]

The discovery of this proposed second "mutator" pathway of carcinogenesis led to numerous studies attempting to establish whether DNA instability leads to accumulation of genetic alterations in oncogenes and tumour suppressor genes similar to those found in the classic pathway. Most early studies did not make the distinction between sporadic MSI-H tumours and HNPCC tumours.[71] A number of differences emerged between Microsatellite stable (MSS) and Microsatellite instability-high (MSI-H) tumours. Mutations involving *TGF-ß type 2 receptor* gene are characteristic of MSI tumours. This mutation causes the cell to express an abnormal TGF-ß receptor on its surface, and thereby fail to bind TGF-ß, which has a potent tumour suppressor activity. Mutations of *TGF-ß* have not been detected in MSS tumours.[16,72] Mutations of *IGF-2R* and *bax* have

also been found at increased frequency in MSI-H cancers. Numerous studies have shown a decreased incidence of *APC*, *p53* and *k-ras* mutations in sporadic MSI-H cancers.[20,59,73,19,71,74] A study by Shitoh et al. on Japanese sporadic colorectal cancers disagreed with this and found a decreased *K-ras* mutation rate, but no difference in *APC* and *p53* mutation rate between MSI-H and MSS cancers.[75] Huang et al. also found a high incidence of *APC* mutations (50%) in MSI-H cancers.[76] This apparent disparity is explained by Jass by dividing MSI-H cancers into those from patients with HNPCC and those occurring in a sporadic setting.[71] The cases in the study of Shitoh et al. were known to include HNPCC cases.[75] The Huang et al. study only included six MSI-H cancers and Jass et al. argues that HNPCC cases may have been unknowingly included.[76,71] Recent studies lend support to this suggestion as studies including only HNPCC cancers have found a 20%[71,77] to 50% mutation rate in *APC*.[78]

Since the main function of APC appears to be regulating beta-catenin, many groups have looked for *beta-catenin* mutations in tumours with preserved *APC*. Experiments on carcinogen-induced rat colon cancers have demonstrated a high incidence of *beta-catenin* mutation, especially in exon 3, in tumours with no *APC* mutation.[79-82] This was thus proposed to be a different method of disruption of the APC/beta-catenin complex, and ultimate activation of wnt signaling, with its downstream effects. Morin et al. showed that mutations rendering beta-catenin insensitive to APC-induced phosphorylation had the same effect as *APC* mutations.[24] Studies of unselected colon cancers have shown beta-catenin mutations in 13% - 50% of cancers without *APC* mutation.[83-85] Salashor et al. looked for *beta-catenin* mutations in unselected MSI-H cancers and found none.[20]

Loving et al. found no mutations in sporadic MSI-H cancers.[74] This is in contrast to Rowan et al. who found a high frequency of *beta-catenin* in MSI-H without *APC* mutations (4/7). It is not known what percentage of these tumours was from HNPCC patients. The sample in the study of Salashor et al. was from unselected cases while Rowan et al. used cell lines. Miyaki et al. studied HNPCC tumours and found *APC* mutations in 20% and *beta-catenin* mutations in 50%.(Miyaki M et al. 1999) Review of this data lends weight to the suggestion that HNPCC tumours have a different genetic profile to MSI-H tumours. The histogenesis also appears to be different. While they are not frequent in comparison to adenomas in FAP, adenomas are seen in HNPCC. Carcinomas can also be seen arising in these adenomas.[71] In contrast, sporadic MSI-H adenomas are rarely seen and these tumours appear to arise through serrated polyps.[87-90] Serrated polyps include hyperplastic polyps, mixed polyps and serrated adenomas. Serrated adenomas are polyps with dysplasia and crypt infolding.[71]

The current evidence, published predominantly by Jass et al, is thus that HNPCC cancers have a similar rate of disruption of the wnt pathway as sporadic lesions, but that sporadic MSI tumours have little evidence of wnt disruption. While it is usually assumed that wnt disruption is an early "gatekeeper" event, very few studies have examined *APC* and *beta-catenin* mutation or beta-catenin shift to the nucleus in HNPCC adenomas. Akiyama et al. studied 14 HNPCC adenomas and found *APC* mutations in 50% and *beta-catenin* mutations in 14% of those without *APC* mutations.[77]

CHAPTER 2: BETA-CATENIN STUDY

The first part of the study aimed to confirm the shift in beta-catenin previously reported in FAP, juvenile and sporadic lesions and to determine if this complex is playing a similar role in the early development of HNPCC lesions. The hypothesis was that beta-catenin shift to the nucleus would occur in HNPCC adenomas at an equal frequency to that occurring in sporadic adenomas.

2.1 AIM

To examine the expression and distribution of beta-catenin in colorectal adenomas and carcinomas in order to

1) confirm the findings of beta-catenin staining in FAP, juvenile and sporadic adenomas and carcinoma.

2) determine if beta-catenin staining is altered in HNPCC

3) determine if beta-catenin alteration is an early or late event in HNPCC

2.2 MATERIALS AND METHOD

A thesis proposal was submitted for approval to the Groote Schuur Hospital/University of Cape Town Ethics Committee and ethical approval was obtained.

2.2.1 MATERIALS

Ten cases each of FAP and sporadic adenomas and carcinomas, and 10 polyps from patients with juvenile polyposis were included in the study. Only one adenoma and/or carcinoma was used from any one patient. For each case a single block was chosen. Where available the section included both tumour and adjacent normal epithelium.

Seven FAP cancers from the Anatomical Pathology Division of Groote Schuur Hospital were included and three additional cases were obtained from the Anatomical Pathology Department of Tygerberg Hospital. The patient ages ranged from 15 to 78 years (mean 45yrs). Ten adenomas were randomly chosen from ten different FAP patients, who had previously undergone polypectomy or colonic resections, at Groote Schuur Hospital. The age range was 29 to 60 years (mean 25yrs). The ten polyps from patients most recently diagnosed with juvenile polyposis, available at the Anatomical Pathology archives at Groote Schuur Hospital, were chosen. All HNPCC cases available at the Anatomical Pathology Division of Groote Schuur Hospital were used in this study. This included 14 adenomas and 13 carcinomas. The ages of the patients with adenomas ranged from 29 to 73 years (mean 36yrs), and carcinomas from 24 to 73 years (mean 39yrs). All the carcinomas were sited in the right side of the colon. Ten sporadic adenomas and ten sporadic carcinomas were selected from sequential cases reported by the investigator and two colleagues, in the period of 2000 to 2002. The age range of patients with adenomas ranged from 31 to 78 years (mean 65yrs). The age range of patients with carcinomas was from 52 to 78 years (mean 64yrs). Eight of the ten carcinomas originated from the left side of the colon.

The FAP and HNPCC cases all had a positive family history and in all but two HNPCC cases proven germline mutations. Cases of juvenile polyposis were selected from children with a history of multiple colonic polyps. No mutation studies were available. The sporadic cases had no family history or morphologic evidence of an inherited syndrome. Testing for hMLH1, hMSH2 and hMSH6 was done in an attempt to exclude any unsuspected HNPCC cases. The method previously described by the investigator's consortium was used.[91]

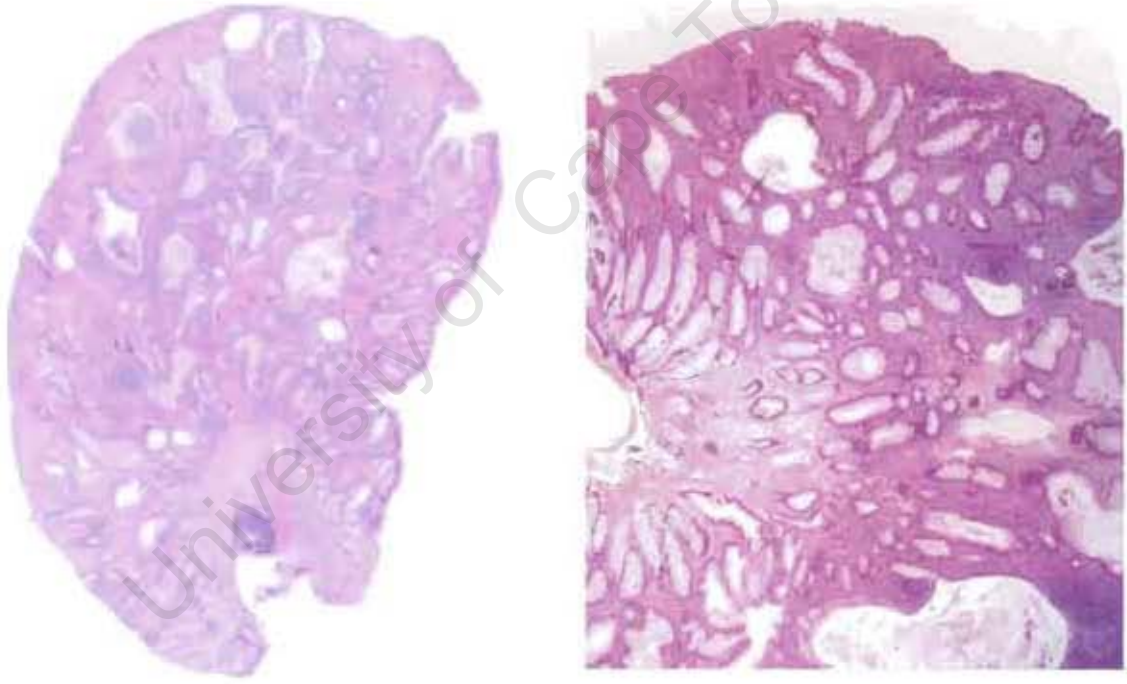

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Figure 2.1:Typical juvenile polyps. (a) Whole mount showing dilated crypts (b) Low power view showing crypt architecture and stromal inflammation.

