Hereditary Colorectal Cancer: Clinical and Biological Consequences of Known and New Genes, as well as Modifiers

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I'll note you in my book of memory"
W. Shakespeare, Henry VI, Act ii, Sc.4

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Abbreviations

AC	Amsterdam Criteria
AFAP	Attenuated Familial Adenomatous Polyposis
APC	Adenomatous Polyposis Coli
ATP	Adenine Tri-Phosphate
BER	Base Excision Repair
BG	Bethesda Guidelines
BRCA	Breast Cancer gene (1 and 2)
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CHRPE	Congenital Hyperpigmentation of the Retinal
CRC	Colorectal Cancer
DGGE	Denaturing Gradient Gel Electrophoresis
dHPLC	Denaturing High Performance Liquid Chromatography
DNA	Deoxyribose Nucleic Acid
Dox	Doxycycline
8-oxoG	7,8-dihydro-8-oxo-guanine
FAP	Familial Adenomatous Polyposis
HE	Hemotoylin and Eosin Stain
HhH	Helix-Hairpin-Helix motif
hMLH	Human MutL Homolog
hMSH	Human MutS Homolog
hPMS	Human Post Meiotic Segregation
HNPCC	Hereditary Non Polyposis Colorectal Cancer
IDLs	Insertion or Deletion Loops
IHC	Immunohistochemistry
LCM	Laser Capture Microdissection
LOH	Loss of Heterozygosity
MCR	Mutation Cluster Region
Min	Multiple Intestinal Neoplasia
MMR	Mismatch Repair
MNNG	N-methyl-N'-nitro-N-nitroguanidine
Mom 1	Modifier of Min 1
Mom 2	Modifier of Min 2
MSI	Microsatellite Instability
PCR	Polymerase Chain Reaction
Pla2g2a	Secretory Phospholipase A2
PTEN	Phosphatase and Tensin Homologue on Chromosome 10
PTT	Protein Truncation Test
RET	Rearranged Transforming
SD	Standard Deviation
SSCP	Single Strand Conformation Analysis
TGFβRII	Tumour Growth Factor β Receptor II

Abstract

Each year 3500 people in Switzerland are diagnosed with colorectal cancer, approximately 51.8 and 34.3 per 100'000 inhabitants for males and females, respectively. Those patients with a familial risk ie. they have 2 or more first or second degree relatives with colorectal cancer, account for approximately 20 percent of all affected patients, whereas roughly 5 to 10 percent of the total annual burden of colorectal cancer is mendelian in nature – that is, it is inherited in an autosomal dominant manner. This thesis has focused on genotype-phenotype correlations in two hereditary colorectal cancer syndromes, hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) in an attempt to optimise the selection criteria for affected individuals, to establish the sensitivity and specificity of different screening methods, to investigate a relatively new gene associated with a multiple colorectal adenoma and carcinoma phenotype and to assess the role of a modifier gene locus on chromosome 1p33-36.

Since only limited data are available which detail the value of the different HNPCC referral criteria in combination with microsatellite instability (MSI) testing and various mutation screening methods, 222 unrelated Swiss patients were studied in order to (i) assess the phenotypic and molecular differences between patients belonging to different referral criteria groups, and (ii) determine the diagnostic accuracy of the criteria and screening procedures employed in identifying individuals with mismatch repair (MMR) gene alterations. The Bethesda Guidelines (BG) proved to be of superior sensitivity and diagnostic accuracy compared to Amsterdam Criteria I/II (AC I/II) alone, in identifying patients with MMR gene alterations. Based on the evaluation of the different screening techniques employed in this study, it is suggested that MSI analysis combined with immunohistochemistry testing and subsequent mutational analysis of the positively scored individuals encompassing both a DNA and a mRNA-based technique, should be conducted for optimal rates of mutation detection.

Investigations subsequently continued in attempts to further characterise the phenotype of Swiss HNPCC patients by comparing 46 MMR gene mutation carriers to 84 gene alteration negative individuals in order to ultimately aid the identification of HNPCC individuals and MMR gene mutation carriers. Ninety-four percent of the mutation positive patients were classified by referral criteria (AC or BG) compared to only 76% of mutation negative individuals. Mutation positive patients were also younger at the time of their CRC diagnosis, had more often proximally located

CRCs, a higher prevalence of syn-/metachronous CRCs and more frequently extracolonic manifestations. Using such phenotypic differences to distinguish mutation positive from mutation negative individuals, clinicians may be aided in their preselection of patients for genetic surveillance, mutation screening and subsequently, genetic counselling.

