

**HEREDITARY NON-POLYPOSIS COLORECTAL CARCINOMA
(HNPCC): MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL
STUDIES.**

BY

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DECLARATION

I, Hannes Holm, hereby declare that the work on which this thesis is based is original (except where acknowledgements indicated otherwise), and that neither the whole work nor part of it has been, or is being, or is to be submitted for a degree in this or any other University.

Signed by candidate

Hannes Holm
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ABBREVIATIONS

AC	Amsterdam criteria
ADP	Adenosine diphosphate
APES	3-Aminopropyltriethoxysilane
CANSA	Cancer Association of South Africa
CIN	Cervical intraepithelial neoplasia
CNS	Central nervous system
COX	Cyclooxygenase
CRC	colorectal carcinoma
CRCRC	Colorectal carcinoma research consortium
DNA	Deoxyribo nucleic acid
FAP	Familial adenomatous polyposis
GSH	Groote Schuur Hospital
HE	Haematoxylin and Eosin stained section
HNPCC	Hereditary non-polyposis colorectal carcinoma
HMRDS	Hereditary mismatch repair deficiency syndrome
HPV	Human papilloma virus
IHC	Immunohistochemical
LOH	Loss of heterozygosity
MGMT	Methylguanine DNA Methyltransferase
MSI	Microsatellite instability
MSI-H	High microsatellite instability
MSI-L	Low microsatellite inst
MSS	Microsatellite stable
NF	Neurofibromatosis
NHLS	National Health Laboratory Service
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PG	Prostaglandin
RNA	Ribo nucleic acid
TNF	Tumour necrosis factor
TXA	Thromboxane alpha
UCT	University of Cape Town
VHL	Von Hippel-Lindau

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CHAPTER ONE: INTRODUCTION

COLORECTAL CARCINOMA IN SOUTH AFRICA

Worldwide, 875 000 new colorectal carcinoma cases, estimated in 1996, representing 8.5% of all newly diagnosed carcinomas are believed to occur [1].

Most of these are considered sporadic (80%). 10-15% are known familial risk and 5-10% are genetic inherited [2]. These subsets of inherited cancer syndromes are well studied in an attempt to understand the carcinogenesis of colorectal carcinoma and ultimately the prevention and treatment thereof.

A great variation of incidence around the world is seen with developed countries such as North and South America, Europe, Australia, New Zealand and part of Asia with still low but rising rates in Malaysia and Korea and relative low incidence in Africa and Asia. Significant differences are also noted within the same countries [3].

Immigrants and their descendants who have relocated from low incidence countries to high incidence countries rapidly reach the incidence rates of the high incidence countries, indicating the importance of environmental factors. The incidence in Korea, Japan and Singapore has rapidly increased probably related to their adopted western life style [4].

An increasing incidence of colorectal carcinoma is noted not only in African Americans but also in African Blacks [5, 6].

The etiology of colorectal carcinoma is multifactorial with both environmental (local toxic effects and mutation) and constitutional factors (e.g. germline mutation of repair gene) playing a role in development and progression of colorectal carcinoma [7, 8]. Many studies on dietary and environmental factors were performed. The difficulty in interpreting these results is multiple variables and inconsistent findings in different populations and circumstances of individuals. Dietary factors such as animal fat, refined carbohydrates, low fiber diet, lack of vegetables and trace elements are potential risk factors for carcinogenesis [9-14]. Epidemiological studies indicate that meat consumption, smoking and alcohol are risk factors whereas a vegetable rich diet, prolonged use of non-steroidal anti-inflammatory drugs, oestrogen replacement therapy and physical activity are protective [1, 15]. Some studies suggest carcinogens produced by bacteria. Others postulate the carcinogenic role of bile acids [16-19]. All these factors certainly may play a role in initiation or progression of carcinoma together with interaction between environment and gene mutation.

Chronic inflammatory conditions of the colon are associated with an increased risk to develop carcinoma (eight to ten fold in longstanding pancolitis in ulcerative colitis) [20]. In ulcerative colitis the risk of developing carcinoma is directly related to the extent of involvement. If more than half the colon affected, the risk of developing carcinoma is 15%. If only the left colon affected, the risk is 5% and isolated proctitis is not associated with an increased risk [21-26]. For Crohn's

disease the risk is reported to be three-fold [27]. Radiation is a known etiological factor with patients at increased risk after therapeutic irradiation [28]. Patients with ulcerative colitis and primary sclerosing cholangitis who undergo liver transplantation are also at increased risk for developing colorectal carcinoma [29].

Several studies on the South African population have been conducted since an interesting mixture of Western descendants, African descendants and African descendants with westernized diets are represented within the Southern African population group.

The incidence of malignancies of the gastrointestinal tract in patients in Grootte Schuur Hospital, Cape Town [30] were as follows in decreasing order:

1. stomach (29.6%),
2. colon and rectum (29.5%) and
3. oesophageal carcinoma (19.8%).

Marked differences in the site of the carcinoma according to race were found; oesophageal carcinoma was the most frequent in black South African patients (62.1%), gastric carcinoma in colored (44.1%) and colorectal carcinoma in white patients (46.8%). Black African patients rarely showed pancreatic (5.1%) or colorectal carcinoma (3.5%).

Poorly differentiated signet and mucinous type adenocarcinoma of the colorectum have often been noted in very young black South African patients (often younger than 25 years of age) showing very aggressive behavior and poor prognosis. No pre-malignant lesions could be identified in these case reports. Several such case reports and small series were published [31].

Two studies done in South Africa show contrasting results: one showing an increase in incidence of colorectal carcinoma in black South African patients [31]. Two periods were evaluated: the one being 1986-87 and the second 1996-97. Also an increase of adenomatous polyps were noted and a younger age of onset (less than 40 years old) was noted [32].

In contrast to the above study a second study showing a still low incidence with occasional associated adenomatous polyps (5.2%) in patients with westernized diets (1991 and 1996). 6% occurred in patients younger than 30 years of age and 22% before the age of 40. More than three quarters arose in sigmoid and rectum with none arising in the right colon with nearly a third of cases were mucinous or signet ring cell carcinoma type [33]. Large bowel carcinomas in black South African patients show a very high percentage (31%) of high grade mucinous and signet ring cell carcinoma with a predilection of younger age and rectal site [34].

Another study shows a high proportion of cases with early onset (32.4% are younger than 40 years old) of which a very high proportion (45%) were right sided neoplasia [35].

Dietary factors were extensively studied in black South Africa with multiple variables and inconclusive results [36].

Another interesting finding in African patients is the low number of adenomatous polyps, synchronous carcinomas and diverticular disease [37].

1. HEREDITARY NON-POLYPOSIS COLORECTAL CARCINOMA (HNPCC)

Inherited cancer syndromes involving the colon include familial adenomatous polyposis (FAP), juvenile polyposis and hereditary non-polyposis colorectal carcinoma (HNPCC).

HNPCC (also known as Lynch syndrome or hereditary mismatch repair deficiency syndrome or HMRDS) is inherited in an autosomal dominant fashion and was originally recognized by Warthin and described by Lynch [38-42]. Today we know at least six mismatch repair genes involved in HNPCC. These have been isolated by cloning (see below section 1.4).

Clinical features

Clinical features of individuals with HNPCC include a high risk of developing colorectal carcinoma (70-85%) and endometrial carcinomas (50%) often at an early age (mean of 45 years old) [43, 44]. The colonic carcinomas are located in the proximal (right sided) colon in more than 65% of cases. Metachronous and synchronous carcinomas are seen in up to 35% of patients often presenting with multiple lesions (but not hundreds as seen in FAP). Therefore the name non-polyposis is used, although polyps are strictly speaking present.

At clinical presentation, the patients are less likely to have lymph node involvement or metastases even if the individuals picked up on screening (surveillance) programs are excluded [45].

Extracolonic neoplasias described include endometrial, ovary, breast, renal pelvis, ureter, stomach, pancreas and small bowel [40, 41, 46, 47].

Glioblastoma associated with colorectal adenomas/carcinomas is referred to as Turcot syndrome whereas sebaceous gland adenomas/carcinomas with colorectal carcinomas is referred to as Muir-Torre syndrome. Both show some similar genetic alterations [41, 48-51].

Macroscopic features

The macroscopic features of colonic adenocarcinomas in HNPCC are in most cases similar to those seen in non-familial carcinomas with a high frequency of microsatellite instability (MSI-H).

Macroscopical characteristics include a predilection for the proximal (right sided) colon [45]. This includes the caecum and ascending colon [41].

The lesions are mostly polypoid, well circumscribed, with ulcers, plaques and seldom show a diffuse infiltrating pattern [52, 53]. Therefore these patients often present only late in disease progression with bowel obstruction.

Adenomas are present but less numerous than in FAP and more prevalent as in age matched controls of the general population [54]. Adenomas are not predominantly present in the right colon in older individual due to development of sporadic adenomas in the distal colon in this age group [54]. The relative low numbers of adenomas is thought to be as a result of the rapid accumulation of genetic alterations and therefore rapid progression from normal vulnerable epithelium (microscopically normal) through adenomas with dysplasia to infiltrating carcinoma [55]. This is also reflected in our own clinical survey (yearly) where individuals at risk had colonoscopies with no macroscopic abnormalities presenting with infiltrating colorectal carcinoma and multiple adenomas a year later reflecting very rapid progression in initial carcinogenesis (unpublished data, Prof. P Goldberg, Department of Surgery, UCT/Groote Schuur Hospital).

Microscopic features

Tubulo-villous or villous adenomas are usually seen adjacent to infiltrating carcinomas. The adenomas more often show often high-grade epithelial dysplasia with rapid progression, as earlier described. Flat adenomas are usually only identified on histological examination showing a single crypt or few adjacent crypts with epithelial dysplasia. These then progress to polypoid adenomas.

Microscopically HNPCC are said to show "typical" but not diagnostic features. These include mucinous carcinomas (more than 50% mucinous component by convention) with well circumscribed borders, poorly differentiated carcinomas (high grade), intratumoral lymphocyte infiltration, marked lymphocytic infiltration surrounding the tumour and lymphoid aggregates in the adjacent stroma ("Crohn-like inflammation") [45, 52, 53, 56].

Individuals with MSH2 mutations more often show mucinous tumours and poorly differentiated carcinomas, whereas MLH1 mutations show more often the histological features of intratumoral lymphocytes and Like-like inflammation [53].

Extracolonic cancers are more commonly seen in patients with MSH2 mutations compared to those with MLH1 mutations [57]. MSH6 mutations are associated with atypical clinical presentation and late onset of disease [58, 59].

1.1. MOLECULAR MECHANISM AND CARCINOGENESIS IN CRC

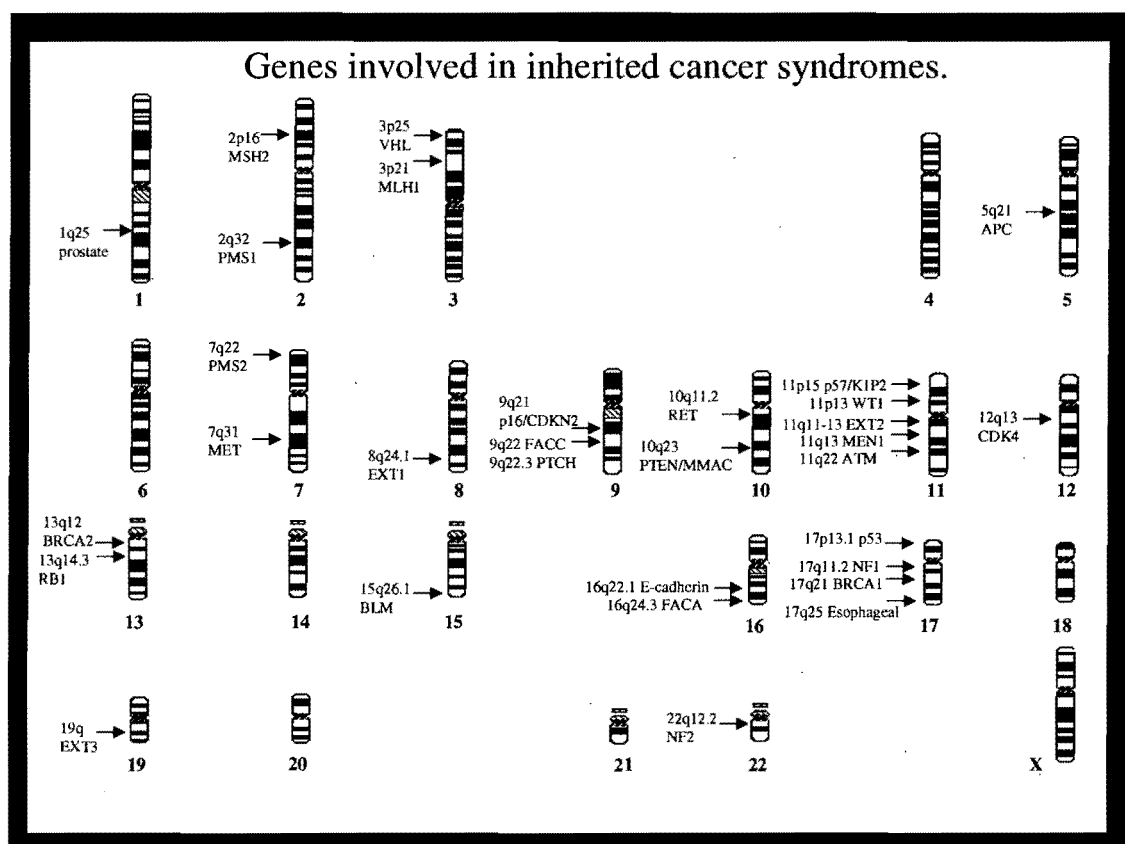
The study of carcinogenesis and molecular mechanism in the field of colorectal carcinoma has been particularly fruitful in our understanding of carcinogenesis in general. This is mostly due to the intensive study of familial cancer syndromes such as FAP and HNPCC, which led to the discovery of important cancer genes. These genes play an important role not only in familial cancers (colonic and extracolonic) but also play important roles in carcinogenesis of sporadic carcinomas, albeit at different stages in carcinogenesis [60].