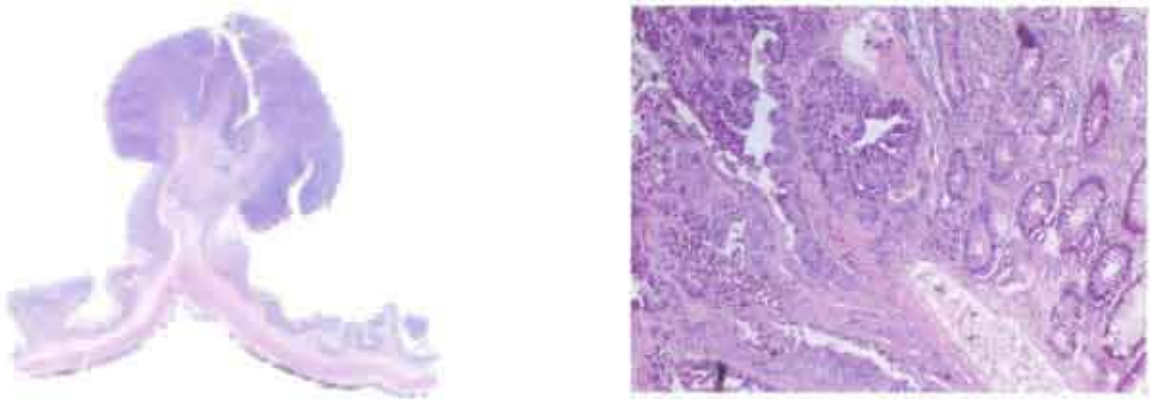

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Figure 2.2: Typical (a) adenomatous polyp and (b) invasive cancer occurring in FAP, HNPCC and sporadic settings.

2.2.2 METHOD: Beta-catenin immunohistochemistry

The chosen method for assessing protein upregulation in this study was through immunohistochemistry. This method has the advantage of giving not only an estimate of the quantity of a protein in a cell but also the subcellular location of the protein. This knowledge is vital with beta-catenin as it is fulfilling different roles at different sites. The stains can also be performed on fomalin-fixed, paraffin wax-embedded tissue, which makes study of archival material possible. A disadvantage is that assessment of protein quantity is based on a visual evaluation of staining intensity and is thus subjective.

Pilot Study

In 2001 a pilot study was performed in the immunohistochemical laboratory at the Division of Anatomical Pathology, Groote Schuur Hospital on seven FAP carcinomas, to assess beta-catenin staining. FAP carcinomas were chosen as these lesions have been well described to have a shift in beta-catenin from the cell membrane to the nucleus in all

cases.[93] The cases were all the FAP cancers currently available in the archives of the Anatomical Pathology Division at Groote Schuur Hospital. A section showing both invasive tumour and normal epithelium was chosen for each case.

Immunohistochemical protocol

2-3micrometer paraffin wax-embedded sections were floated onto APES-coated slides and incubated overnight in a 60°C incubator. Sections were dewaxed in xylene, rehydrated in graded ethanols and washed in water. The sections were blocked for endogenous peroxidase in a 3% H2O2 water solution for 5 minutes. After washing in water, heat-mediated antigen retrieval with a citrate buffer and pressure-cooking was performed at pH6. Sections were rinsed in phosphate buffered saline (PBS) pH7.6. Non-specific binding was blocked using a 5% goat serum solution at room temperature for 10 minutes (Dako X0907). The goat serum was drained off and the sections were incubated with the beta-catenin antibody (NCL-beta-catenin;Novocastra,UK) at a dilution of 1:100 for 2hrs at room temperature. Sections were well washed in PBS and then treated with goat anti-mouse immunoglobulins labeled with horseradish peroxidase (Envision:Dako D4001) at room temperature for 30 minutes. Sections were washed in PBS and positivity developed by applying 3.3'-diaminobenzidine (Dako K3466) at room temperature for 5-10 minutes. Sections were washed well in water and counter-stained in haemotoxylin, followed by dehydration using graded ethanols and xylene before mounting with entallan.

The protocol was established after trying the commercially recommended protocol and making adjustments. Sections of normal colonic epithelium and colon cancers were stained at various dilutions for different lengths of incubation. The results in normal epithelium were then compared with that reported in previous studies and the dilution and length of incubation selected to produce similar results.

Normal epithelium: Staining was isolated to the cell membrane. No cytoplasmic or nuclear staining was present. This is in accordance with previous studies.[21,92-94] Loss of membrane staining and positive staining in the cytoplasm and nucleus were seen in some adenomas and carcinomas.

Immunohistochemical scoring

Staining was evaluated according to the presence of membranous, cytoplasmic and nuclear staining. The scoring system described by Jass et al. was used in this study.[95]

Membrane staining	**Cytoplasmic staining**	**Nuclear staining**
0 = no loss	0 = absent	0 = absent
1 = loss	1 = weak	1 = weak
	2 = strong	2 = strong

The three scores were then added to give a total out of five, with four or five considered a positive result.

Beta-catenin staining has been shown to be greater at the invasive front of tumours.[95] If this area was available it was evaluated. In the case of biopsies the highest scoring area of the available tissue was scored.

The tumours were coded and scored by the investigator, who was blinded to the origin of each tumour. A random sample of the tumours was then scored by a second pathologist.

The results were statistically analysed using the chi-squared and Mann-Whitney tests.

2.3 RESULTS

All of the FAP adenomas had some shift in beta-catenin staining. 7/10 cases had a score of four or five (Table 2.1). All of the FAP carcinomas had complete shift with a score of five. (Table 2.2)

Table 2.1: Beta-catenin score in FAP adenomas

Code	Membrane	Cytoplasm	Nucleus	Total
FA 1	1	1	2	4
FA 2	1	2	2	5
FA 3	1	2	2	5
FA 4	1	1	1	3
FA 5	0	1	0	1
FA 6	0	1	1	2
FA 7	1	2	2	5
FA 8	0	2	2	4
FA 9	1	2	2	5
FA 10	1	2	2	5

Beta-catenin staining scored according to the presence of membranous, cytoplasmic and nuclear staining. Membrane staining scored 0 if present and 1 if absent. Cytoplasmic and nuclear staining scored 0 if absent, 1 if weak and 2 if strong. The three scores are then added to give a total out of five, with four or five considered a positive result.

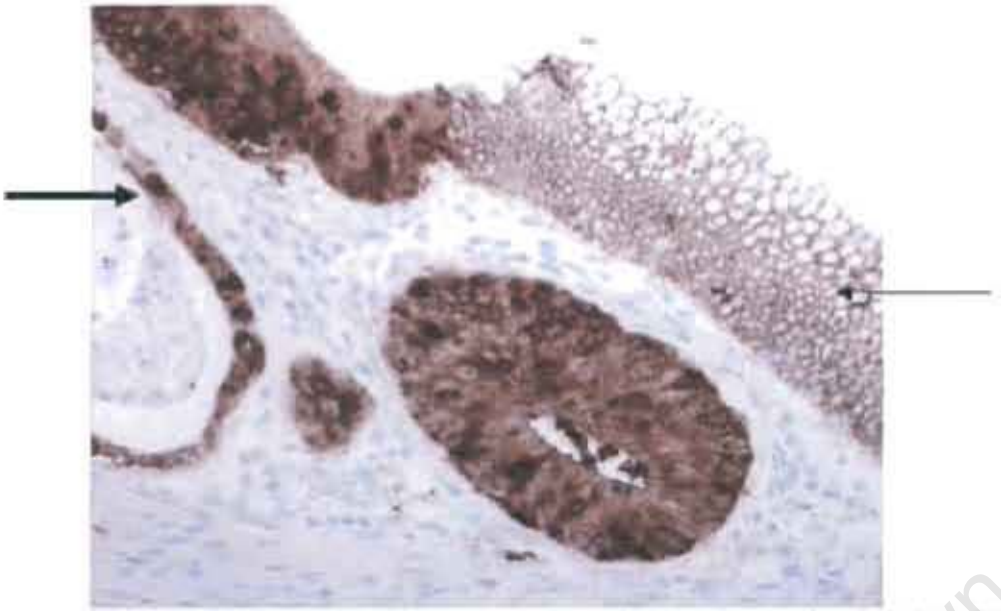

Figure 2.3: Membranous beta-catenin staining in normal epithelium (thin arrow), nuclear beta-catenin staining in FAP adenoma (thick arrow).