In light of results from recent studies, implicating germline mutations in *MYH* with a multiple colorectal adenoma and carcinoma phenotype, it was the purpose of this study to further correlate *MYH* germline mutations with Swiss APC-negative individuals (n=65) and establish any genotype-phenotype correlations to aid in the optimisation of clinical screening and prevention strategies. An optimised protocol for the rapid and sensitive mutation analysis of *MYH* via high performance liquid chromatography (DHPLC) was established. Thirteen (20%) individuals were identified as *MYH* mutation carriers, 7 (54%) of which had biallelic mutations. Aside from previously reported mutations, 3 apparently novel gene alterations were established in 3 patients with a multiple adenoma phenotype. The phenotypical characteristics of all patients investigated were similar, with no statistically significant correlations to genotype, hence, clinicians and counsellors are advised to screen for MYH mutations in patients displaying tens to hundreds of colorectal adenomas, and a family history consistent only with recessive inheritance.

FAP patients typically display considerable inter- and intra-familial phenotypic heterogeneity, which represents a major problem in genetic counselling of *APC* mutation carriers. The *Min* mouse model indicated a putative disease modifier locus on chromosome 4, which is syntenic to human chromosome 1p35-36. Furthermore, germline mutations in the base-excision repair gene *MYH*, which maps to the 1p33-34 region, have been described in patients with multiple adenomas, pointing to a possible role as disease modifier in FAP. Here, the re-assessment of one of the largest FAP kindreds published, which was previously used in linkage mapping of 1p35-36, is documented. Using the latest available clinical information, additional mutation carriers and polymorphic markers, fine-mapping of the critical region as well as mutation analysis of the *MYH* gene were performed. These investigations significantly excluded (i) the 1p33-36 region as a modifier locus and (ii) *MYH* as a modifier gene for extracolonic disease in this FAP kindred. The results indicate that linkage analysis of further putative candidate regions is necessary to identify a disease modifier locus in FAP.

General Introduction

Colorectal Cancer Incidence:

Today's global population is approximately 6.1 billion people, with 133 million being born and 52 million dying each year. World-wide, about 8 million people develop cancer each year. Approximately 876 000 of these are diagnosed with colorectal cancer, the third most frequently occurring cancer after that of lung and stomach (http://home.swipnet.se/crc/crc.htm). The lifetime risk in the general population for developing colorectal cancer is 5%, but this figure rises dramatically with age and by 70 years, almost half the Western population will have developed an adenoma. In general, the incidence of colorectal cancer is high in developed countries (Jemel et al., 2002). However, incidence rates vary up to 20-fold between low- and high-risk geographical areas throughout the world, probably due to environmental and dietary factors (Lothe et al., 1993). Each year 3500 people in Switzerland are diagnosed with colorectal cancer, approximately 51.8 and 34.3 per 100'000 inhabitants for males and females, respectively (Swiss Cancer Registries' Association Database, 2003).

Since only 37% of cancers are detected in the early, most treatable stages (Dukes A or B), almost 50% of the patients with a new diagnosis of colorectal cancer are expected to die within 5 years of diagnosis (Jass et al., 1992).