The multistep carcinogenesis (adenoma-carcinoma sequence) concept is well described and accepted in sporadic carcinomas and supported by clinical, pathological and epidemiological data [61]. In the histological normal epithelium genetic alteration may already be present. This might be either in the form of a germline mutation or an acquired somatic mutation resulting in epithelium at risk. A second mutation resulting in the inactivation of the wild-type allele results in clonal proliferation of cells. Each step is initialized by a genetic change with clonal proliferation, different characteristics and biological behavior [7, 62].

Two different pathways have been described for genetic changes in carcinogenesis: classic (or "gatekeeper") and alternative (or "caretaker") pathways. "Gatekeeper" genes are those initializing the first mutations and genetic alteration leading the epithelium into the first steps of carcinogenesis (entry into multistep carcinogenesis). Examples of such genes are APC, Rb, VHL

and NF-1 [7]. See figure 1.1 below of examples of inherited cancer genes and their loci.

Fig 1.1: Examples of genes involved in inherited cancer syndromes. On this figure the gene name and its location is indicated next to the gene. Note gene number 2 (MSH2 and PMS1), gene 3 (MLH1) and gene 7 (PMS2) often mutated in HNPCC.



Contrasted to this, “caretaker” genes repair altered DNA and prevent small genetic alterations to proliferate and expand resulting in genomic instability. Examples of such genes include hMSH-2, hMLH-1, BRCA-1 and BRCA-2.

For carcinogenesis via this pathway, four steps are needed to enter clonal proliferation. A first somatic mutation of one allele of the caretaker gene, then a second hit to the other allele with inactivation of the caretaker gene. This per se is not enough to result in clonal expansion but does cause genomic instability. Both copies of gatekeeper genes (e.g. APC) need to be inactivated resulting in clonal expansion. In contrast, patients who already have one germline gene defect require only three more genetic alterations to enter carcinogenesis, therefore being at much higher risk to develop early and multiple carcinomas.

In sporadic carcinomas the multistep carcinogenesis can be summarized as follows (see also table 1.1):

APC mutation is most often the first alteration in at least 80% of sporadic colorectal carcinoma with less frequent mutations of mismatch repair genes.

Early hypomethylation occurs resulting in activation of oncogenes. These include K-ras (chromosome 12p12), which plays a role in intracellular signal transduction. This gene is mutated in 50% percent of large adenomas and infiltrating carcinomas.

DCC (deleted in colon cancer, a cell adhesion molecule, chromosome 18q21) is normally widely expressed in normal colonic epithelium and reduced in 70-75% of colon carcinomas [60].

P53 (chromosome 17p13) mutations in 70-80% in infiltrating carcinomas are seen and infrequently present in adenomas suggesting a late mutation [60].

In the progression from adenoma to carcinoma, subsequent late mutations and loss of heterozygosity occur with inactivation of the second allele ('second hit') in p53 and DCC genes [60].

Late changes associated with infiltrating carcinoma and progression includes multiple additional mutations and gross chromosomal alterations [60].

A few topical concepts in colorectal carcinogenesis are discussed below.

1.1.1. Methylation

Both hyper- and hypomethylation of DNA occur in carcinogenesis of colorectal carcinoma although the exact cause is not fully understood. Methylation occurs at the CpG sites of genes. Genes rich in CpG sites are therefore prone to be silenced by methylation [63]. Two types of methylation (type A associated with ageing and in some colorectal carcinomas and type C associated only with carcinoma) have been described [64]. MLH-1 gene is one mismatch repair gene associated with methylation thus resulting in microsatellite instability in sporadic colorectal carcinoma [65].

Methylguanine DNA methyltransferase (MGMT), also affected by methylation, and *K-ras* are linked to MSI-L pathway in which *K-ras* mutation is frequent [66, 67]. Other genes inactivated by methylation include oestrogen receptor, HPP1, COX-2, p14 and p16 [63,68-72].

1.1.2. Microsatellite instability

Microsatellites are tandem repeats of either mononucleotide (AAAAA) or dinucleotide (ACACAC) base pairs in the non-coding regions of the genome. These changes often happen during replication and are particularly prone in tissue with high turnover of cells and constant replication. With normal repair genes, these "spelling errors" are repaired. If the repair mechanism is impaired, accumulation of satellites occurs resulting in an unstable genome –the microsatellite instability. The grade of instability is measured by bandshifts in a panel of microsatellite markers. For high microsatellite instability (MSI-H), at least 40% of the markers should show a bandshift whereas less than 40% are considered microsatellite stable (MSS or MSI-L) [73].

Mixed hyperplastic polyps, serrated adenomas, adenomas and carcinomas in HNPCC interestingly show MSI-H contrasted to sporadic adenomas, which are MSS [74, 75]. MSI-H carcinomas show reduced mutation of APC, p53 and *K-ras* and commonly show LOH at 5q, 17p and 18q [52, 66, 76-79]. Contrastd to this, mutations are commonly encountered in TGFRII, IGF2R, BAX, E2F-4, MSH3, MSH6 and caspase 5 [78, 80].

DNA microsatellite instability is an important marker in identifying HNPCC carcinoma. A panel of five markers is recommended currently and includes BAT25, BAT26, D2S123, D5S346 and D17S250 [73, 81]. About 60% of adenomas in patients with HNPCC are MSI-H [82]. MSI-H is seen in all HNPCC and in up to 30% (but in the range of 10-15% [73]) of sporadic carcinomas, thus useful in identifying possible HNPCC cases.

1.1.3. The hyperproliferation concept and flat adenomas

Pioneering work by Lipken and Deschner [86] on cell kinetic studies, evaluating the cell proliferation in normal colonic crypts and adenomas of FAP families, was done. Two phases were identified: phase 1: proliferation limited to cells of the lower compartment, phase 2: cycling cells in the upper and surface compartment progressing to adenomas [83, 84]. Several problems occur with this theory amongst others that anatomical site, gender, bowel preparation, age, diet, chronic diseases and other variables influence the changes noted [85-88]. Other changes noted are fission of crypts and decreased apoptosis in the upper crypt zones and surface epithelium, reversible by COX-2 inhibitors [89, 90].

Unicryptal adenomas start as a small bud from the side of a normal crypt, forming a tubule migrating to the surface of the luminal epithelium. Fission occurs with expansile growth forming an adenomatous polyp [89, 91]. Occasionally a

few crypts in a vicinity are affected resulting in a flat adenoma. These might progress to a polyp and are often polyclonal proliferations [92-94].

1.1.4. Aberrant crypt foci and adenoma progression

Experimental animal studies [95] and routine specimens show changes other than typical adenomas or hyperplastic polyps with crypt branching and widening in the absence of cytological atypia [96, 97]. These hyperplastic foci are clonal and show K-ras mutations (possibly relating to Lipkin and Drescher phase 1) whereas microadenomas show APC mutations (possibly relating to Lipkin and Drescher phase 2) [97]. Flat adenomas lack K-ras mutation whereas polypoid adenomas (like the hyperplastic polyps) most commonly have the K-ras mutation reflecting possible different pathways of carcinogenesis and tumour progression [98-100]. Therefore flat-, depressed- or microadenomas are now well recognized and genetically characterized [101, 102].

Progression of adenomas seems to follow the initial model of Fearon and Vogelstein with mutations of APC, TP53, DCC and K-ras [103]. This is true for most sporadic and some FAP cases but does not explain all FAP, non-polyposis, villous adenomas to mucinous carcinoma, tumours with low p53 or K-ras mutations [98, 104-108].

DNA microsatellite instable carcinomas progress through a distinct pathway involving mutations of TGF-BRII receptor, GF2R and BAX [80, 109, 110].

The final conversion from adenomas to carcinoma is statistically a very unlikely event considering the abundance of adenomas compared to the actual infiltrating carcinomas. Multiple changes occur at this stage including metabolic pathways, growth factor production with stromal response and angiogenesis, proteolytic enzyme activity for invasion, alteration in adhesion molecules, increased telomerase activity, alteration to glycoproteins of membranes as well as aneuploidy [111-120].

1.2. DIAGNOSTIC CRITERIA (AMSTERDAM CRITERIA)

In 1990 the International Collaborative Group on HNPCC proposed a set of diagnostic criteria to set a uniform base for further studies (Amsterdam Criteria I) [121, 122]. These were initially not universally accepted and did not allow extracolonic presentation of carcinomas as an inclusive criterion.

A new set of criteria (Amsterdam Criteria II) has been formulated in 1999 to accommodate the extracolonic neoplasias [57].

These are summarized as follows:

There should be at least three relatives with an HNPCC-associated cancer and include colorectal carcinoma, endometrial carcinoma, small bowel carcinoma, carcinoma of the ureter or renal pelvis.

1. One patient should be a first degree relative of the other two
2. At least two successive generations should be affected

3. At least one tumour should be diagnosed before the age of 50
4. Familial adenomatous polyposis should be excluded in cases of colorectal carcinoma
5. Tumours should be verified by histopathological examination

It is noted that not all families fulfilling these criteria have mismatch repair gene defects indicating a percentage of families with alternative forms of inherited predisposition to develop colon carcinoma [123].

In one study of a Korean cohort, the mutation detection rate is hardly affected by the change of criteria from ACI to ACII. The reason being the wide selection of individuals at risk for genetic screening [124]. HNPCC families not complying with the ACII show extremely low frequency (8% versus 49% of patients fulfilling the ACII) of mismatch repair gene defects and microsatellite instability [125].

ACII is reliable and shows reproducible and practical results. However, genetic testing is important in patients suggestive of non-polyposis colorectal carcinoma falling short the diagnostic criteria [126].

1.3. IMMUNOHISTOCHEMICAL STUDIES ON PROTEIN PRODUCTS IN TUMOURS

Expression of gene products is a normal phenomena, and is in particular prominent in normal epithelium of the gastrointestinal tract, testis and ovary [127-

129]. Normal staining for gene products is increased in the replicating compartment of the colonic epithelium, namely in the base of crypts.

An unpublished study of immunohistochemical staining for gene products MLH1 and MSH2, performed in our laboratory using patients with colorectal carcinomas under the age of 45 years was undertaken. Of the 51 tumours stained, 12 (23.5%) showed negative staining for MLH1 and 7 (14%) showed negative staining for MSH2, therefore suggesting mismatch repair gene defects. In this study, all cases of known HNPCC (germline screened) were identified therefore proving, as in other studies, immunohistochemical staining for mismatch repair genes to be successful and sensitive [130].

1.4. GENETIC STUDIES AND GENE PROFILES

Genes involved in carcinogenesis (cancer genes) are the imbalance of activation or inactivation of proto-oncogenes as well as tumour suppressor genes (see table 1.1).

Proto-oncogenes (oncogenes) promote the proliferation of cells resulting in uncontrolled growth. These are usually up regulated in neoplasia (over expression). In contrast tumour suppressor genes inhibit uncontrolled growth of cells. In neoplasias these are usually silenced by mutation resulting in down regulation (under expression).

Mutation of proto-oncogenes leads to unregulated growth whereas loss of tumour suppressor genes result in uncontrolled cell cycling, reduced DNA repair and abnormal signaling pathways [7, 131-140].

Table 1.1: Summary table of genetic alterations involved in colorectal carcinoma. The table indicates the gene involved, the microsatellite stability status of the particular gene and the alteration (mutation, LOH or methylation) undergone to result in a defective gene function. Adapted from [141].