Table 2.2: Beta-catenin score in FAP cancers

Code	Membrane	Cytoplasm	Nucleus	Total
FC 1	1	2	2	5
FC 2	1	2	2	5
FC 3	1	2	2	5
FC 4	1	2	2	5
FC 5	1	2	2	5
FC 6	1	2	2	5
FC 7	1	2	2	5
FC 8	1	2	2	5
FC 9	1	2	2	5
FC 10	1	2	2	5

A shift in staining was seen in the three juvenile polyposis polyps with dysplasia. The shift was seen in the dysplastic cells. None of the polyps without dysplasia showed a shift one of the polyps without dysplasia showed a shift (Table 2.3) Nine of the sporadic adenomas had some shift in staining, five had nuclear staining and four a score of five (40%). (Table 2.4) All of the sporadic carcinomas had some shift in staining with nuclear staining and six had a score of four or five (60%). (Table 2.5)

Table 2.3: Beta-catenin score in juvenile polyposis polyps

Code	Dysplasia	Membrane	Cytoplasm	Nucleus	Total
JP 1	Yes	1	2	2	5
JP 2	Yes	1	2	2	5
JP 3	Yes	1	1	1	3
JP 4	No	0	0	0	0
JP 5	No	0	0	0	0
JP 6	No	0	0	0	0
JP 7	No	0	0	0	0
JP 8	No	0	0	0	0
JP 9	No	0	0	0	0
JP 10	No	0	0	0	0

Table 2.4: Beta-catenin score in sporadic adenomas

Code	Membrane	Cytoplasm	Nucleus	Total
SA 1	0	1	0	1
SA 2	1	2	2	5
SA 3	1	2	2	5
SA 4	0	1	0	1
SA 5	1	2	2	5
SA 6	0	1	0	1
SA 7	0	1	2	3
SA 8	0	1	0	1
SA 9	0	0	0	0
SA 10	1	2	2	5

Table 2.5: Beta-catenin score in sporadic carcinomas

Code	Membrane	Cytoplasm	Nucleus	Total
SC 1	1	2	2	5
SC 2	1	2	2	5
SC 3	1	2	2	5
SC 4	1	2	1	4
SC 5	1	1	1	3
SC 6	1	1	1	3
SC 7	0	1	2	3
SC 8	1	1	1	3
SC 9	1	1	2	4
SC 10	1	2	2	5

The HNPCC adenomas showed some shift in 13/14 cases, nuclear staining in 7/14 cases (50%), and four had a score of five (28.6%). (Table 2.6) The HNPCC carcinomas all had some shift with 11/13 (84.6%) having nuclear staining and 9/13 (69.2%) having a score of four or five. (Table 2.7)

Table 2.6: Beta-catenin score in HNPCC adenomas

Code	Membrane	Cytoplasm	Nucleus	Total
HA 1	0	1	0	1
HA 2	1	2	0	3
HA 3	1	2	0	3
HA 4	0	1	0	1
HA 5	0	2	1	3
HA 6	1	2	2	5
HA 7	0	0	0	0
HA 8	1	2	2	5
HA 9	0	1	0	1
HA 10	1	2	2	5
HA 11	0	1	0	1
HA 12	1	1	1	3
HA 13	1	1	1	3
HA 14	1	2	2	5

Table 2.7: Beta-catenin score in HNPCC carcinomas

Code	Membrane	Cytoplasm	Nucleus	Total
HC 1	1	2	1	4
HC 2	1	1	2	4
HC 3	1	2	1	4
HC 4	1	2	2	5
HC 5	1	1	2	4
HC 6	0	2	1	3
HC 7	1	2	2	5
HC 8	0	1	0	1
HC 9	1	2	2	5
HC 10	1	2	1	4
HC 11	1	2	2	5
HC 12	0	2	1	3
HC 13	1	2	0	3

HNPCC adenomas were compared with sporadic adenomas and no difference in beta-catenin total score was found. The similarity was significant with a Mann-Whitney score of 0.89. The lesions were then stratified into positive shift and negative shift. A score of one to three was viewed as negative and a score of four or five as positive. There was once again no difference between the two groups. The chi-squared test was used and the p-value is 0.88. The same comparison was done with the sporadic and HNPCC

carcinomas and no difference was found. Mann-Whitney value 0.9 and chi-squared test 0.88.

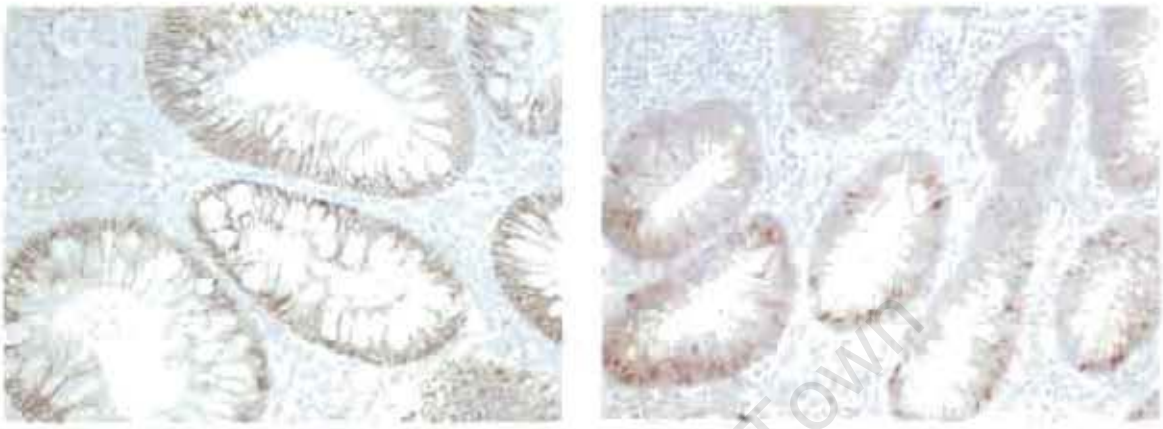

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Figure 2.4: (a): membranous beta-catenin staining in a juvenile polyp with no dysplasia (b): Nuclear beta-catenin staining in an area of dysplasia in a juvenile polyp.

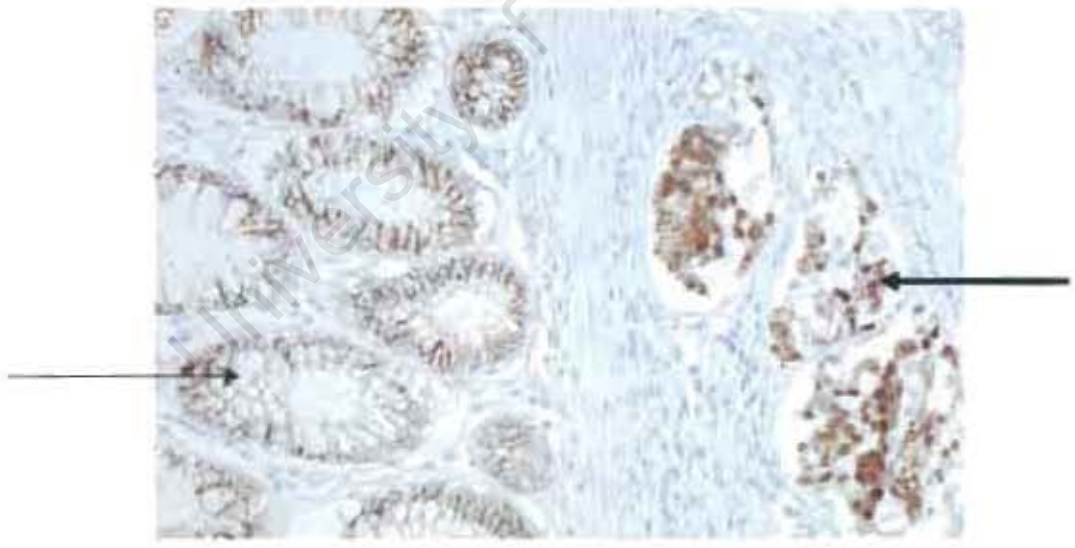

Figure 2.5: Surface epithelium with normal membranous beta-catenin staining (thin arrow), HNPCC carcinoma with nuclear beta-catenin staining (thick arrow).