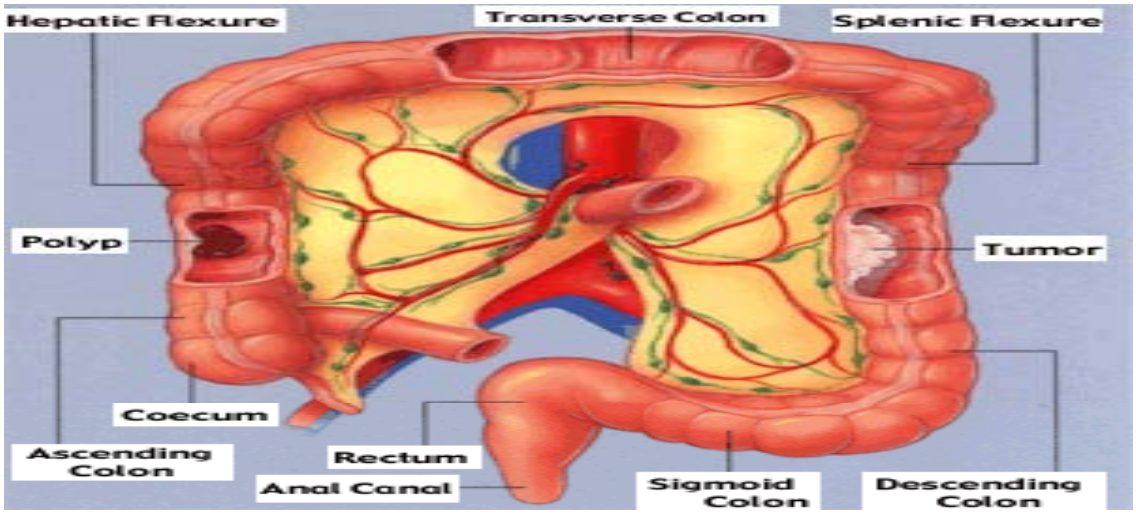


Figure 1: The colon and examples of an adenoma and carcinoma

Colorectal Carcinogenesis:

It is a common understanding that cancer cells are derived from normal stem cells. Only such stem cells have the natural capacity for extensive proliferation and the ability to differentiate along several directions, factors that define cancer (Campbell et al., 1998). Detailed morphological, biochemical and physiological studies have provided clear evidence for the existence of stem cells near the base of the crypts (Sancho et al., 2003). The progeny of stem cells migrate up the crypts, continuously dividing, until they reach the mid-section. Here, the migrating epithelial cells cease to divide and subsequently differentiate to mature cells, the majority being mucous-secreting globlet cells and absorptive epithelial cells. On reaching the top of the crypt, the differentiating cells undergo apoptosis and are engulfed by stromal cells or shed into the lumen (Brittan et al., 2002).

However, in the initial stages of tumorigenesis, dysplastic cells are commonly found at the luminal surface of the crypts and are found to be mutant clones, genetically unrelated to the cells at the base of the crypt. This dysplastic eptithelium, forming the top portion of the crypt, proliferates in a manner identical to that observed in advanced neoplasms (Schon, 2003). In addition, the dysplastic epithelium harbours such genetic alterations at the *APC* locus that are associated with functional changes in beta-catenin expression and localisation (Michor et al., 2004). These histologic, biochemical and genetic features are virtually always detected, in almost every crypt of every small adenoma investigated and suggest that adenomatous polyp development proceeds via a top-bottom mechanism (Vogelstein et al., 1998). The genetically altered cells spread laterally and downward to form new crypts that primarily attach themselves to pre-existing normal crypts and subsequently replace them.

By the time the cancer cell and its progeny have divided 30 times, the resulting tumour could contain 1 billion cells and weigh about 1 gram, and it could be

detectable by X-rays or endoscopy. However, the growth rate of a tumour is greatly affected by cell death, in the form of apoptosis or necrosis.

Additional mutations in oncogenes and tumour suppressor genes give rise to clonal expansion and the adenoma gaining the ability to invade surrounding tissue and metastasize to other organs as adenocarcinoma (see Figure 2, Vogelstein et al., 1993, 1998). It is thought that at least 4 sequential genetic changes are necessary to ensure colorectal cancer evolution. One oncogene (*KRAS*) and three tumour suppressor genes (*APC*, *SMAD4* and *TP53*) are the primary targets for these genetic changes (Weinberg, 1994). The dominant and recessive nature of these genes predicts that at least 7 mutations are required: one oncogenic mutation at *KRAS* and six further mutations to inactivate both alleles of the *APC, SMAD4* and *TP53* tumour suppressor genes. Tumour suppressor gene mutations are determined in the majority of tumours, however *KRAS* mutations are found in approximately 50-60% of cases (Lipton et al., 2003).