Alteration	Gene	MSS	MSI-L	MSI-H
Mutation	APC	+++	+++	+
	K-ras	++	+++	+
	TP53	+++	+++	+
	TGFBetaRII	+	+	+++
	IGF2R	-	-	+
	BAX	-	-	++
	Caspase-5	-	-	+
	CDX-2	-	-	+
	BCL-10	-	-	+
	E2F-4	-	-	+
LOH	5p (APC)	+++	++	-
	17p (TP53)	+++	+++	-
	18q (DCC)	+++	+++	-
Methylation		+	+++	+++

Oncogenes are either activated by mutation (e.g. K-ras) [7, 131] or dysregulation of signaling pathway (e.g. c-myc in wnt signaling pathway) [142]. A single allelic

change may manifestate in partial abnormality and does relate to an increase risk of carcinoma.

Inactivation of the one allele of tumour suppressor genes is either an inherited or acquired mutation. The second allele may be either by mutation or often loss of heterozygosity (LOH) as seen in chromosomes 22q, 17q, 14q, 8p and 1p in colorectal carcinoma [143-150].

Five mismatch repair genes in HNPCC are identified showing an autosomal dominant transmission and are listed and described in table 1.2 [151].

Table 1.2: Characteristics of known HNPCC-associated DNA mismatch repair genes [151].

Gene	Chromosome	Length	Exon number	Genomic size
MSH2	2p21	2.8kb	16	73kb
MLH1	3p31-p23	2.3kb	19	58-100
PMS1	2q31-q33	2.8kb	?	?
PMS2	7p22	2.6kb	15	16
MSH6	2p21	4.2kb	10	20

The function of mismatch repair genes can be summarized as follows:

Two heterodimeric complexes do mismatch recognition: MSH2-MSH3 and MSH2-MSH6 complexes. MSH3 and MSH6 are interchangeable whereas normal functioning MSH2 is mandatory for recognition of insertion-deletion mismatches.

Once mismatch binding has occurred, the heterodimeric complex proteins MLH1-PMS2 and MLH1-MLH3 are mobilized with multiple enzymatic action to repair the mismatched base pairs. Once again, MLH1 is essential in this complex for successful repair.

Most mutations in all parts of the world are either MLH1 or MSH2: the essential proteins in both identification and repair complexes. Hot spots were identified in exon 12 in MSH2 and exon 16 in MLH1 [152]. Less common mutations include MSH6, PMS1 and PMS2 [153].

Mutations in these DNA mismatch repair genes account for only two-thirds meeting the ACII and showing MSI-H [154] and less than 30% in HNPCC kindreds not meeting the ACII [125, 155]. Some patients meeting the ACII, showing colonic carcinoma and extracolonic cancers do not show mismatch repair gene mutations but TGFbeta-RII and E-cadherin and no MSI [156, 157].

1.5 COLORECTAL CARCINOMA RESEARCH CONSORTIUM (CRCRC)

In 1999 a consortium was formed using expertise in the Western Cape to study colorectal carcinomas: the epidemiology, demography, pathology and genetics.

Colorectal surgeons (Prof. Paul Goldberg and Dr. Fayaz Hameed), gastrointestinal physicians (Prof. Japie Louw and Dr. Trevor Winter), anatomical pathologists (Prof. Pauline Hall, Dr. Hannes Holm and Dr. Jenny Watkins), human geneticists (Prof. Raj Ramesar and Rebecca Felix) and research nurses (Ursula Algar) all form part of an integrated team to establish the extent of disease in the Western and Northern Cape regions.

Financial support and epidemiological data is provided by Cancer Association of South Africa (CANSA) and partially funded by THRIP.

HNPCC patients have been identified in these areas, particularly in the Northern Cape province, in villages which often are remote and isolated. See figure 1.2 showing the distribution of MLH1 mutation families. This again results in a small genetic pool with often high prevalence of mutations within these communities.

These groups of families are in particular of importance for surveillance and prevention of advanced carcinomas. 1079 individuals within 294 families are identified to be at risk. So far 291 of these were genetically screened for mutations. Those with positive results and those not yet tested undergo regularly (yearly) colonoscopic surveillance.

In the year 2000, 156 colonoscopies were done on the yearly visit to the remote clinics. 137 of these showed endoscopically normal bowel and 19 showed mucosal lesions. Of these, two were mutation negative, one showing a

hyperplastic polyp and the other, non-specific inflammation. The remainder (17) of these showed 9 adenomatous polyps, 3 hyperplastic polyps and five other non-neoplastic pathologies (inflammation, melanosis coli, lymphoid, etc.). 14% of mutation positive individuals developed an adenoma.



Fig 1.2: Distribution of families is shown in this map in the Western and Northern Cape along the west coast. The size of the star represents the number of individuals with known MLH1 mutation. The Clanwilliam star represents approximately 25 individuals as a rough guide (courtesy Prof. R Ramesar, Human Genetics, UCT).

Two studies were undertaken in an attempt to understand and characterize HNPCC cases in South Africa:

1. The pathological characteristics of these tumours were evaluated and compared to sporadic tumours in South Africa.
2. COX-2 expression of HNPCC tumours were evaluated compared to sporadic adenomas and carcinomas.

These results will then be compared to other studies around the world demonstrating similarities and differences.

CHAPTER TWO: INTRODUCTION

CYCLOOXYGENASE ENZYME (COX-1 and COX-2)

2.1 STRUCTURE AND FUNCTION

Two isoenzymes are described which have very similar crystalline structures: COX-1 and COX-2. The gene encoding COX-1 is located on chromosome 9 at q32-q33.3 whereas COX-2 in chromosome 1 at q25.2-25.3 [158-160]. COX-2 has a slightly smaller gene intron of 8kb in COX-2 compared to the 22kb in COX-1. The reason for this is that COX-2 has a single exon in the place of exons 1 and 2 in COX-1 [160, 161]. COX-1 and COX-2 have a molecular weight of 71kb, a length of 600 amino acids and 63% identical sequencing [158].

COX-2 is found on the nuclear- and endoplasmic reticulum membrane contrasted to COX-1 which is only found attached to the endoplasmic reticulum [160, 162]. COX-1 and COX-2 have no transmembrane domain but are an integral part of membrane proteins attached with three amphipathic alpha helices.

Cyclooxygenase (COX) is the enzyme catalyzing the initial steps of transforming arachnidonic acid (the substrate) via prostaglandin G2 and prostaglandin H2 to the prostanoid end products PGD2, PDE2, PGF2, PGI2 and TXA2 [158, 163].

COX is classified into two isoenzymes: constitutive (COX-1) enzyme expressed in nearly all cell types at a constant level or induced (COX-2) which is only

expressed by inflammatory stimuli such as lipopolysaccharide, interleukins (1 and 2) and tumour necrosis factor alpha (TNF) [159, 160, 163-167]. Unlike COX-1, COX-2 expression can be rapidly reduced once stimuli are subside or become absent.

See figure 2.1 for a simplified illustration of the function and metabolism of COX and the metabolism of Arachnidonic acid.

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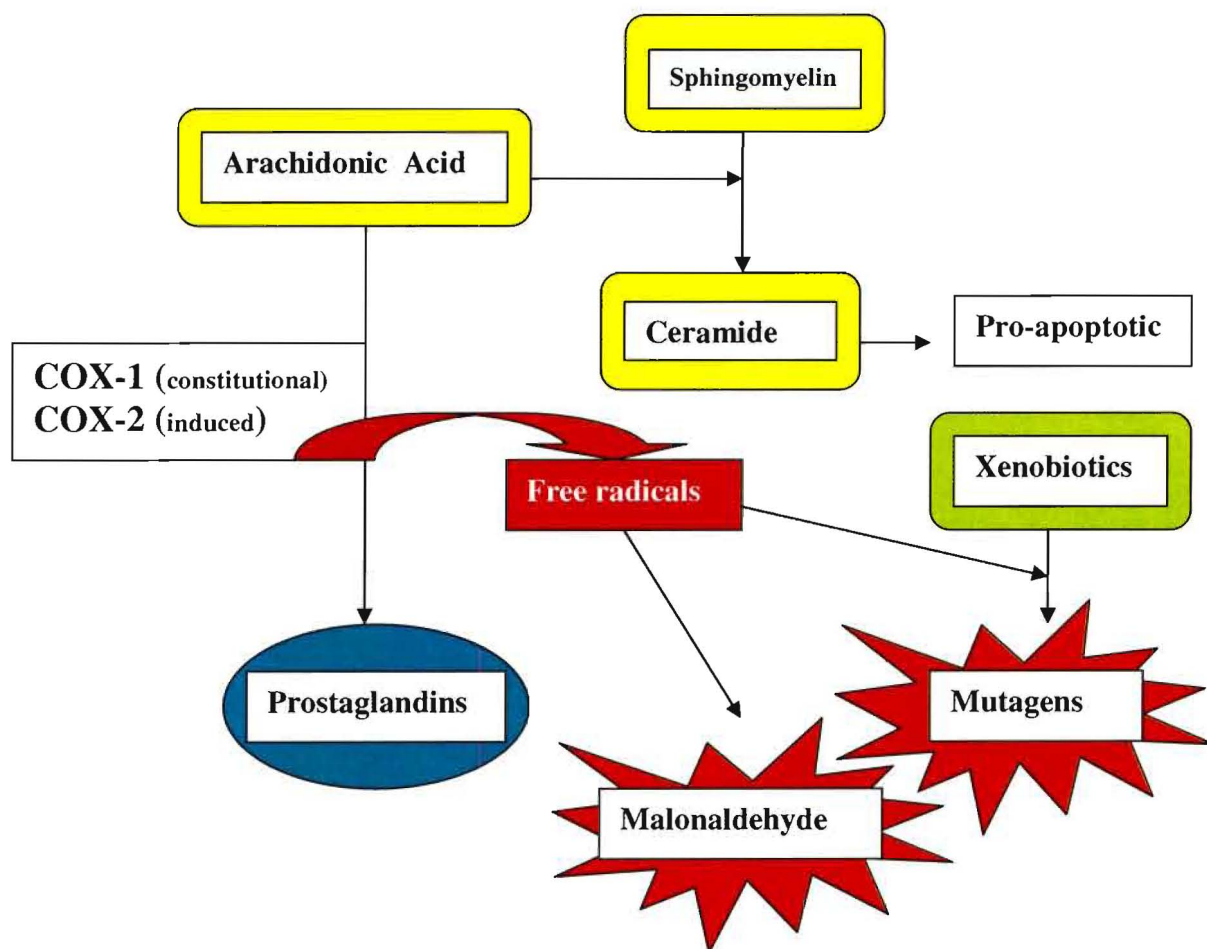


Fig 2.1: Function and metabolism of COX in the metabolism of Arachidonic acid, the break down products and the postulated mechanism of genetic alteration. Adapted from [168].

Implantation, foetal and placental development

COX is expressed in the endometrial epithelium, present at implantation (mediation with prostaglandins) of the embryo and establishment of the placental vasculature [158, 169, 170]. Animal models show multiple female reproductive failures in COX-2-deficient mice [171]. Premature labour is also suppressed by COX-2 inhibition, particularly if the labour is associated with infection and release of cytokines [172]. COX-2 is of importance in fertility (ovulation, implantation, decidual change) [171, 173-175].

Haematological and vascular effects

Platelet adhesion is mediated by von Willebrand factor binding to specific platelet surface receptors. Adenosine diphosphate (ADP) and thromboxane A2 (TXA2) are the two most pivotal platelet-activating factors [176]. Inhibition of COX-1 leads to reduced TXA2 and therefore reduces platelet aggregation and vasoconstriction [176, 177]. COX-1 inhibition on platelets is irreversible and the effect lasts as long as the lifespan of platelets (8-10 days). This mechanism and the effect of COX-1 is used in the treatment of coagulative disorders.

COX-2 has a lesser effect on platelets and is therefore also not used in coagulative preventative therapy [178].

COX enzyme in the central nervous system

Both COX-1 and COX-2 are expressed in the central nervous system: COX-1 widely and generally expressed and COX-2 mainly in the forebrain cortex, hypothalamus, hippocampus and the spinal cord [158, 169, 179-181]. Prostaglandins in the brain are mainly PGE2 and PGD2 subtypes. Lipopolysaccharides induce release of cytokines and activate COX-2 in CNS endothelial cells. PGE2 affects the temperature sensitive neurons and results in fever induction. COX-2 is also induced in sensory neurons resulting in inflammatory pain [158, 169, 180, 182].

Microglial cells also express COX-2 and prostaglandins particularly in the vicinity in neuritic plaques (amyloid containing) seen in Alzheimer diseases. This resulted in the postulated partial preventative effect of COX-2 inhibitor therapy [158, 169, 181, 183].

Gastrointestinal effects

COX-1 has a vasodilator effect and therefore enhances mucosal blood flow. COX-1 inhibition therefore results in erosion, ulceration and the complications such as anaemia, perforation and malabsorption [158, 169, 177]. This is not the only mechanism involved resulting in mucosal damage. Prostaglandins are essential in gastric mucosal regeneration and if suppressed result in suppressed epithelial regeneration and repair [184].