2.4 DISCUSSION

The majority of sporadic colorectal cancers are known to occur via the classic pathway of carcinogenesis with progression from normal epithelium, via adenomas, to carcinomas.[61] Mutation of the *APC* gene plays a crucial "gatekeeper" role in these tumours. Mutation of the *APC* gene leads to decreased posphorylation and degradation of beta-catenin, and a shift of beta-catenin from the cell membrane to the nucleus. Beta-catenin then binds to TCF transcription factors and leads to increased cell proliferation and decreased apoptosis.[23]

Tumours occurring in the setting of HNPCC, and a small subset of sporadic tumours, have loss of DNA mismatch repair genes as the initiating event and follow the so-called "mutator" phenotype. The role of the APC/beta-catenin complex in these lesions is not as well defined.

The results of this study show a shift in beta-catenin in 7/10 FAP adenomas and 10/10 of the FAP carcinomas. Inomata et al. showed beta-catenin shift in all their FAP adenomas and carcinomas.[93] The finding of beta-catenin shift in all the cases in this study is in accordance with this result. They did not attempt to score cases beyond positive or negative for shift.

In the juvenile polyposis cases beta-catenin shift was found only in the polyps with dysplasia. The remainder of the lesion had membranous beta-catenin staining. A similar

finding of beta catenin shift in polyps of juvenile polyposis has been previously reported.[96] The finding of focal beta-catenin nuclear staining in those lesions with dysplasia indicates that wnt pathway disruption is probably implicated in the development of carcinomas in these lesions.

A shift in beta-catenin staining to the nucleus was seen in five out of ten sporadic adenomas. Previous immunohistochemical studies of beta-catenin in sporadic adenomas have shown very varied results. Inomata et al. found no nuclear staining, Hao et al. found nuclear staining in 50% of adenomas, Valizadeh et al. in 65% and Iwamoto et al. in 100%.[93,92,25,94] In carcinomas Hugh et al. showed beta-catenin shift in 75% with nuclear staining in 50%.[97] Hao et al. showed nuclear staining in 78.8%.[92] Wang et al. in 83% and Iwamoto et al. in 100%.[50,94] The scoring system for each of these studies was different. Hao et al. viewed nuclear staining in >5% of nuclei as positive and Iwamoto required nuclear staining in >10% of tumour cells. [92,94] Wang reported any nuclear staining as positive and >50% staining as diffuse.[50] Hugh et al. divided beta-catenin score into negative (no staining), focal (less than 75%) and diffuse (>75%).[97] This variability in scoring highlights the difficulty experienced when attempting to evaluate and compare the results of different immunohistochemistry studies.

The tumours in these above mentioned studies were not stratified according to microsatellite instability. Microsatellite evaluation was not available for the current study. Sporadic lesions should ideally have been stratified into MSI-H and MSS lesions to allow comparison between these two groups and the HNPCC tumours. An attempt was made to

do this by immunohistochemically testing for loss of MLH1, MSH2 and MSH6. This staining was done using the method previously devised by the investigators research consortium.[91] All the cases chosen had preserved mismatch repair staining and are thus unlikely to be from unsuspected HNPCC or sporadic MSI-H cases.

Nuclear beta-catenin staining was demonstrated in seven of 14 HNPCC adenomas and 11 of 13 HNPCC carcinomas. Despite the small sample size, these results show statistically significant similarity to the results for the sporadic adenomas and carcinomas, thus supporting the hypothesis.

Mutational analysis of HNPCC cancers has shown that the combination of *beta-catenin* or *APC* mutation occurs with approximately equal frequency in these tumours as in sporadic cancer. Immunohistochemical localization of beta-catenin can be expected to reflect this. This is supported by this study. The study also demonstrates that the situation in the South African cases is similar to that found in European and Japanese studies. The immunohistochemical demonstration of a beta-catenin shift at the adenoma stage is, to the investigator's knowledge, a new finding. A single Japanese study showed *beta-catenin* and *APC* mutations to be present in a percentage of these adenomas. The study was based on 14 adenomas from Japanese patients with HNPCC.[78] The finding of beta-catenin shift in the HNPCC tumours led to the second part of the study looking at the "down stream" effects of this shift.

CHAPTER 3: E-CADHERIN, CYCLIN D1 AND C-MYC STUDY

The hypothesis was that the E-cadherin, cyclin D1 and c-myc staining in HNPCC adenomas and carcinomas would be the same as that reported in sporadic cases. A further hypothesis was that the decrease in E-cadherin and increase in cyclin D1 and c-myc would be positively correlated with nuclear beta-catenin staining.

3.1 AIM

To examine the expression and distribution of E-cadherin, cyclin D1 and c-myc in HNPCC adenomas and carcinomas to

1) determine if E-cadherin membranous expression is decreased in HNPCC
2) determine if cyclin D1 and c-myc nuclear expression is increased in HNPCC
3) determine the relationship between beta-catenin distribution and the expression of E-cadherin, cyclin D1 and c-myc.

3.2 MATERIALS AND METHOD

The same HNPCC adenoma and carcinoma blocks used in the beta-catenin study were used. Immunohistochemical staining for E-cadherin, cyclin D1 and c-myc was performed using the same immunohistochemistry protocol that was used in the beta-catenin study, with the following antibody dilutions and incubation times:

	AG retrieval	Ab dilution	Time	Buffer
E-cadherin(code081223)Zymed	pH6	1:2	1hr	citrate
C-myc (Clone 9E10)DAKO, USA	pH8	1:500	2hr	EDTA
Cyclin D1 (codeM7155)DAKO,Denmark	pH6	1:150	1hr	citrate

Background staining occurred with the cyclin D1 (codeM7155;DAKO,Denmark) and c-myc (Clone 9E10;DAKO, USA) antibodies. Blocking for endogenous peroxidase and treatment with goat serum improved, but did not totally remove, this staining. The staining did not however obscure the morphology of mucosal staining.

3.2.1 E-CADHERIN

Normal epithelium: baso-lateral membrane staining was present.

Loss of membrane staining was evaluated and a score of 1 awarded for <30% loss (focal) and 2 for >30% loss (diffuse). This scoring system was adapted from that used by Gagliardi et al.[31]

The coded sections were scored by the investigator and a second pathologist.

The E-cadherin results were correlated with the beta-catenin results.

3.2.2 CYCLIN D1

Weak membranous and cytoplasmic staining was present in the normal epithelium. No nuclear staining was observed.

Staining could not be performed on one adenoma as the tissue had cut away. Nuclear staining was assessed and a score of 1 awarded for positive staining in <30% of tumour nuclei and 2 for staining in >30% of tumour nuclei.

The sections were scored by the investigator and a second pathologist.

The cyclin D1 results were correlated with the beta-catenin results.

3.2.3 C-MYC

Strong nuclear staining was seen in the crypts and surface epithelium in the normal mucosa. Weak cytoplasmic staining was also observed. The staining was uniform throughout to the length of the epithelium. Occasional inflammatory cells in the stroma also stained positive. The nuclear and cytoplasmic staining in the carcinomas was compared to the normal epithelium and reported as weaker, the same or stronger staining. The adenomas were not evaluated as the tissue had cut away in 5/14 and only two polyps had adjacent normal epithelium for comparison. Attempts were made to quantify the difference in staining in terms of percentages, but this was found to yield too much intra-observer and inter-observer variation. Good intra-observer and inter-observer reproducibility was found using the simple more, less or the same staining system.

3.3 RESULTS

3.3.1 E-CADHERIN

Normal membrane staining was seen in 10/14 HNPCC adenomas (71.4%). Focal loss of staining (<30% loss) was seen in four cases (28.6%). No adenomas had diffuse loss of staining. 9/13 (69.2%) HNPCC carcinomas had loss of E-cadherin staining. Staining was diffusely lost (>30% loss) in 6/13 (46.1%) and focally lost (<30% loss) in three cases (23%).

The beta-catenin score was then correlated with the score for loss of E-cadherin staining. A strong positive correlation was found (correlation co-efficient 0.7).