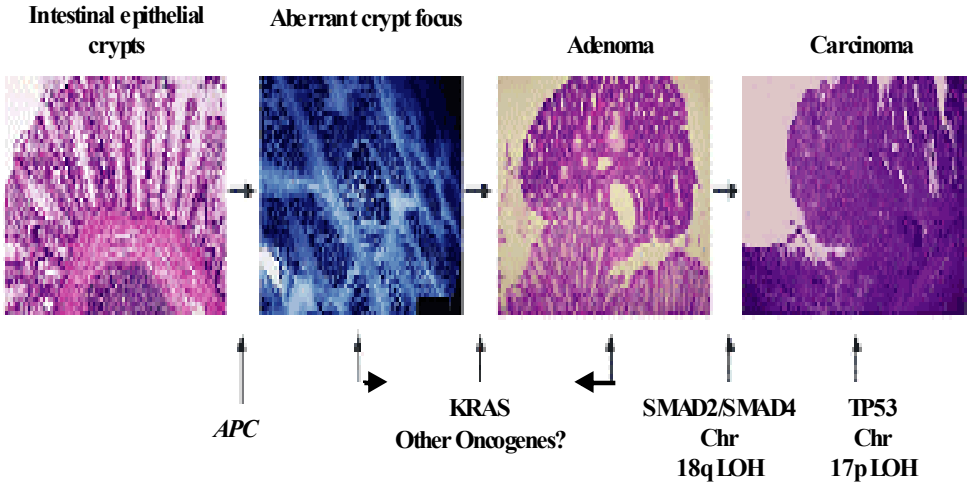


Figure 2: Histopathology and genetic hits in the progression of normal epithelial cells to carcinoma (taken from Nature Reviews: Cancer, October 2001, Vol 1).

Genetic Factors of Colorectal Cancer:

Colorectal cancer usually arises sporadically due to environmental or dietary factors, but can also stem from a hereditary pre-disposition.

Approximately 80% of patients with colorectal cancer appear to have sporadic disease with no evidence of having inherited the disorder, whilst 20% seem to be attributed to a definable genetic component (Cannon-Albright et al., 1998). Evidence for a genetic factor playing a role in colorectal cancer includes increased risk of colorectal malignancy in persons with a family history and familial aggregation of colorectal cancer consistent with autosomal dominant inheritance. In 5-6% of all colorectal cancer cases a germline genetic mutation, conferring high lifetime risk of colorectal cancer in carriers, has been found (Lynch et al., 2003). Additional gene mutations, some with lower lifetime risks, are continuing to be characterized (Narayan et al., 2003).

Colon cancer can be effectively prevented through timely removal of adenomatous polyps by endoscopy (recto-sigmoidoscopy or colonoscopy). Once a carcinoma has developed, surgery is the primary treatment for most patients, sometimes in combination with a 5-fluorouracil-based adjuvant chemotherapy.

Inherited Genetic Susceptibility to Colorectal Cancer:

Approximately 15-20% of all colorectal adenocarcinomas are familial in origin. The best-defined inherited syndromes are Hereditary Non-Polyposis Colorectal Cancer (HNPCC) (and its rare variants Muir-Torre and Turcot syndromes) (Lynch, 2000) and Familial Adenomatous Polyposis (FAP) (Beech et al., 2001), which are estimated to account for 2-5% and less than 1% of all colorectal cancers in Western countries, respectively. Other, albeit very rare, inherited cancer predisposition syndromes include Juvenile Polyposis, Gardner's syndrome and Peutz-Jeghers syndrome. Although many familiar aggregations of colon cancer remain etiologically undefined, HNPCC appears to be the most

frequently inherited cancer syndrome in humans. The main focus points of the chapters to come, are HNPCC and FAP.

Hereditary Non Polyposis Colorectal Cancer (HNPCC):

The clinical definition of HNPCC describes a syndrome with an excess of colon cancer and a defined spectrum of extracolonic manifestations, diagnosed at an early age and inherited via an autosomal dominant mechanism. Individuals with a HNPCC gene mutation have a 70-80% lifetime risk of developing colorectal cancer (Lynch et al., 2003). The renown international diagnostic criteria for HNPCC, known as the Amsterdam Criteria I (primarily concerned with colorectal cancers only) and Amsterdam Criteria II (concerning cancers of the colon and rectum, endometrium, small bowel, ureter and renal pelvis) rely on these clinical characteristics (Vasen et al., 1991).