COX-2 is induced in the gastrointestinal tract following inflammatory stimuli such as infection or idiopathic inflammatory bowel disease. Therefore COX-2 inhibition worsens healing and aggravates acute idiopathic inflammatory bowel disease in general [184, 185].

Renal effects

COX-1 induction produces PGI₂, PGE₂ and PGD₂ resulting in reduced vascular resistance through dilatation of renal vessels and increased organ perfusion. Blood is shunted from the renal cortex to the juxtamedullary region [169, 186, 187].

COX-2 is associated with renal salt excretion and upregulation of COX-2 in the maculae densa of the juxtaglomerular apparatus and adjacent epithelial cells [188, 189]. This is illustrated by sodium retention, generalized oedema and increased in blood pressure when inhibiting COX-2 [187, 190].

2.2 ROLE IN INFLAMMATION

The discovery of COX-2 and the initial suggestion that the enzyme is only expressed in acute and chronic inflammation raised the possibility of selective treatment without side effects of NSAID such as gastric erosion and renal toxicity [191, 192]. This prompted development and production of highly selective COX-2 inhibitors. Today more than 70% of people in the USA over the age of 65 years

(over 17 million) take NSAIDs for different types of arthritis of which osteoarthritis is the most common [193].

Many animal models show the over expression of COX-2 in acute and chronic inflammation [194, 195]. Injecting cotton oil resulting in foreign body reaction and chronic inflammation simulates pannus formation and inflammation similar to that seen in rheumatoid arthritis [196]. An initial peak in PGE₂ at two hours corresponds with the affect of acute inflammation in a pleurisy induced animal model [196]. This proves the role of COX-2 expression in acute inflammation.

Multiple studies show that COX-2 has a protective role in the gastrointestinal tract including those showing increased expression of COX-2 at the edge of gastric ulcers and delayed healing of ulcers when treated with COX-2 inhibitors [197-199].

2.3 ROLE OF COX-2 IN CARCINOGENESIS

The ability to potentially treat or prevent neoplasia with COX-2 inhibitors led to the investigation of multiple neoplasms and their expression of COX-2.

Carcinogenesis is a multistep process of long-term accumulation of genetic and epigenetic alterations eventually resulting visible changes in the form a neoplasia. Understanding these steps will help in the design of targeted therapy with strategies for prevention and/or treatment. Epidemiological and experimental studies illustrate the preventative effect of NSAIDs, which, as explained reduces

prostaglandins. COX-2 is often over expressed in many neoplasia and therefore is of particular interest for chemoprevention [200]. It is not fully understood how COX inhibitors prevent carcinoma but some mechanism can be explained, at least partially. COX-2 is an important mediator of angiogenesis and tumour growth expressed in tumour endothelial cells, immune cells, and stromal fibroblast. TXA₂, PGE₂ and PGI₂ all result in endothelial growth and proliferation, promotion of vascular sprouting, migration and tube formation, enhanced endothelial cell survival via bcl-2 expression and Akt signaling [201], induction of metalloproteinases, activation of epidermal growth factor receptor mediated angiogenesis and suppression of interleukin-12 production. COX-2 inhibitors are shown to suppress angiogenesis [202, 203].

COX-2 and gastrointestinal neoplasia

Normal intestinal mucosa has no expression of COX-2 contrasted to sporadic colorectal carcinoma which expresses COX-2 at a high rate [158, 166, 204].

Gastric adenocarcinoma [157, 182] and oesophageal squamous cell carcinoma [212, 213] show increase COX-2 expression and in the case of gastric carcinoma correlates with lymphatic invasion and lymph node metastases.

COX-2 in oral, laryngeal and lung carcinoma

Squamous cell carcinomas of the head and neck region show over expression of COX-2 through activation of epidermal growth factor receptor which is effectively

prevented by NSAIDs in animal models [205]. It is also suggested that radio resistant laryngeal squamous cell carcinomas show increased expression of COX-2 with a potential benefit of inhibitory therapy [206].

COX-2 is also over expressed in lung carcinoma, particularly squamous cell and adenocarcinoma with a very low expression in small cell carcinoma. Expression also correlates with the degree of differentiation in adenocarcinomas with low levels of expression in poorly differentiated adenocarcinomas. Atypical alveolar and hyperplastic bronchial epithelium does also show increased expression suggesting a neoplastic potential [207, 208].

COX-2 expression in breast carcinoma

COX-2 expression is associated with an aggressive phenotype in ductal carcinoma in situ [209]. COX-2 inhibitors are promising but under investigated as a potential chemotherapy [210].

COX-2 and urogenital neoplasia

COX-2 is over expressed only in poorly differentiated endometrial carcinomas and known be expressed in ovarian neoplasia [211, 212]. In a study done in our department COX-2 expression was evaluated on normal, HPV, CIN I, II and III and infiltrating squamous cell carcinoma of the uterine cervix. The results indicated no expression in the ectocervix of normal cervix biopsies. All cases of squamous cell dysplasia showing increased expression of COX-2 with highest

expression in CIN I, II and squamous cell carcinoma. Infection by two types of HPV correlated with the highest expression of COX-2 [213]. Some COX-2 inhibitors have an inhibitory effect on cancer cell proliferation in cell lines of prostatic adenocarcinoma [214].

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CHAPTER THREE

MORPHOLOGY STUDY OF COLORECTAL CARCINOMA

3.1 AIM

1. The purpose of this section of this study was to blindly evaluate a range of the pathological features (intratumoral lymphocytes, stromal lymphocytes, lymphoid aggregates, type of necrosis, percentage intra- and extracellular mucin and tumour grade) and where possible grade them in histological sections of HNPCC tumours.
2. To compare these findings with genetic germline testing for specific mutations and determine whether pathological features *per se* are characteristic of HNPCC colonic carcinomas or not.
3. To determine which pathological features are useful in predicting the likeliness of a colorectal carcinoma being an HNPCC lesion.

Proposal

If a tumour is suspected to be of HNPCC origin on the basis of pathology, tumours could be immunohistochemically stained (MLH1, MSH2) and tumour tissue and blood could be genetically tested. New cases and families at risk could be identified with this approach and this could lead to regular screening and hopefully prevention of colorectal carcinoma in family members at risk.

3.2 MATERIALS AND METHODS

Materials

Blocks and slides were retrieved from the archives of Groote Schuur Hospital, division of Anatomical Pathology, National Health Laboratory Service (NHLS).

Three groups of patients with CRC were included:

Group 1: Sporadic colorectal carcinoma (Sporadic CRC)

Sixty-five (65) most recent (2001 and 2002), consecutive cases of left sided colorectal carcinomas in patients older than 65 years were selected. This was done to minimize the possibility of including cases of inherited cancer syndromes. Cases with family history of colorectal carcinoma, prior malignancies, histories of previous malignancies or where tissue blocks were not found in the archives were excluded. Seven cases were excluded from this study for the above-mentioned reasons and the remaining of 58 cases were studied.

Group 2: Early onset colorectal carcinoma (less than 45 years of age)

Ninety three (93) cases of colorectal carcinoma in patients presenting at less than 45 years of age were selected over a period of eight years (1993-2001) [130]. These cases were diagnosed clinically and confirmed histologically. Cases of known MLH1 and MSH2 mutations, known and tested by the division of human genetics at UCT, were excluded from this group. This group of cases is the same used in a study on MLH1 and MSH2 immunohistochemical staining in identifying mismatch repair gene defects in the absence of staining [130].

Group 3: Genetically proven HNPCC

This group consisted of thirty (30) cases of known HNPCC carcinomas proven by genetic testing for mismatch repair gene (MLH1 and MSH2) germline mutations by the division of human genetics, UCT.

Cases of known FAP, hyperplastic polyposis, juvenile polyposis and colorectal neoplasias other than adenocarcinomas were excluded (group 3).

Methods

The slides and paraffin blocks were retrieved from the archives. The sections were recut if not available or technically sub-optimal, stained with H&E, and coded.

All sections for each cases were evaluated and graded on the following features:

1. intratumoral lymphocytes (tumour infiltrating lymphocytes)
(Fig. 3.1 and 3.2)
2. stromal lymphocytes (Fig. 3.3 and 3.4)
3. lymphoid aggregates (Crohn-like inflammation) (Fig. 3. and 3.4)
4. "dirty" necrosis (Fig 3.5)
5. % mucin per surface tumour evaluated (intra- and extracellular mucin)
(Fig. 3.6 to 3.8)
6. Histological differentiation (grade) (Fig. 3.9 and 3.10)

The figures following under the headings of pathological features assessed are representative of the typical morphology seen in these cases and illustrate the pathology range encountered (Fig. 3.1 to 3.10).

See appendix 1 for raw data (table 1) for pathological evaluation.

1. Intratumoral lymphocytes

Intratumoral lymphocytes were scored as present (positive) or absent (negative). A positive result would be tumours where lymphocytes are diffusely present in the neoplastic epithelium ("peppered epithelium") and within normal limits (less than 20 intraepithelial lymphocytes per 100 epithelial cells) in the non-neoplastic epithelium away from the tumour. Small foci of intratumoral lymphocytes often in the vicinity of lymphoid follicles, epithelial ulceration or associated necrosis were considered negative. Lymphocytes were identified by their typical morphology, often showing peri-lymphocytic clearing when present in the epithelium.

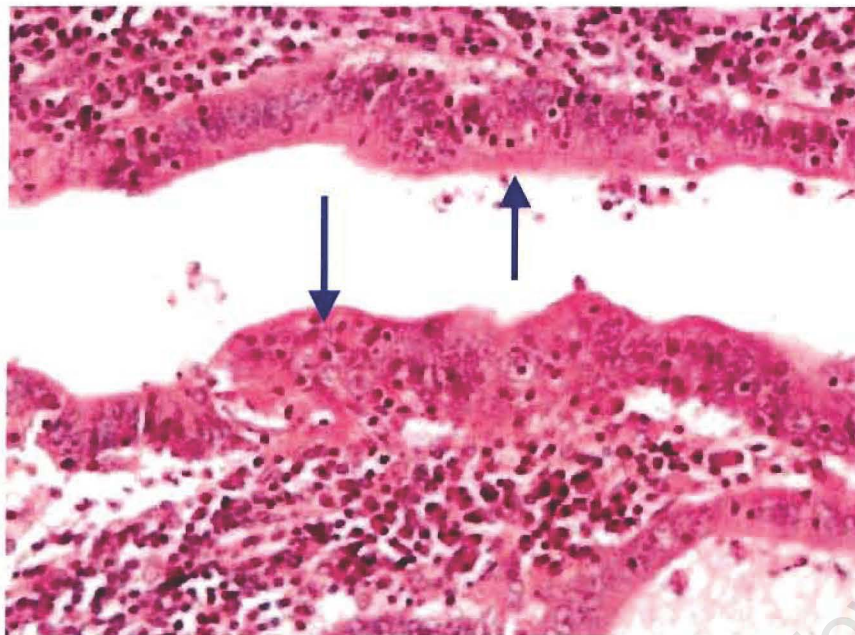


Fig 3.1: Medium power view of multiple intratumoral lymphocytes in neoplastic epithelium (positive result).

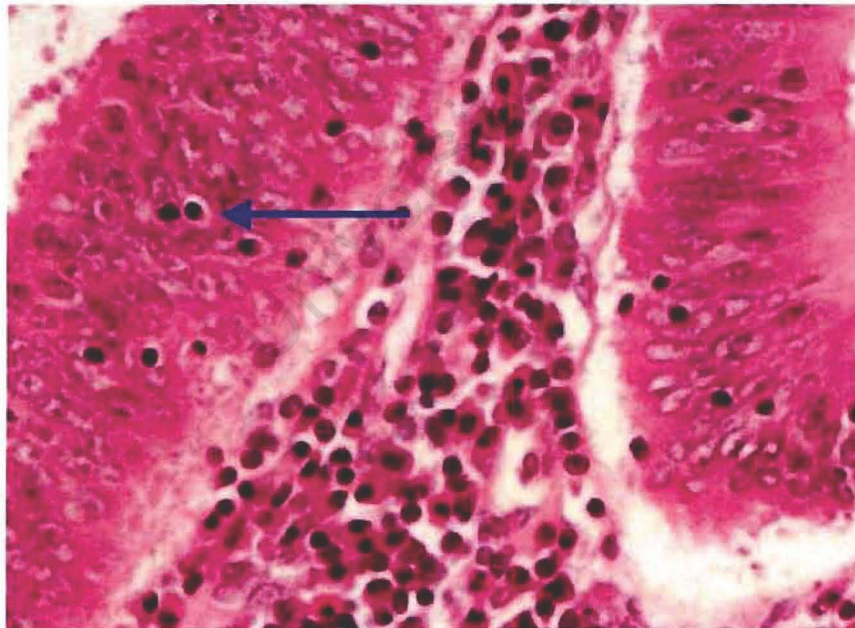


Fig 3.2: High power view of lymphocyte within a colon cancer, some of which show peri-lymphocytic clearing (arrow). An example of a positive result.