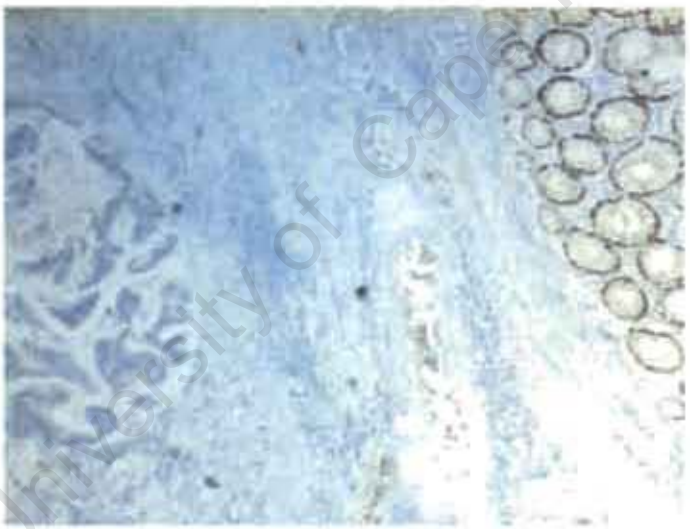

Figure 3.1: E-cadherin immunohistochemistry – positive staining in normal epithelium (right) and negative staining in the tumour (left)

3.3.2 CYCLIN D1

9/13 cases had no nuclear staining (69%). 4/13 (30.8%) showed focal nuclear staining (<30% of tumour cells). No adenomas had diffuse nuclear staining. 4/13 HNPCC

carcinomas (30.8%) had no nuclear staining. 5/13 (38.5%) had focal staining and 4/13 (30.7%) had diffuse nuclear staining.

The beta-catenin score was then correlated with the score for cyclin D1 staining. A strong positive correlation was found (correlation co-efficient 0.63). Division of adenomas and carcinomas into separate groups did not affect the significance of the correlation. Stratification of beta-catenin into groups of negative (0-3) and positive (4-5), and cyclin D1 into no nuclear staining (0) and nuclear staining (1-2), also had no effect on the significance

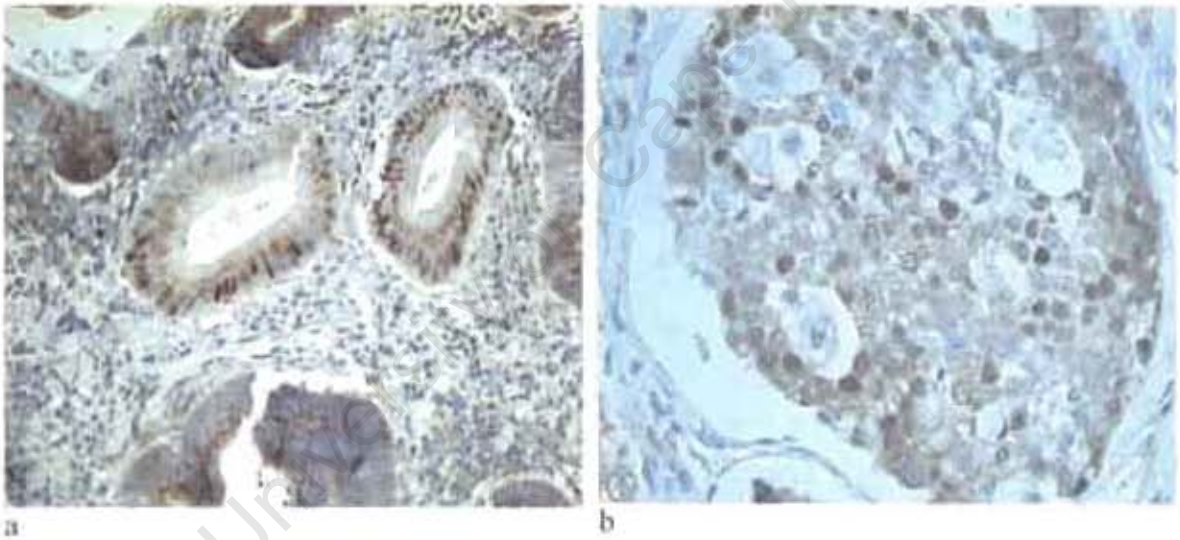

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Figure 3.2: nuclear cyclin D1 staining in an adenoma (a) and carcinoma (b)

Table 3.1: E-cadherin and cyclin D1 scores in (a) HNPCC adenomas and (b) HNPCC carcinomas

Code	E-cadherin	Cyclin D1
HA 1	0	0
HA 2	0	0
HA 3	0	1
HA 4	0	0
HA 5	0	0
HA 6	1	1
HA 7	0	0
HA 8	1	1
HA 9	0	0
HA 10	1	0
HA 11	0	0
HA 12	0	c/away
HA 13	0	0
HA 14	1	1

a

code	E-cadherin	Cyclin D1
HC 1	2	2
HC 2	2	1
HC 3	2	2
HC 4	1	2
HC 5	2	0
HC 6	0	0
HC 7	2	2
HC 8	0	0
HC 9	1	1
HC 10	1	1
HC 11	2	1
HC 12	0	1
HC 13	0	0

b

Loss of E-cadherin membrane staining: a score of 1 awarded for <30% loss (focal) and 2 for >30% loss (diffuse). Cyclin D1 nuclear staining: a score of 1 awarded for positive staining in <30% of tumour nuclei and 2 for staining in >30% of tumour nuclei.

3.3.3 C-MYC

Staining was heterogeneous within the tumours, with most lesions showing areas of increased and decreased nuclear staining. When the entire section was evaluated 3/13 tumours showed an overall increase in nuclear staining. Four tumours showed no difference in nuclear staining compared to the adjacent normal mucosa and six tumours had decreased nuclear staining. Increased cytoplasmic staining was seen in five tumours including the three cases with increased nuclear staining.

No relationship between beta-catenin and nuclear c-myc staining could be demonstrated. Nuclear staining showed no relationship to tumour grade.

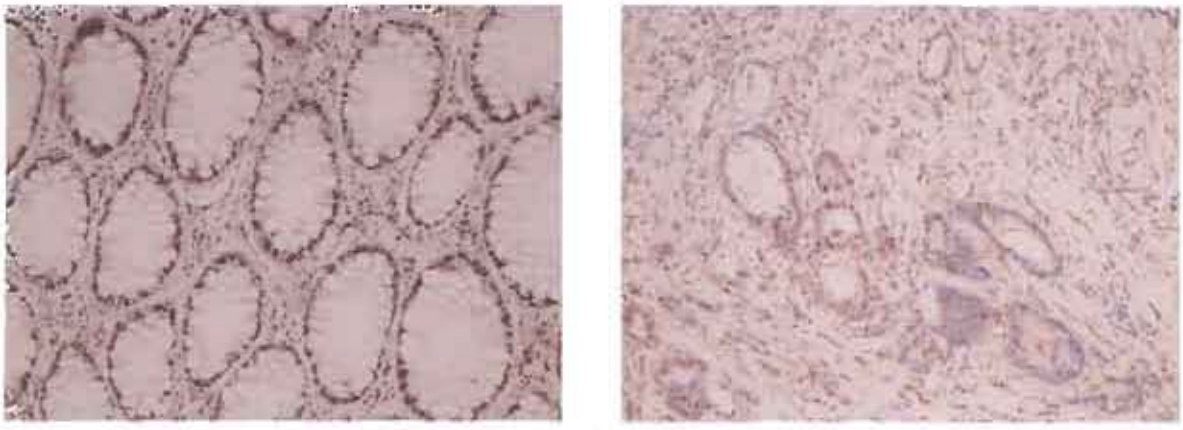

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Figure 3.3: a) c-myc expression in normal mucosa b) c-myc in HNPCC carcinoma of same patient.

Table 3.2: Nuclear and cytoplasmic c-myc expression in an HNPCC carcinoma compared to the adjacent normal epithelium

Code	Nucleus	Cytoplasm
HC 1	Decreased	No change
HC 2	Increased	Increased
HC 3	Increased	Increased
HC 4	No change	Increased
HC 5	Decreased	No change
HC 6	Decreased	No change
HC 7	No Change	No change
HC 8	No change	No change
HC 9	Increased	Increased
HC 10	Decreased	Increased
HC 11	No change	No change
HC 12	Decreased	No change
HC 13	Decreased	No change

3.4 DISCUSSION

Evidence of beta-catenin shift to the nucleus in HNPCC tumours led to the second part of the study looking at the down-stream effects of this shift. Studies in sporadic carcinomas have linked beta-catenin/wnt to upregulation of cyclin D1 and c-myc. Loss of membranous beta-catenin has also been linked to a decrease in E-cadherin. These

changes have been shown to be present at the adenoma and carcinoma stage in FAP and sporadic lesions. To the investigator's knowledge the expression of these proteins has not been evaluated in HNPCC adenomas.