2. Stromal lymphocytes

This feature was scored either positive when stromal lymphocytes were present in a dense peritumoral sheet or negative when moderate or sparse stromal lymphocytic infiltration was seen [53]. See figures 3.3 and 3.4 below.

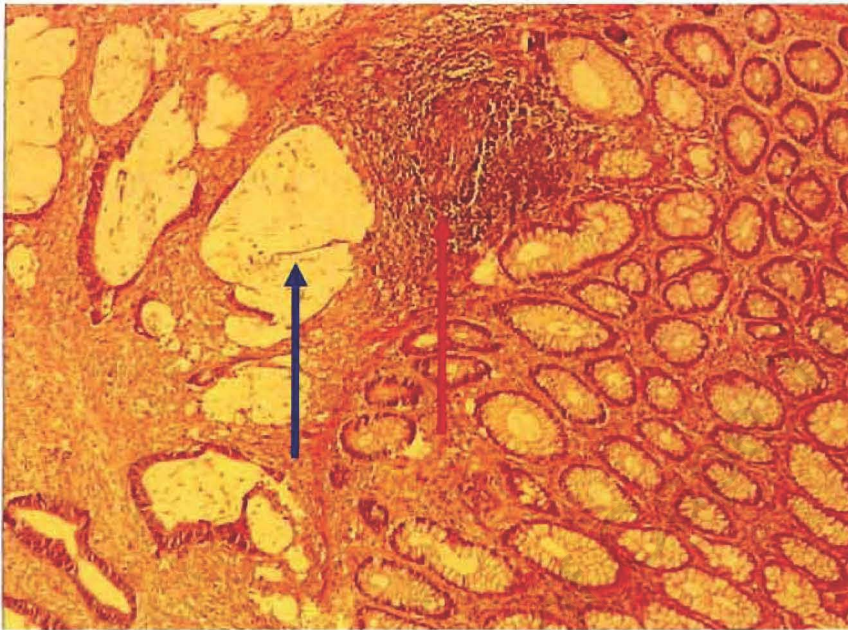


Fig 3.3: Medium power view of a reactive lymphoid aggregate with germinal center formation (red arrow) in the vicinity of an infiltrating adenocarcinoma (blue arrow). An example of a positive result.

3. Lymphoid aggregates

A Crohn-like inflammatory pattern was looked for and was considered positive if more than one deep lymphoid aggregate per ten high power fields were apparent or negative when less than one lymphoid aggregate per ten high power field was present. See figure 3.4 showing three lymphoid aggregates in one field.

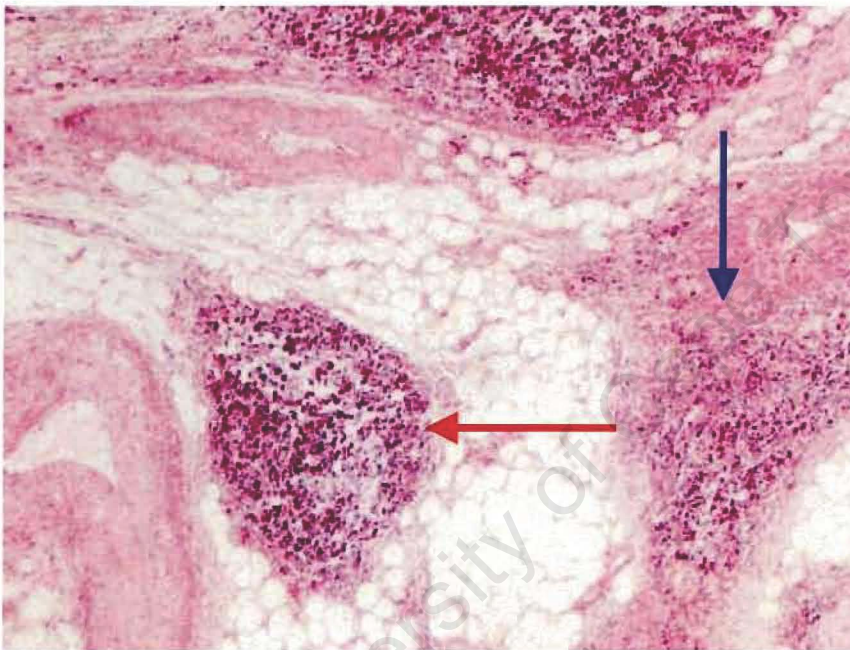


Fig 3.4: Deep lymphoid aggregates (red arrow) and dense stromal lymphocytic inflammation (blue arrow) in the vicinity of infiltrating tumour and into mesenteric adipose tissue. An example of a positive result.

4. Necrosis

Tumour necrosis resulting in areas of cellular tumour debris (so-called “dirty” necrosis) was scored positive. Coagulative necrosis and infarcted bowel as well as surface epithelium ulceration and necrosis were considered negative.

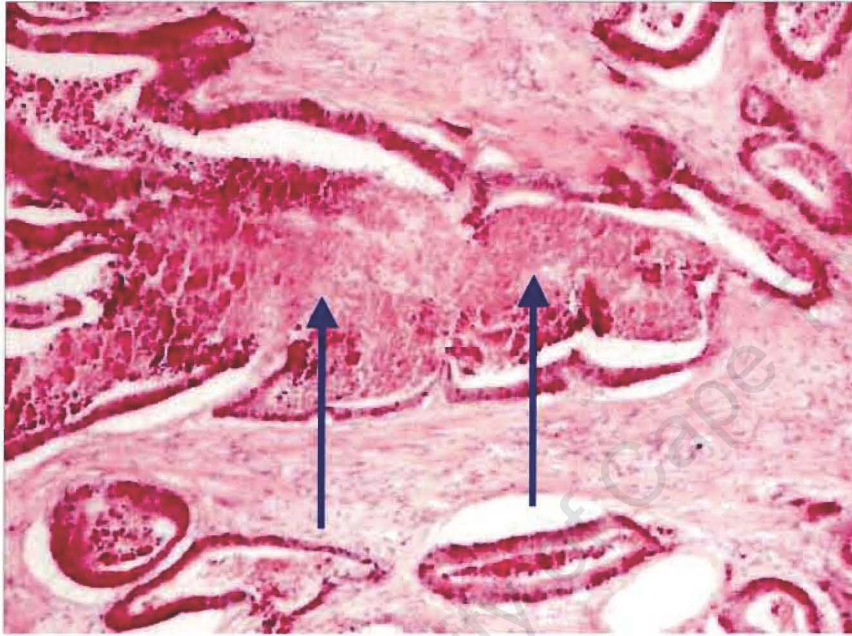


Fig 3.5: Neoplastic epithelium with large areas of “dirty” necrosis (arrows).

A negative result for HNPCC but more typical of sporadic CRC.

5. Percentage of mucin

On evaluating intra- and extracellular mucin the tumours were grouped into five categories depending of the amount of mucin present:

1. 0% (Fig. 3.6)
2. < 25% (Fig. 3.7)
3. 25-50%
4. 50-75%
5. > 75% (Fig. 3.8).

This was estimated by the presence of intra- and extracellular mucin expressed in percentage of surface area of tumour examined (amount of mucin production expressed in a percentage of surface area evaluated) [53].

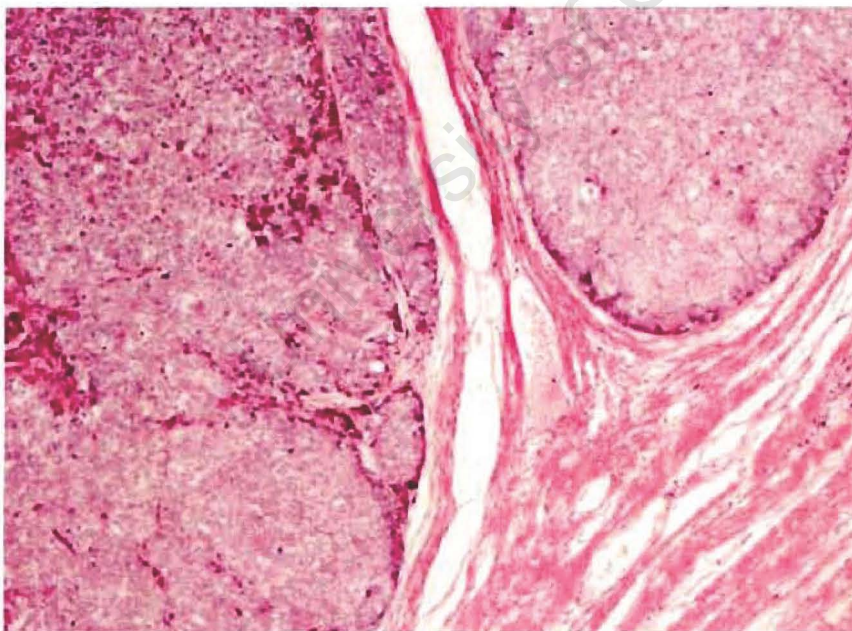


Fig 3.6: Solid type CRC, poorly differentiated adenocarcinoma with 0% mucin in the tumour.

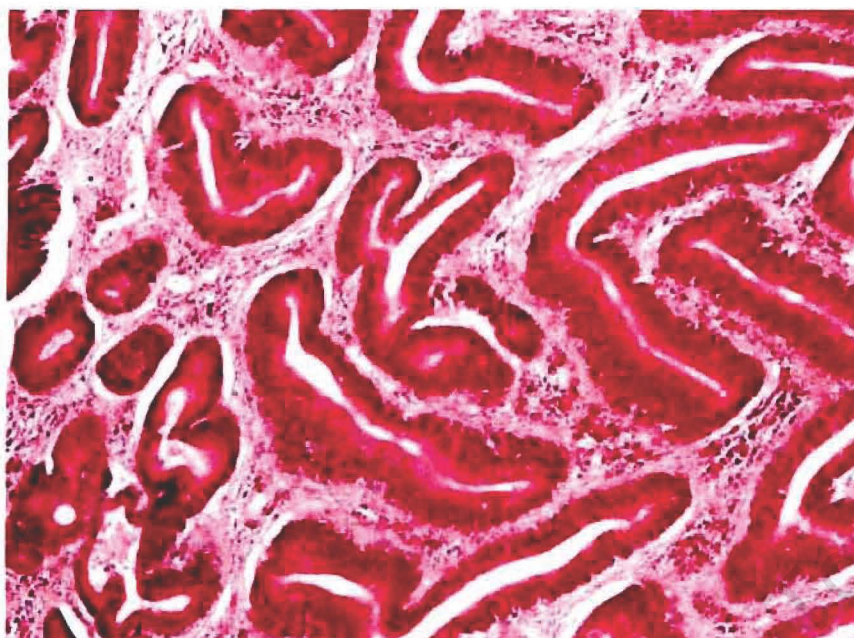


Fig 3.7: Well-differentiated CRC, non-mucinous adenocarcinoma (mucin expression < 25%)



Fig 3.8: Poorly differentiated mucinous CRC (mucin expression >75%) adenocarcinoma

6. Grading (differentiation)

A four-tiered grading system of the WHO [151] was used. The percentage of the tumour showing formation of gland-like structures was evaluated:

Grade I: well differentiated (more than 95%) (Fig 3.9)

Grade II: moderately differentiated (50-95%)

Grade III: poorly differentiated (5-50%)

Grade IV: undifferentiated carcinomas (less than 5%) (Fig 3.10)

In the conventional three-tiered grading system well, moderate and poorly differentiated grades are used. Grade I and II of the WHO system correlate with well- and moderately differentiated tumour grades in the three-tiered system. Both the grades III and IV of the WHO grading system correlate with poorly differentiated tumours in the three-tiered grading system.