This study found focal E-cadherin loss in 30% of HNPCC adenomas. This is the same percentage as that found by Valizadeh et al. (30%) and similar to the findings of Gagliardi et al. (25%) in unselected colon tumours.[25,31] 46% of HNPCC cancers showed diffuse loss of staining. This is the same as the findings of Dorudi et al. (46%) and very similar to the results of Gagliardi et al. (44%) in unselected cases. [25,29,31] The loss of E-cadherin showed a significant correlation with a shift of beta-catenin from the membrane to the nucleus. The hypothesis regarding E-cadherin was thus supported.

Increased expression of nuclear cyclin D1 was found focally in 30% of the HNPCC adenomas. This is similar to that reported by Arber et al. (34%).[38] 69% of HNPCC carcinomas in this study showed nuclear cyclin D1 staining. This is higher than that found in the Arber et al. study (30%). The comparison of the two studies is made difficult by the difference in scoring of staining. Arber et al describe positive staining as nuclear staining present in >5% of one microscopic field.[38] The magnification is however not stated making this statement meaningless. Another study has disagreed with their findings and showed positive nuclear staining in all of their sporadic colorectal carcinomas.[50] A positive correlation was found between nuclear beta-catenin and increased cyclin D1 staining. The hypothesis regarding cyclin D1 was thus supported.

C-myc staining was found to be highly heterogeneous in the carcinomas studied. 3/13 showed increased nuclear staining when compared to the adjacent epithelium. The remaining 10 showed equal or decreased staining. Five tumours showed increased cytoplasmic staining, including the three with increased nuclear staining. This is in apparent conflict with studies performed in the late eighties, that showed increased c-myc RNA and protein in all colonic cancers using Western blotting, Northern blotting and immunohistochemical staining.[45-47] This elevated c-myc could however not be shown by Wang et al. in their recent study. Using immunohistochemistry and PCR they demonstrated that while c-myc m-RNA was increased in 2/3 of tumours, c-myc nuclear protein expression as detected by immunohistochemistry, was not increased. They did not comment on the presence or absence of cytoplasmic staining. The decreased nuclear staining was substantiated by Western blot which also showed an unaltered or decreased c-myc level. The Wang et al. study was however small, including only 12 colonic cancers. Furthermore five of the twelve were tumours of the right colon, raising the possibility that these may represent unsuspected HNPCC or sporadic MSI-H lesions.[50] Royds et al. also found decreased nuclear staining in carcinomas, but noted an increase in cytoplasmic staining. They used electron microscopy to demonstrate that this was genuine binding to polyribosomes and not staining artifact. The finding of cytoplasmic c-myc may explain the increase in c-myc found in studies using Western and Northern blotting and not immunohistochemistry. The major function of c-myc is thought to be in the nucleus where it is involved in progression through the cell cycle. It is possible that cytoplasmic c-myc performs an as yet unknown function.[49]

No relationship was found in this study between beta-catenin and c-myc expression. This is in contrast to cell culture studies, using human colon cancer cell lines, which have shown that nuclear beta-catenin leads to increased expression of c-myc.[41] Wang et al. however found no relationship between beta-catenin and c-myc expression. They thus hypothesised that nuclear localization of beta-catenin and c-myc expression are not linked in-vivo.[50] The cell culture experiments of Kollings et al. showed that gamma-catenin activation of the TCF transcription factor leads to an increase in c-myc expression. This function was independent of beta-catenin, which they found to have no effect on c-myc expression.[43] Gamma-catenin expression is increased in about 85% of sporadic colon cancers.[42]

One explanation for the low incidence of c-myc increase in the current study may be due to the fact that the tumours are from HNPCC patients and not sporadic cancers. Rothberg et al. showed a significant correlation between increased c-myc expression and tumours of the left colon, with significantly less c-myc expression in tumours of the right colon They thus suggested that increased c-myc is less prominent in HNPCC carcinomas compared to FAP and sporadic tumours.[98] The studies showing an increase in c-myc in all tumours do not specify whether their cases are sporadic or familial, or from the right or left colon.[45-47] It is, however, likely that most originated from the left as these tumours are more common. A possible explanation for this finding of decreased c-myc expression in tumours from the right side of the colon, is that the level of *APC* mutation is thought to be lower in tumours from HNPCC patients, with an increased contribution by *beta-catenin* mutations.[77] Since gamma-catenin is regulated by APC, the lower incidence of

APC mutations may also lead to less gamma-catenin expression, with possible effects on the expression of c-myc.

In the absence of a relationship to beta-catenin staining, a correlation with tumour differentiation was sought. No correlation with nuclear staining could be demonstrated, but increased cytoplasmic staining was positively correlated with poor differentiation. Sikora et al. showed a similar result with increased cytoplasmic staining in poorly differentiated tumours.[45]

CHAPTER 4: CONCLUSION

This study demonstrates that beta-catenin plays as significant a role in HNPCC adenomas and carcinomas, as it does in sporadic tumours. Chemopreventative and chemotherapeutic strategies targeting beta-catenin, which are currently the focus of much attention, are thus appropriate in these individuals.

4.1 THERAPEUTIC IMPLICATIONS

The demonstration of the role and importance of beta-catenin/TCF signals has raised the possibility of developing cancer therapies based on targeting this pathway. Kim et al. demonstrated that the inhibition of the oncogenic form of beta-catenin is sufficient to reverse the transformed properties of human cancer cell lines. [99] They thus provided evidence that pharmacological inhibition of oncogenic beta-catenin is likely to be an effective strategy for reversing the malignant properties of advanced human tumours with upregulated beta-catenin.

Various laboratories have used different methods to target this pathway. Chen et al. introduced a cell death gene *fadd* under the control of wild-type TCF/LEF into colon cancer cell lines. They were able to selectively target tumour cells that had defects in the wnt/beta-catenin/TCF signal transduction pathway.[100] Kwong et al. achieved a similar result using gene therapy in nude mice. They introduced a recombinant adenovirus, which carries a herpes simplex virus thymidine kinase gene under the control of a beta-

catenin/TCF-response promoter linking to a minimum CMV promoter. A combination of the adenovirus and gancyclovir suppressed the growth of colon cancer cells with up regulated beta-catenin in the mice.[101]

A simpler approach is the use of anti-inflammatories. Non-steroidal anti-inflammatories reduce beta-catenin/TCF transcription. They do this via the inactivation of a phosphatase thus leading to an increase in phosporylated beta-catenin and greater beta-catenin degradation.[102]

4.2 FUTURE STUDIES

1) Sporadic MSI-H tumours were not included in this study. It has been suggested that the wnt pathway is less involved in these lesions. A study, similar to the current one, but focusing on these sporadic cases would be useful.
2) Evaluation of gamma-catenin expression in HNPCC tumours, and the possible link between gamma-catenin expression and c-myc expression.

CHAPTER 5: REFERENCES

1. Yang VW. *APC* as a checkpoint gene: the beginning or the end? Gastroenterology 2002;123(3):935-938.
2. Bienz M, Clevers H. Linking colorectal cancer to wnt signaling. Cell 2000;103:311-320.
3. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. New England journal of medicine 2003;348(10):919-932.
4. Segal I. Continuing low incidence of colon cancer in African populations. The American journal of gastroenterology 2000;95(4):859-860.
5. O'Keefe JD, Kidd M, Espitalier-Noel G, Owira P. Rarity of colon cancer in Africans is associated with low animal product consumption, not fiber. The American journal of gastroenterology 1999;94(5):1373-1380.
6. Cancer registry. http://www.cancer.co.za/registry_colorectal.asp.
7. Dreyer I, Angelo N. Colorectal carcinoma-a new threat to black patints? South African medical journal 2001;91(8)
8. Jass JR. Familial colorectal cancer: pathology and molecular characteristics. The Lancet 2000;1:220-226.
9. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159-170.
10. Bright-Thomas RM, Hargest R. APC, ß-catenin and hTCF-4; an unholy trinity in the genesis of colorectal cancer. European journal of surgical oncology 2003;29:107-117.
11. Chung DC. The genetic basis of colorectal cancer: insights into critical pathways of tumourigenesis. Gastroenterology 2000;119:854-865.
12. Jass JR. Epithelial tumours of the large intestine. In: Morson and Dawson's gastrointestinal pathology. Blackwell publishing. 2003:551-610.
13. Jass JR, WC, Bussey HJR, Morson BC. Juvenile polyposis-a precancerous condition. Histopathology 1988;13:619-630.
14. Woodford-Richens KL, Rowan AJ, Poulsom R et al. Comprehensive analysis of *SMAD4* mutations and protein expression in juvenile polyposis. American Journal of Pathology 2001;159(4):1293-1300.
15. Aaltonen LA, Jass JR. Juvenile polyposis. In: Hamilton SR editor. WHO Tumours of the digestive system. IARC press. 2000:130-232.
16. Anwar S, Hall C, White J, Deakin M, Farrell W, Elder JB. Hereditary non-polyposis colorectal cancer: an updated review. European journal of surgical oncology 2000;26:635-645.
17. Edmonston TB, Cuesta KH, Burkholder S. Colorectal carcinomas with high microsatellite instability: defining a distinct immunologic and molecular entity with respect to prognostic markers. Human Pathology 2000;31(12):1506-1514.

18. Lynch HT, Smyk T, Lynch JF. Molecular genetics and clinical pathology features of hereditary non polyposis colorectal cancer. (Lynch syndrome). Oncology 1998;55:103-108.
19. Konishi M, Kikuchi-Yanoshita R, Tanaka K et al. Molecular nature of colon tumours in hereditary non-polyposis colon cancer, familial polyposis and sporadic colon cancer. Gastroenterology 1996;111:307-316.
20. Salashor S, Kressner U, Pahlman L, Glamelius B, Lindmark G, Lindblom A. Colorectal cancer with and without microsatellite instability involves different genes. Genes, chromosomes and cancer 1999;22:247-252.
21. Brabletz T, Jung J, Kirchner T. ß-Catenin and the morphogenesis of colorectal cancer. Virchows Archives 2002;441:1-11.
22. Takayama T, Shiozaki H, Shibamoto S et al. ß-Catenin expression in human cancers. American Journal of Pathology 1996;148(1):39-46.
23. Peifer M. *Beta-catenin* as oncogene: The smoking gun. Science1997;275:1752-1753.
24. Morin PJ, Sparks A, Korinek V et al. Activation of ß-Catenin-TCF signaling in colon cancer by mutations in *ß-Catenin* or *APC*. Science 1997;275:1797-1799.
25. Valizadeh A, Karayiannakis AJ, El-Hariry, Kmiot W, Pignatelli M. Expression of E-cadherin associated molecules (Alpha-, Beta-,Gamma-catenins and p120) in colorectal polyps. American Journal of Pathology 1997;150(6):1977-1984.
26. Shiozaki H, Tahara H, Oka H. Expression of immunoreactive E-cadherin adhesion molecules in human cancers. American Journal of Pathology 1991;139:17-23.
27. Ghadimi BM, Behrens J, Hoffmann I, Haensch W, Birchmeier W, Schlag PM. Immunohistological analysis of E-cadherin, alpha, beta- and gamma-catenin expression in colorectal cancer: implications for cell adhesion and signaling. European journal of cancer 1999;35(1):60-65.
28. Hiscox S, Jiang WG. Expression of E-cadherin, alpha, beta and gamma-catenin in human colorectal cancer. Anti cancer-research. 1997;17(2B):1349-1354.
29. Dorudi S, Sheffield J, Poulsom R, Northover JMA, Hart IR. E-Cadherin expression in colorectal cancer. American Journal of Pathology 1993;144(4):981-986.
30. Herminston MC, Wong MH, Gordon J. Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautomomous regulation of cell-fate in a self-renewing system. Genes and development. 1996;10:985-996.
31. Gagliardi G, Kandemir O, Liu D et al. Changes in E-Cadherin immunoreactivity in the adenoma-carcinoma sequence of the large bowel. Virchows Archives 1995;426:149-154.
32. Wagenaar RA, Crawford HC, Matrisian LM. Stabilised ß-Catenin immortalises colonic epithelial cells. Cancer Research 2001;61:2097-2104.

33. Akiyama T. Wnt/ß-Catenin signaling. Cytokine and Growth factor reviews 2000;11:273-282.
34. Kawasoe T, Furukawa Y, Daigo Y et al. Isolation and characterisation of a novel human gene, *DRCTNNB1A*, the expression of which is down-regulated by ß-Catenin. Cancer Research 2000;60:3354-3358.
35. Crawford JM. The gastrointestinal tract. In: Cotran RS, Kumar V, Collins T. Robbins Pathological Basis of Disease. WB Saunders company. 1999:775-845.
36. Tetsu O, McCormick F. ß-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422-426.
37. Arber N, Hiboshoosh H, Moss FH et al. Increased expression of Cyclin D1 is an early event in multistage colorectal carcinogenesis. Gastroenterology 1996;110:669-674.
38. Borre A, Cultraro CM, Segal S. C-myc inactivation by mutant Max alters growth and morphology of NCI-H-630 colon cancer cells. Journal of cellular physiology 1996;169(1):200-208.
39. Erisman MD, Scott JK, Astrin SM. Evidence that the familial adenomatous polyposis gene is involved in a subset of colon cancers with a complementable defect in c-myc regulation. Proceeding of the national academy of science, USA 1989;86(11):4264-4268.
40. He TC, Sparks AB, Rago C et al. Identification of c-myc as a target of the APC pathway. Science 1998;281:1509-1512.
41. El-Bahrawy MA, Talbot IC, Poulsom R, Jeffery R, Alison MR. The expression of E-cadherin and catenins in colorectal tumours from familial adenomatous polyposis patients. The journal of pathology 2002;198(1):69-76.
42. Kollings FT, Hajra KM, Hu G et al. Gamma-catenin is regulated by the APC tumour suppressor and its oncogenic activity is distinct from that of beta-catenin. Genes and development 2000;14(1):1319-1331.
43. El-Bahrawy MA, Poulson R, Jeffery R, Talbot IC, Alison MR. The expression of E-cadherin and catenins in sporadic colorectal carcinoma. Human pathology 2001;32(11):1216-1224.
44. Sikora K, Chan S, Evan G et al. *c-myc* oncogene expression in colorectal cancer. Cancer 1987;59:1289-1295.
45. Stewart J, Evan G, Watson J, Sikora K. Detection of *c-myc* oncogene product in colonic polyps and carcinomas. British journal of Cancer 1986;53:1-6.
46. Imaseki H, Hayashi H, Taira M et al. Expression of *c-myc* oncogene in colorectal polyps as a biological marker for monitoring malignant potential. Cancer 1989;64:704-709.
47. Melhem HF, Meisler IA, Finley GG et al. Distribution of cells expressing myc proteins in human colorectal epithelium, polyps, and malignant tumours. Cancer Research 1992;52:5853-5864.
48. D'Orazio D, Muller PY, Heinimann K et al. Overexpression of Wnt target genes in adenomas of familial adenomatous polyposis patients. Anticancer research 2002;22(6A):3409-3414.

49. Royds JA, Sharrard M, Wagner B, Polacarz SV. Cellular localisation of c-myc product in human colorectal epithelial neoplasia. Journal of Pathology 1992;166:225-233.
50. Wang HL, Wang J, Xiao SY et al. Elevated protein expression of cyclin D1 and FRA-1 but decreased expression of c-myc in human colorectal adenocarcinomas overexpressing ß-Catenin. International Journal of Cancer 2002;101:301-310.
51. Mann B, Gelos M, Siedow A et al. Target Genes of ß-Catenin-T cell-factor/Lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proceedings of the National Academy of Science 1999;96:1603-1608.
52. Zang T, Otevrel T, Gao Z et al. Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Research 2001;62:8664-8667.
53. Yamada T, Takaoka AS, Naishiro Y et al. Transactivation of the *multidrug resistance 1* gene by T-cell factor 4/ß-Catenin complex in early colorectal carcinogenesis. Cancer Research 2000;60:4761-4766.
54. Ougolkov AV, Yamashita K, Mai M, Minamoto T. Oncogenic ß-Catenin and MMP-7 (Matrilysin) cosegregate in late stage clinical colon cancer. Gastroenterology 2002;122:60-71.
55. Brabletz T, Jung J, Dag S, Hlubek F, Kirchner T. ß-Catenin regulates the expression of matrix-metalloproteinase-7 in human colorectal cancer. American Journal of Pathology 1999;155(4):1033-1038.
56. Hlubek F, Jung A, Kotzor N, Kirchner T, Brabletz T. Expression of the invasion factor laminin gamma-2 in colorectal carcinomas is regulated by ß-Catenin. Cancer Research 2001;61:8089-8093.
57. Zhang X, Gaspard JP, Chung DC. Regulation of vascular endothelial growth factor by the wnt and k-ras pathways in colonic neoplasia. Cancer Research 2001;61:6050-6054.
58. Mariadason JM, Bordonaro M, Aslam F. Down-regulation of ß-Catenin TCF signaling is linked to colonic epithelial cell differentiation. Cancer Research 2001;61:3465-3471.
59. Fujita M, Tsunoda T, Tanaka T et al. Up-regulation of the *ectodermal-neural cortex* (*ENC1*) gene, a downstream target of the beta-catenin/T-cell factor complex, in colorectal carcinomas. Cancer Research. 2001;61:7722-7726.
60. Stevens a, Lowe J. Pathology. Mosby. 1995:232-233.
61. Vogelstein B, Fearon ER, Hamilton SR et al. Genetic alterations during colorectal tumour development. New England journal of medicine 1988;319(9):525-532.
62. Kubuta O, Kino I. Minute adenomas of the depressed type in Familial Adenomatous Polyposis of the colon. Cancer 1993;72:1159.
63. Thibodeau SN, French AJ, Cunningham JM et al. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of *hMLH1*. Cancer Research 1998;58:1713-1718.