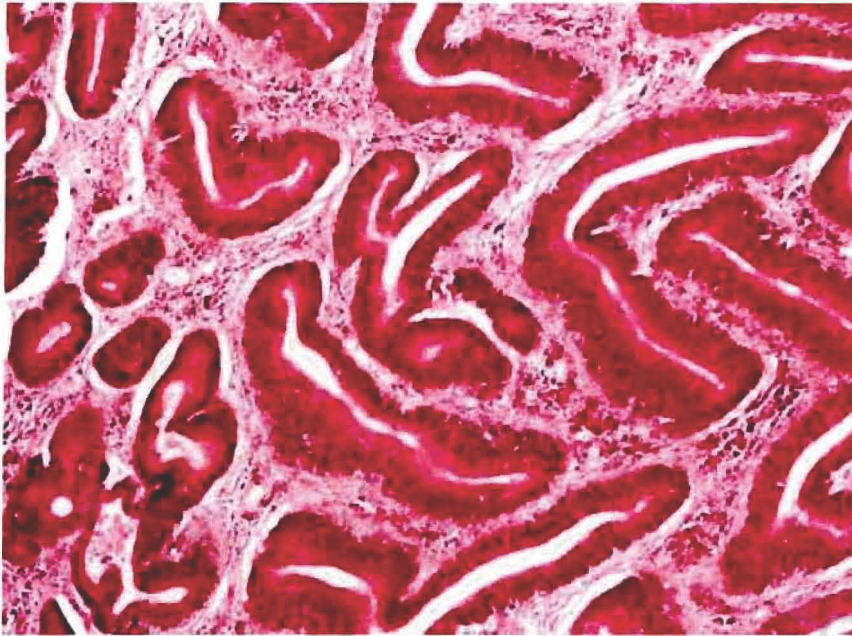


Fig 3.9: Well differentiated adenocarcinoma (WHO grade I and well-differentiated in three-tiered system)

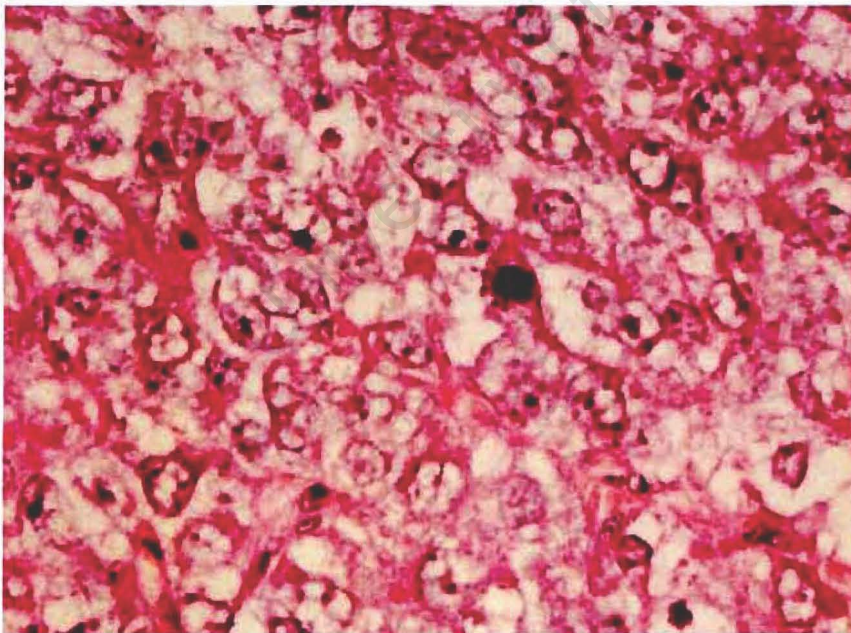


Fig 3.10: Poorly differentiated colorectal adenocarcinoma (WHO grade IV and poorly differentiated in three-tiered system)

3.4 RESULTS

See appendix 1, raw data table 1.

1. Intratumoral lymphocytes

Most of the HNPCC tumours contain intratumoral lymphocytes. This feature on its own identified 70% of HNPCC. 22% of sporadic CRC show this feature while only 15% of CRC in patients less than 45 years of age show intratumoral lymphocytes. See figure 3.1 below.

Table 3.1: Percentage intratumoral lymphocytes

Group	% intratumoral lymphs
Sporadic CRC	22%
CRC less than 45 years	15%
HNPCC	70%

2. Stromal lymphocytes

90% of HNPCC cases show stromal lymphocytes. However, a very high percentage (65%) of sporadic CRC and tumours in the under 45-year-old age group (78%) show this pathological feature. This is therefore a sensitive but less specific pathological features in identifying HNPCC. See figure 3.2 below.

Table 3.2: Stromal lymphocytes in CRC

Group	% stromal lymphs
Sporadic CRC	65%
CRC less than 45 years	78%
HNPCC	90%

3. Lymphoid aggregates

Lymphoid aggregates in the stroma surrounding infiltrating carcinoma are a common finding in all carcinomas but are slightly more prevalent in HNPCC (73% contrasted to 53% in sporadic CRC. See table 3.3 below.

Table 3.3: Lymphoid aggregates in CRC

Group	Lymphoid aggregates
Sporadic CRC	53%
CRC less than 45 years	50%
HNPCC	73%

4. Necrosis

'Dirty' necrosis is a common histological feature in CRC. 50% of HNPCC cases show this feature. Necrosis is more frequent in sporadic carcinomas (62%) and early onset CRC (76%) than in HNPCC. See table 3.4 below.

Table 3.4: Percentage 'dirty' necrosis in CRC

Group	% necrosis
Sporadic CRC	62%
CRC less than 45 years	76%
HNPCC	50%

5. Mucin percentage

The amount of mucin production is of no discriminating use in identifying HNPCC tumours (30% mucinous) compared to those of sporadic CRC (26%) or early onset CRC (30%). See summary tables 3.5 to 3.10 below.

Table 3.5: Percentage cases showing 0% mucin in CRC

Group	0% mucin
Sporadic CRC	6%
CRC less than 45 years	17%
HNPCC	12%

Table 3.6: Percentage cases showing less than 25% mucin in CRC

Group	less than 25% mucin
Sporadic CRC	38%
CRC less than 45 years	41%
HNPCC	27%

Table 3.7: Percentage cases showing 25-50% mucin in CRC

Group	25-50% mucin
Sporadic CRC	30%
CRC less than 45 years	12%
HNPCC	31%

Table 3.8: Percentage cases showing 50-75% mucin in CRC

Group	50-75% mucin
Sporadic CRC	12%
CRC less than 45 years	7%
HNPCC	11%

Table 3.9: Percentage cases showing more than 75% mucin in CRC

Group	More than 75% mucin
Sporadic CRC	14%
CRC less than 45 years	23%
HNPCC	19%

Table 3.10: Summary table showing conventional percentage non-mucinous carcinomas (less than 50% mucin) and mucinous adenocarcinoma (more than 50% mucin).

Group	% Non-mucinous	% Mucinous
Sporadic CRC	74%	26%
CRC less than 45 years	70%	30%
HNPCC	70%	30%

7. Differentiation

The four tiered system (WHO) initially used was later (see appendix 3, raw data table1) consolidated to a three-tiered grading system, since no statistical differences were noted in either grading systems. Grading is not a useful discriminating histological factor in identifying HNPCC carcinomas and show a very similar distribution of numbers in all groups of tumours. See tables 3.11 to 3.15 below.

Table 3.11: Percentage cases showing grade I differentiation

Group	Grade I
Sporadic CRC	17%
CRC less than 45 years	13%
HNPCC	25%

Table 3.12: Percentage cases showing grade II differentiation

Group	Grade II
Sporadic CRC	66%
CRC less than 45 years	54%
HNPCC	50%

Table 3.13: Percentage cases showing grade III differentiation

Group	Grade III
Sporadic CRC	13%
CRC less than 45 years	33%
HNPCC	25%

Table 3.14: Percentage cases showing grade IV differentiation

Group	Grade IV
Sporadic CRC	4%
CRC less than 45 years	0%
HNPCC	0%

Table 3.15: Summary table showing percentage cases in the conventional classification based on a three-tiered system.

Group	Well differentiated	Moderately differentiated	Poorly differentiated
Sporadic CRC	17%	66%	17%
CRC less than 45 years	13%	54%	33%
HNPCC	25%	50%	25%

3.4 DISCUSSION

In this study it is shown that some histopathological features are useful in prompting further investigation when considering HNPCC. Documentation of detailed histopathological findings in the surgical report of resected CRC is warranted and enables us, together with the clinical history and presentation, to decide on further immunohistochemical and molecular testing.

The most useful pathological findings in suggesting an HNPCC are:

Intratumoral lymphocytes (70%) as well as lymphoid aggregates (73%), but on their own they are not specific histological indicators for HNPCC. The presence of intratumoral lymphocyte infiltration is the most suggestive histological indicator of HNPCC of all the histological markers. Stromal lymphocytes are a more sensitive marker (present in 90% in HNPCC) but less specific since these findings are present in 65% of sporadic carcinomas and in 78% of the under 45 year old group. 'Dirty' necrosis is a more typical feature for sporadic carcinomas and the under 45-year-old group. Similar findings have been found in another

study [53]. However, half of the HNPCC cases also show this feature, which makes it a less useful discriminating histological feature. This is in contrast to a previous study where 'dirty' necrosis was much less common in HNPCC [53].

The categorization of mucinous or non-mucinous tumours is of no discriminatory value in identifying HNPCC versus non-HNPCC carcinomas in this study. This is in contrast to multiple previously published studies where mucinous tumours were more numerous in HNPCC [53, 215]. The reason for this might be the high prevalence of MLH1 mutation and less common MSH2 in our South African population group.

The least helpful feature in identifying HNPCC carcinomas was the histological grading of tumour; the grading of the tumour (whether three- or four-tiered) was of no distinguishing value in this study aimed at distinguishing HNPCC tumours from 'sporadic' tumours. Minor differences occurred but had no statistical significance.

CHAPTER FOUR

CYCLOOXYGENASE-2 STUDY

Normal intestinal mucosa has no expression of COX-2 contrasted to sporadic colorectal carcinoma which expresses a high rate [158, 166, 204]. In familial adenomatous polyposis (FAP) numbers and size of adenomas are reduced in patients on NSAID treatment [158, 165, 166, 204, 216]. In clinical practice this has led to the use of NSAIDs in patients with known FAP in preventative therapy before polyps develop and in patients with polyps preventing progression to carcinoma. It has also been proven that colon cancer cells with high invasive potential have a susceptible of apoptosis by means of COX-2 inhibitors [217]. In contrast to sporadic tumours, expression of COX-2 in HNPCC is reduced [218, 219]. COX-2 expression is in some studies regarded as an indicator to poor outcome since more advanced tumours often show higher levels of COX-2 expression [220]. However, this is still controversial since other studies did not show such a correlation [221]. Chronic inflammatory conditions such as ulcerative colitis and Crohn's disease predispose to development of colorectal carcinoma. It is assumed, and supported by rodent models, that chronic inflammation with bacterial colonization, prostaglandin and interleukins production which leads to dysplasia and malignancy [222-224].

Normal colonic mucosa does not express COX-2 in contrast to sporadic and FAP colorectal carcinomas where over expression is marked. It is important to note that some cells in normal epithelium, including inflammatory cells, dendritic cells

and some fibroblastic cells may express COX-2 and this may lead to misinterpretation. Positive staining is interpreted as tumour cells showing cytoplasmic staining often with perinuclear distribution [158, 166].

4.1 AIM

1. To evaluate COX-2 expression in colonic adenomas and carcinomas in South African patients with genetically proven HNPCC.
2. To compare COX-2 expression in HNPCC cases with that in sporadic CRC.

Hypothesis

HNPCC will express COX-2 (as do sporadic CRC) and consequently COX-2 inhibitor therapy will be warranted.

4.2 MATERIAL AND METHODS

Coded sections of formalin fixed, paraffin embedded, archived tissue from patients with known hMLH1 or hMSH2 mutations (6 polyps and 17 carcinomas) and consecutive sporadic tumours (5 polyps and 39 carcinomas), received during the year of the study (2001), where tissue blocks were available were immunohistochemically stained with an antibody to COX-2 (Santa Cruz).

Expression of COX-2 in the tumours was scored on intensity where:

0 = staining,

1 = weak staining,

2 = intermediate staining and

3 = intense dark staining.

Distribution was scored where:

0 = no staining,

1 = very focal staining,

2 = moderate staining and

3 = diffusely positive staining.

The adjacent normal epithelium was evaluated and compared. This was then expressed in a combined score (intensity + distribution) and scored out of maximum score of 6.

A second pathologist did a random audit looking at a number of slides scoring COX-2 staining independently showing reproducibility.

Immunohistochemical method

1micrometer sections were cut onto APES-coated slides and heat-fixed overnight at 60 degree Celsius followed by dewaxing. Endogenous peroxidase reactivity was blocked by treating slides with a 1% H₂O₂ in water solution for 15 minutes.

Antigen retrieval was performed by pressure-cooking in citrate buffer for 2 minutes at full pressure and immediately immersed in water followed by rinsing with phosphate buffered saline solution (PBS pH 7.6). Non-specific binding was blocked by treating slides with a 5% goat serum solution (DAKO #X0907).

The sections were incubated with the primary antibody (1:200 dilution at room temperature for 4 hours and washed with PBS buffer followed by incubation with DAKO envision labeled polymer, HRP (DAKO #K4001) for 30 minutes at room temperature and again washed with PBS buffer.

Positivity was developed by applying 3,3'-diaminobenzidine (DAKO K3466) for 10 minutes. The slides were then washed in water, immersed in 1% CuSO₄ solution for 5 minutes and washed in water again.

The slides were then counterstained with haematoxylin stain and blued in Scott's tap water. Finally the slides were washed in water, dehydrated, cleared and coverslipped.

4.3 RESULTS

The normal colonic epithelium does not show any COX-2 expression (See fig.4.1 below). 1/6 adenomas (17%) of HNPCC showed positive expression and 7/17 carcinomas (41%) expressed COX-2 (See figures 4.3, 4.4 and table 4.1).

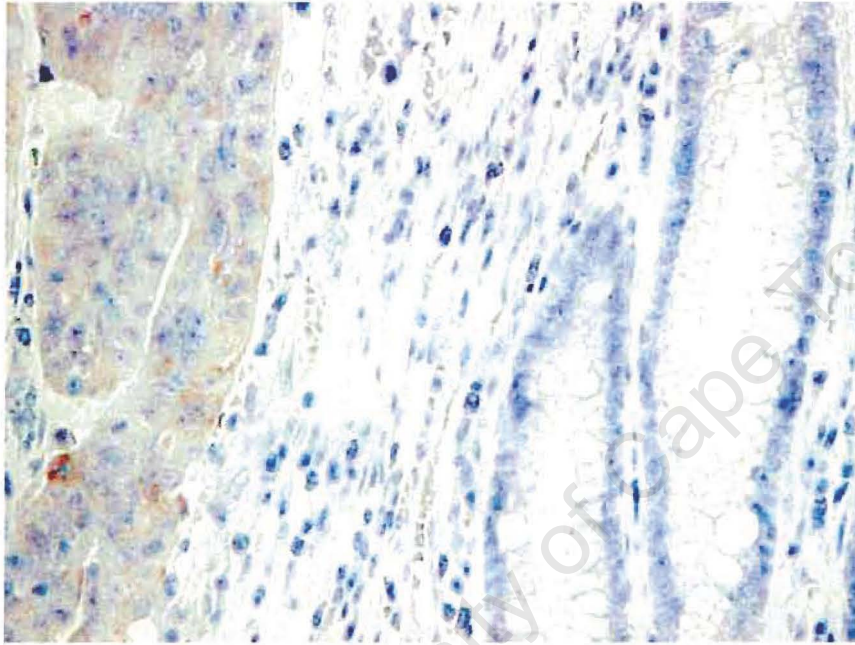


Fig 4.1: COX-2 expression in an HNPCC case with no staining of normal colonic mucosa (right) and weak staining of carcinoma (left)

In sporadic carcinomas 3/5 adenomas (60%) and 26/39 (67%) of carcinomas express COX-2 (see fig. 4.2 below).

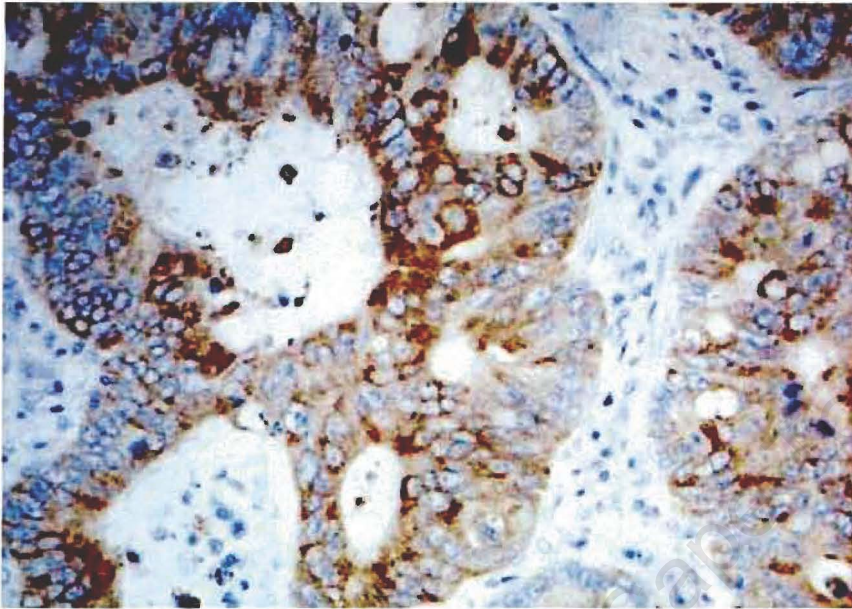


Fig 4.2: Strong COX-2 expression in sporadic colorectal carcinoma

Only sporadic carcinomas showed high levels of expression (4/6 and 5/6)(see figure 4.2) in contrast to HNPCC cases, which showed highest expression with a combined (distribution and intensity) score of 3/6 (only three cases).

See figures 4.3 and 4.4 below.

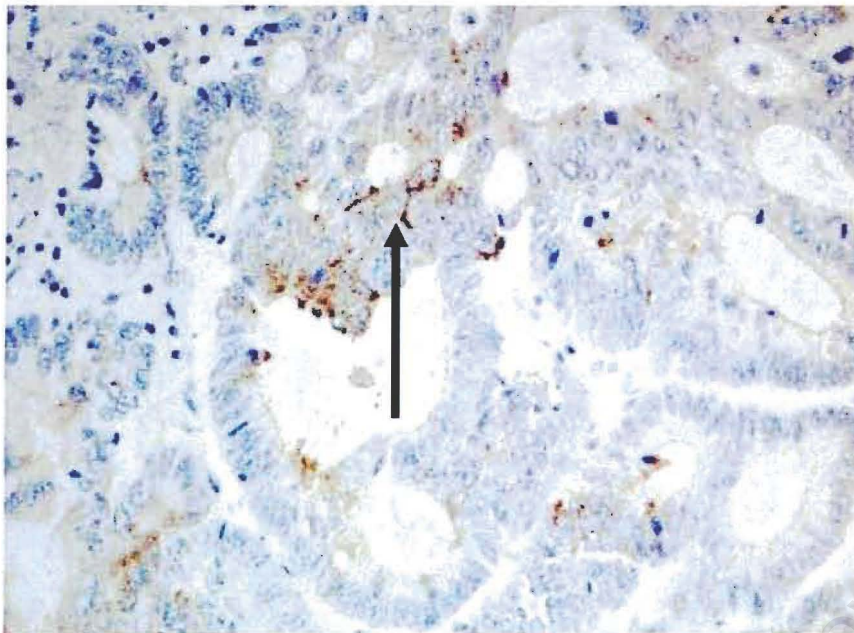


Fig 4.3: Weak and focal COX-2 expression in an HNPCC colorectal carcinoma

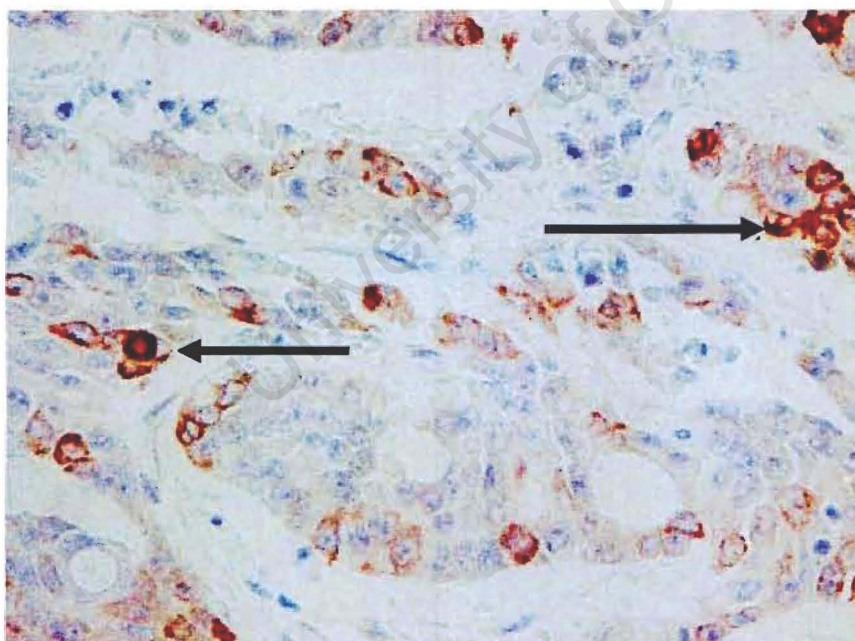
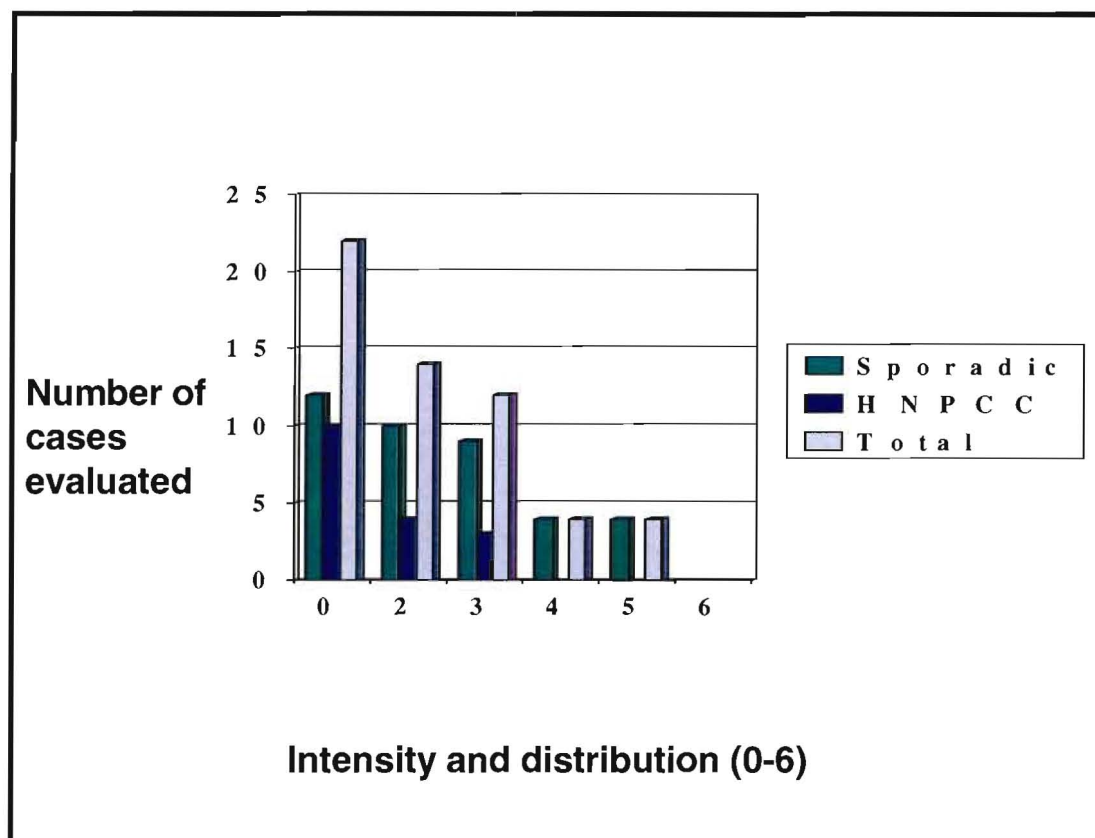


Fig 4.4: Focal strong expression in an HNPCC case

Table 4.1: Intensity and distribution of COX-2 staining in HNPCC and sporadic cases of carcinoma.



4.4 DISCUSSION

In this study, known HNPCC cases showed less expression of COX-2 than in sporadic CRC. Adenomatous polyps show similar results compared to colorectal carcinomas but too few cases were evaluated to have any statistical significance. Adenomas showed generally less expression and therefore COX-2 expression might be a late manifestation in progression to invasive malignancy. This is also very suggestive of different pathways of carcinogenesis in HNPCC compared to sporadic colorectal carcinomas.

In summary the following could be extrapolated from the above results:

1. Adenomas

Only 17% of HNPC adenomas express COX-2 in contrast to 60% of sporadic adenomas.

2. Carcinomas

Only 41% of HNPCC express COX-2 in contrast to 67% of sporadic CRC.

HNPCC and adenomas do not express COX-2 as strongly as sporadic CRC and adenomas in this study.

These results suggest chemoprevention with COX-2 inhibitors (NSAIDs) may have a place only in selected patients with HNPCC. COX-2 inhibitor therapy might not be as successful in delaying carcinogenesis and preventing infiltrating carcinomas as in sporadic and FAP cases.

CHAPTER FIVE

SUMMARY AND FUTURE DIRECTIONS

The two studies and described in this thesis gave insight and understanding in the pathogenesis, genetics and morphological features of South African HNPCC cases. This enables us to construct a profile of these unique cases based on epidemiology, genetics, morphology, clinical outcome and therapy –ultimately leading to effective management with effective screening, early diagnosis, prevention and effective therapy. All these observations will hopefully benefit patients and relatives in HNPCC families who are at risk of developing carcinomas with associated morbidity and mortality.

Some, but not all, of the findings in these studies are in keeping with published findings of other study groups around the world.

The morphological study confirmed other studies of the diagnostic usefulness of certain histological features. Intratumoral lymphocytes, stromal lymphocytes and lymphoid aggregates suggested HNPCC cases (or MSI-H). The presence of “dirty” necrosis is a more common histological finding suggesting sporadic rather than HNPC carcinomas.

In contrast to most other studies, mucinous tumours were not more commonly seen in HNPCC in South African patients in this study. Also, the grade of the tumour (differentiation) was of no discriminating use.