64. Boland CR, Thibodeau S, Hamilton SR et al. A national cancer institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Research 1998;58:5248-5257.
65. Dietmaier W, Wallinger S, Bocker T, Kullman F, Fishel R, Ruschoff J. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Research 1997;57:4749-4756.
66. Young J, Biden K, Simms LA et al. HPP1: a transmembrane protein-encoding gene commonly methylated in colorectal polyps and cancers. Proceedings of the National Academy of Science 2001;98(1):265-270.
67. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JPJ. CpG island methylation phenotype in colorectal cancer. Proceedings of the national academy of science, USA 1999;96:8681-8686.
68. Whitehall VLJ, Walsh MD, Young J, Leggett BA, Jass JR. Methylation of O-6-methylguanine DNA methyltransferase characterises a subset of colorectal cancer with low-level DNA microsatellite instability. Cancer Research 2001;61:826-830.
69. Ahuja N, Qing L, Avinash L, Mohan AL, Baylin SB, Issa JPJ. Aging and DNA methylation in colorectal mucosa and cancer. Cancer research 1998;58:5489-5494.
70. Jass JR, Young J, Leggett BA. Hyperplastic polyps and DNA microsatellite-unstable cancers of the colorectum. Histopathology 2000;37:295-301.
71. Markovitz S, Wang J, Myeroff L et al. In activation of the type 2 TGF-ß receptor in colon cancer cells with microsatellite instability. Science 1995;268:1336-1338.
72. Olschwang S, Hamelin R, Laurent-Puig P et al. Alternative genetic pathways in colorectal carcinogenesis. Proceedings of the National Academy of Science 1997;94:12122-12127.
73. Loving T, Meling GI, Diep CB et al. *APC* and *CTNNB1* mutations in a large series of sporadic colorectal carcinomas stratified by the microsatellite instability status. Scandinavian journal of gastroenterology 2002;37(10):1184-1193.
74. Shitoh K, Konishi F, Miyaki M et al. Pathogenesis of non-familial colorectal carcinomas with high microsatellite instability. Journal of Clinical Pathology 2000;53:841-845.
75. Huang J, Papadopoulos N, McKinley A et al. *APC* mutations in colorectal tumours with mismatch repair deficiency. Proceedings of the National Academy of Science 1996;93:9049-9054.
76. Jass JR, Walsh MD, Barker M, Simms LA, Young J, Leggett BA. Distinction between familial and sporadic forms of colorectal cancer sowing DNA microsatellite instability. European journal of cancer 2002;38:858-866.

77. Miyaki M, Iijima T, Kimura J et al. Frequent mutation of *ß-Catenin* and *APC* genes in primary colorectal tumours in patients with hereditary non-polyposis colorectal cancer. Cancer Research 1999;59:4506-4509.
78. Akiyama Y, Nagasaki H, Yaji, Nomizu T, Yuasa Y. *ß-Catenin and adenomatous polyposis coli* (*APC*) mutations in adenomas from hereditary non-polyposis colorectal cancer patients. Cancer Letters 2000;157(2):185-191.
79. Yamada Y, Yoshimi N, Hirose Y et al. Frequent *ß-Catenin* gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. Cancer Research 2000;60:3323-3327.
80. Yamada Y, Yoshimi N, Hirose Y et al. Sequential analysis of morphological and biological properties of ß-Catenin accumulated crypts, provable premalignant lesions independent of aberrant crypt foci of rat colon carcinogenesis. Cancer Research 2001;61:1874-1878.
81. Takahashi M, Fukuda K, Sugimura T, Wakabayashi K. *ß-Catenin* is frequently mutated and demonstrates altered cellular location in azoxymethane induced rat colon tumours. Cancer Research 1998;58:42-46.
82. Dashwood RH, Suzui M, Nakagama H, Sugimura T, Nagao M. High frequency of *ß-Catenin* (*Ctnnb1*) mutations in the colon tumours induced by two heterocyclic amines in the F344 rat. Cancer Research 1998;58:1127-1129.
83. Iwao K, Nakamori S, Kaneyama M et al. Activation of *ß-Catenin* gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without *APC* mutations. Cancer Research 1998;58:1021-1026.
84. Sparks AB, Morin P, Vogelstein et al. Mutational analysis of the APC/ß-Catenin/TCF pathway in colorectal cancer. Cancer Research 1998;58:1130-1134.
85. Mirabelli-Primdahl L, Gryfe R, Kim H et al. *ß-Catenin* mutations are specific for colorectal carcinomas with microsatellite instability but occur in endometrial carcinomas irrespective of mutator pathway. Cancer Research 1999;59:3346-3351.
86. Rowan AJ, Lamlum H, Iiyas M et al. *APC* mutations in sporadic colorectal tumours: A mutational "hotspot" and interdependence of the "two hits". PNAS 2000;97(7):3352-3357.
87. Iino H, Jass J, Simms LA et al. DNA microsatellite-instability in hyperplastic polyps, serrated adenomas, and mixed polyps: a mild mutator pathway of colorectal cancer ? Journal of Clinical Pathology 1999;52:5-9.
88. Jass JR. Hyperplastic-like polyps as precursors of microsatellite-unstable colorectal cancer. American Journal of Clinical Pathology 2003;119:773-775.
89. Goldstein NS, Bhanot P, Odish E, Hunter S. Hyperplastic-like colon polyps that preceded microsatellite-unstable adenocarcinomas. American Journal of Clinical Pathology 2003;119:778-796.

90. Young J, Simms LA, Biden KG et al. Features of colorectal cancers with high-level micro-satellite instability occurring in familial and sporadic settings. American Journal of Pathology 2001;159(6):2107-2116.
91. Hameed MF, Barnard R, Ramesar RS et al. Immunohistochemical detection of mismatch repair deficit in patients under 45 years with colorectal cancer. Journal of Gatroenterology and Hepatology 16, Suppl. A17, 53
92. Hao X ,Tomlinson I, Ilyas M, Palazzo JP, Talbot IC. Reciprocity between membranous and nuclear expression of ß-Catenin in colorectal tumours. Virchows Archives 1997;431:167-172.
93. Inomata M, Ochiai A, Akimoto S, Kitano S, Hirohashi S. Alteration of ß-Catenin expression in colonic epithelial cells of familial adenomatous polyposis patients. Cancer Research 1996;56:2212-2217.
94. Iwamoto M, Ahnen DJ, Franklin WA, Maltzman TH. Expression of ß-Catenin and full length APC protein in normal and neoplastic colonic tissues. Carcinogenisis 2000;21(11):1935-1940.
95. Jass JR, Barker M, Fraser L et al. *APC* mutation and tumour budding in colorectal cancer. Journal of Clinical Pathology 2003 Jan;56(1):69-73.
96. Back W, Loff S, Jennie D, Bleyl U. Immunolocalisation of ß-Catenin in intestinal polyps of Peutz-Jeghers and juvenile polyposis syndromes. Journal of Clinical Pathology 1999;52:345-349.
97. Hugh TJ, Dillon SA, Taylor BA, Pignatelli M, Poston GJ, Kinsella AR. Cadherin-catenin expression in primary colorectal cancer: a survival analysis. British journal of Cancer 1999;80(7):1046-1051.
98. Rothberg PG, Spandorfer JM, Erisman MD. Evidence that c-myc expression defines two genetically distinct forms of colorectal carcinoma. British Journal of Cancer 1985;52:629-632.
99. Kim JS, Crooks H, Foxworth A, Waldman T. Proof of Principle:oncogenic ß-Catenin is a valid molecular target for the development of pharmacological inhibitors. Molecular cancer therapeutics 2002;1:1355-1359.
100. Chen RH, McCormick F. Selective targeting to the hyperactive ß-catenin/T-cell factor pathway in colon cancer calls. Cancer Research 2001;61:4445-4449.
101. Kwong KY, Zou y, Day CP, Hung MC. The suppression of the colon cancer cell growth in nude mice by targeting beta-catenin/TCF pathway. Oncogene 2002;21(54):8340-8346.
102. He TC, Chan TA, Vogelstein B, Kinzler KW. PPAR-delta is an APC-regulated target of non-steroidal anti-inflammatory drugs. Cell 1999;99:335-345.