The results of this study will be usefully applied in our daily laboratory practice illustrated by the following example:

A right-sided colectomy for colorectal carcinoma/lesion from a 38-year-old male colored patient was submitted for routine histological examination. The histology showed occasional adenomas and an infiltrating carcinoma showing marked intratumoral lymphocytes, stromal lymphocytes and lymphoid aggregates. These findings were suggestive of MSI-H carcinoma and immunohistochemical studies (MLH1, MSH2) for protein products were performed (already tested and proven useful in our IHC laboratory [130]).

Absence of staining suggested dysfunctional mismatch repair gene. However, this could represent either a germline or somatic mutation. Germline testing (blood sample) for that specific gene locus (e.g. MLH1) was performed at the department of Human Genetics giving a definitive positive result on the germline status. In the future this type of investigation could identify new families or family members at risk. A preventative and early diagnosis colonoscopy-screening program is the adhered to. In future, screening programs for endometrial and breast carcinomas are also planned.

The results of the COX-2 expression by the tumours in this study suggests that there may be a place for chemoprevention with NSAIDs in HNPCC cases since only selected cases express this protein. Since COX-2 inhibitors are not without

side effects (potential adverse effect on the cardiovascular system), it is probably worthwhile testing the CRC for COX-2 expression before commencing chemoprevention with NSAIDs [225-227].

Future directions

Future studies in the investigation of HNPCC are of considerable promise; some of these studies have been initiated already in selected pilot studies. These include mainly molecular analysis of the tumour itself and both the techniques and the analysis by microsatellite profiling of tumours and CGH of the genome of normal epithelium, adenomas, infiltrating carcinomas and metastasis (please see preliminary results on pilot studies in the appendix, raw data, table 3 and figure 1). Hopefully, such studies will provide insight in the molecular changes associated with each stage of carcinogenesis in HNPCC. Once these analyses have been completed a correlation with the known mutations, the presence of additional mutations and specific oncogenes and whether tumor suppressor genes are up- or down regulated will be seen.

Tissue samples continue to be collected and frozen for future microarray analysis (RNA), giving us even more detailed information on exact molecular and genetic changes.

COX-2 expression should be evaluated and compared pre-therapy (at biopsy and initial histological diagnosis) and post-therapy (at formal resection of lesion).

COX-2 inhibitors would be given between the time of original biopsy and formal resection (1-2 weeks usually). This will give as a clear indication of the effect of therapy on HNPCC cases.

Chemopreventative therapy probably will be effective if the COX-2 expression is reduced after initial treatment.

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APPENDECES

Appendix 1

Abstract:

Oral presentation at the National Pathology Congress, Johannesburg 2004

MORPHOLOGICAL FEATURES OF HEREDITARY NON-POLYPOSIS COLORECTAL CARCINOMAS COMPARED TO OTHER EARLY ONSET AND SPORADIC COLORECTAL CARCINOMAS.

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Background:

Families with hereditary non-polyposis colorectal carcinoma (HNPCC) are not uncommon along the West-Coast of South Africa. These patients present with early onset carcinomas mostly colorectal, predominantly in the right colon. They may develop tumours of other organs, including uterus, breast, stomach and skin.

Objectives:

1. To evaluate and compare the microscopic characteristics of three groups of colorectal carcinomas (HNPCC, early onset colorectal carcinomas and sporadic colorectal carcinomas).
2. To determine the features most characteristic of the group.

Methods:

Coded sections of formalin-fixed paraffin-embedded tissue from patients with

- (1) sporadic colorectal carcinomas (58 cases)
- (2) early onset (<45 years) colorectal carcinomas (93 cases) and
- (3) HNPCC (30 cases) are evaluated and graded on the following features:

Intratumoral lymphocytes (**TL**), stromal lymphocytes (**SL**), lymphoid aggregates in the vicinity of the tumour (**LA**), presence and type of necrosis (**Ne**), percentage of mucin (**M0** = 0%, **M1** = <25%, **M2** = 25-50%, **M3** = 50-75%, **M4** = >75%) and histological grade based on the standard criteria of gland formation (**G1** to **G4**).

Results:

Group	TL	SL	LA	Ne	M0	M1	M2	M3	M4	G1	G2	G3	G4	
Sporadic	22	65	53	62	6	38	30	12	14	16	58	12	4	%
<45 year	15	78	50	76	17	41	12	7	23	13	54	32	0	%
HNPCC	70	90	73	50	12	27	31	11	19	23	47	23	0	%

Conclusions:

Intratumoral lymphocytes and lymphoid aggregates are fairly sensitive markers for HNPCC, the former being more specific than the latter. Stromal lymphocytes are a third, even more sensitive marker for HNPCC, but less specific than the other two. The other parameters tested are of no help in the differential diagnosis except for presence of necrosis, which is more typical of sporadic and early onset colorectal carcinoma than of HNPCC.

Appendix 2

Abstract:

Poster presentation at the National Pathology Congress, Bloemfontein 2002 and the annual research day at UCT 2003.

CYCLOOXYGENASE-2 (COX-2) EXPRESSION IN POLYPS AND CARCINOMAS OF HEREDITARY NON-POLYPOSIS COLORECTAL CARCINOMA (HNPCC) AND SPORADIC COLORECTAL CARCINOMAS.

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Background:

COX-2 inhibitors are being used as chemoprevention in familial adenomatous polyposis. If COX-2 is also expressed in HNPCC there might be a role of COX-2 inhibitor treatment for patients with known a mismatch repair gene defect - either to inhibit progression of tumours or to prevent the onset of carcinogenesis.

Objectives:

1. To evaluate COX-2 expression in colonic adenomas and carcinomas in patients with genetically proven HNPCC.
2. To compare COX-2 expression in HNPCC cases with that in sporadic tumours.

Methods:

Coded sections of formalin fixed paraffin embedded tissue from patients with known hMLH1 or hMSH2 mutations (6 polyps and 12 carcinomas) and sporadic tumours (5 polyps and 39 carcinomas) were immunohistochemically stained with an antibody to the COX-2 (Santa Cruz). Expression of COX-2 in the tumours was scored on both intensity and distribution and compared to the adjacent normal epithelium.

Results:

Normal colonic epithelium does not express COX-2. **HNPCC:** 1/6(17%) polyps and 5/17(41%) carcinomas express COX-2. **Sporadic tumours:** 3/5(60%) polyps and 26/39(67%) carcinomas express COX-2.

Conclusions:

COX-2 is generally more expressed in sporadic compared to HNPCC tumours. Furthermore COX-2 expression is more often expressed in infiltrating lesions compared to the expression in polyps. These results support the theory of different pathways in carcinogenesis in colorectal carcinoma. Chemoprevention is probably indicated in a selected number of HNPCC individuals.

RAW DATA

Table 1: An example of a histological scoring data table as used for the study on morphological features of CRC.

Name	Number	Grading	Necrosis	% Mucin	Intrat L	Stromal L	Lymph foll
1978	6022	2	0	>75	0	1	1
1978	6176	2	0	<25	1	1	1
1989	6412	2	1	<25	0	1	1
1989	6412	2	0	25-50	1	1	1
1989	14509	2	0	<25	0	1	1
1989	16342	2	0	<25	0	1	1
1991	2089	2	1	<25	1	0	1
1991	9053	1	1	<25	1	1	1
1992	5740	2	0	<25	1	1	1
1992	11950	2	1	<25	0	1	0
1993	6951	1	0	<25	1	1	0
1993	10500	1	1	25-50	0	0	0
1993	13457	2	0	<25	0	1	0
1994	1085	1	0	25-50	1	1	1
1994	1362	2	0	<25	0	0	0
1994	6525	1	0	50-75	0	1	0
1994	8065	2	0	<25	1	0	0
1994	8667		0	>75	1	1	0
1994	9148	2	0	25-50	1	1	1
1994	13027	3	1	25-50	0	0	1
1994	13027	3	1	>75	0	1	1
1994	13187		0	<25	0	1	1

Table 2: Example of data sheet used for scoring COX-2 staining. Both intensity and distribution is scored (0-3).

Number	Year	Distribution	Intensity	Ca/polyp	Group
607	2001	1	1	1	1
1362	1994	0	0	2	1
1894	2002	1	3	2	1
2065	2002	2	3	2	1
2534	1997	2	1	1	1
3111	2001	0	0	2	1
4389	2001	1	2	2	1
4972	1996	0	0	2	1
5373	2001	2	1	2	1
5374	2001	0	0	1	1
5857	2002	1	1	2	1
6525	1994	2	2	2	1
6703	2001	0	0	2	1
6837	2001	1	1	2	1
7841	2001	1	1	2	1
9150	2001	1	1	2	1
9258	2001	1	1	2	1
10113	2001	3	2	2	1
10331	2001	1	2	2	1
10974	2000	0	0	2	1
11865	2000	1	1	2	1
309	1997	1	1	2	2
791	1996	0	0	2	2
1615	1994	2	2	2	2
1662	1987	0	0	2	2
2213	1994	0	0	1	2
2977	2002	2	2	2	2
3444	2002	1	2	2	2
3478	1995	1	2	2	2
4067	1990	0	0	2	2
4069	1992	1	2	2	2
5158	1995	1	1	2	2
5515	2001	2	3	2	2
5987	1994	2	3	2	2
6733	1995	2	1	1	2
7980	1997	0	0	2	2
8976	1995	1	1	2	2
10118	1998	1	2	2	2
10253	1999	0	0	2	2
365	1992	1	1	2	3
1882	1995	0	0	2	3

1951	1990	0	0	1	3
2960	2001	0	0	1	3
4648	1996	1	2	2	3
5237	2001	1	1	1	3
6412	1989	0	0	2	3
9053	1995	1	1	1	3
9054	1995	0	0	2	3
10182	2000	0	0	2	3
10356	1998	0	0	2	3
10496	2001	1	1	2	3
11036	2001	0	0	2	3
12367	1991	1	2	2	3
12737	1999	0	0	1	3
13057	1994	2	1	2	3
18240	1989	0	0	1	3

Table 3: MSI profile results of a pilot study on 12 of genetically proven HNPCC cases. MSI- markers used are listed on the left column with their loci and gene involved.

MSI RESULTS																
Marker	Type	Repeat	Locus	Gene	13027P1	10496Ca	10496Ad	6412AO	11036Ca	11036Ad	9054A	4557T	4887T	8275T	4987T	969T
TGFRB11*	INTRAGENIC	POLY A	3pter-3p24.2	TGFRBII	S	S	S	S	S	S	MSI	/	S	S	S	S
IGFR11R*	INTRAGENIC	POLY A	6q25.3	IGFR11R	S	/	S	S	S	S	MSI	/	S	S	S	S
BAX*	INTRAGENIC	POLY A	19q13.3-13.4	BAX	MSI	S	S	S	MSI	MSI	MSI	S	S	S	S	S
BAT25*	INTRONIC	MONUC	4q12	KIT	MSI	MSI	MSI	MSI	MSI	MSI	/	/	S	S	/	S
BAT26*	INTRONIC	MONUC	2p16	hMSH2 intron5	MSI	/	MSI	MSI	MSI	MSI	MSI	/	/	S	/	S
BAT40*	INTRONIC	MONUC	1p13.1	hsd3b1	/	MSI	MSI	/	MSI	MSI	MSI	S	/	S	/	S
MYB*	INTRONIC	MONUC	6q23.3-6q24	myb	/	/	/	MSI	MSI	MSI	MSI	/	S	S	S	S
D5S346*	GENOMIC	DINUCLEO	5q21-22	unknown	/	S	SLOW?	MSI	/	S	MSI	/	/	S	S	S
D5S107*	GENOMIC	DINUCLEO	5q11-13	unknown	MSI	MSI	MSI	MSI	S	MSI	MSI	S	S	S	S	S
D17S250*	GENOMIC	DINUCLEO	17q11-12	unknown	MSI	MSI	MSI	MSI	MSI	MSI	MSI	/	/	/	MSI	MSI
D5S123	GENOMIC	DINUCLEO	2p15-16	close hMSH2	MSI	MSI	MSI	MSI	MSI	MSI	MSI	MSI	S	S	S	S

NCI ≥2 HIGH 1 LOW 0 STABLE	NCI PANEL n=5	Overall Score	4/4	3/4	4/5	5/5	4/5	3/4	4/4	1/1	0/2	0/4	1/3	1/5
		Classification	HIGH	HIGH	HIGH	HIGH	HIGH	HIGH	HIGH	?	?	?	?	LOW

MSouthey 0-1 STABLE 2-5 LOW 6-10 HIGH	MSOUTHEY PANEL * n=10	Overall Score	5/7	4/7	5/9	6/8	6/9	6/9	9/9	0/3	0/6	0/9	1/7	1/10
		Classification	?	?	?	HIGH	HIGH	HIGH	HIGH	?	?	S	?	S

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Figure 1: Selected example of a pilot study of Comparative Genomic Hybridization (CGH) on genetically proven HNPCC cases.