Hereditary Non Polyposis Colorectal Cancer, an autosomal dominant disorder, represents 1-5% of all colorectal cancers, has a frequency of between 1:2000 and 1:200 and is hence one of the most commonly observed cancer syndromes in humans (Lynch et al.,1998; Aaltonen et al., 1998). It is characterised by a number of criteria:

1. the involvement of several family members. In accordance with the Amsterdam criteria (Vasen et al.,1991 and 1999), at least 3 family members in 2 generations should be affected (by colon or endometrial cancer, see below), with one being a first degree relative of the other two.

2. diagnosis made at or below 50 years of age in at least one of the affected family members. Typically, HNPCC tumours occur at an average age of 45 years compared to 65 for sporadic colon cancer.

3. a higher frequency of tumours in other organs, primarily the endometrium, followed by the ovaries, stomach, small bowel, ureter, and renal pelvis.

4. an 80% and 60% lifetime risk for developing colorectal and endometrial cancer, respectively, compared to 6% and 1-2% in the general population.

5. tumours that are more commonly located in the right (proximal) portion of the colon

6. an increased incidence of synchronous (more than 1 primary colon cancer occurring at the same time) and metachronous (more than one primary colon cancer occurring at different times) cancers.

7. tumours that demonstrate an increased rate of transformation of the benign polyp, but a better prognosis.

8. germline mutations in ***mismatch repair (MMR)*** genes

In addition, skin tumours (sebaceous adenomas, sebaceous carcinomas and karatoacanthomas) are apparent in a few families affected by Muir-Torre syndrome (Coldron and Reid, 2001) and brain tumours (glioblastomas or medulloblastomas) in families with Turcot syndrome (Hampel and Peltomaki, 2000).

Due to the increasing number of small families in Western countries with high migration rates, the criteria for HNPCC diagnosis have been relaxed. Today, families with only 2 colon or endometrial cancers occurring before the age of 50 are also screened for HNPCC.

HNPCC results from germline mutations in one of the four major HNPCC-associated mismatch repair (MMR) genes: *hMSH2* (human mutS homolog 2) on chromosome 2p16 (Aaltonen et al.,1993; Peltomaki et al., 1993), *hMLH1* (human mutL homolog 1) on chromosome 3p21 (Lindblom et al., 1993), *hMSH6* (human

mut S homolog 6) on chromosome 2p16 (Palombo et al., 1995) and *hPMS2* (human postmeiotic segregation 2) on chromosome 7q11 (Nicolaides et al., 1994). An excess of 400 different predisposing MMR gene mutations are known to date with germline mutations of *hMSH2* (frameshift = 60%, or nonsense mutations = 23%) and *hMLH1* (frameshift = 40% and missense alterations = 31%) accounting for more than 95% of the mutations found in HNPCC families; they are distributed throughout the 16 and 19 exons of these two genes, respectively (International Collaborative Group on HNPCC Web site: http://www.n-fdht.nl). Less than 5% of *hPMS2* mutations attribute to the HNPCC syndrome. Recently, the newly established MMR gene *hMSH3* has been shown to play a small role in HNPCC, although additional data regarding prevalence, pathogenicity and clinical correlations, is required to reinforce its part as an HNPCC predisposition gene (Hienonen et al., 2003).

Microsatellite instability (MSI) is a phenomenon detected in the colorectal tumour DNA of individuals with mismatch repair gene mutations. Tumours developing through this pathway have alterations in the length of short, repeated mononucleotide or dinucleotide sequences of DNA ie. microsatellites, caused by the insertion or deletion of repeated units. MSI has been found in most cases (>90%) of HNPCC that fulfil the Amsterdam Criteria and 15% of sporadic colorectal cancers. This phenomenon reflects the underlying defect in the DNA mismatch repair gene system (Dietmaier et al., 1997).

Germline mutations in *hMLH1* or *hMSH2* generally lead to a classical HNPCC phenotype with families fulfilling the Amsterdam Criteria and tumours displaying a high degree of microsatellite instability (Peltomaki et al., 1993; Boland et al., 1998). Mutations in *MSH6* and *PMS2* however, are less frequently observed in the classical HNPCC families and present themselves in a more atypical HNPCC phenotype (Table 1). Severe MSI has been occasionally observed in conjunction

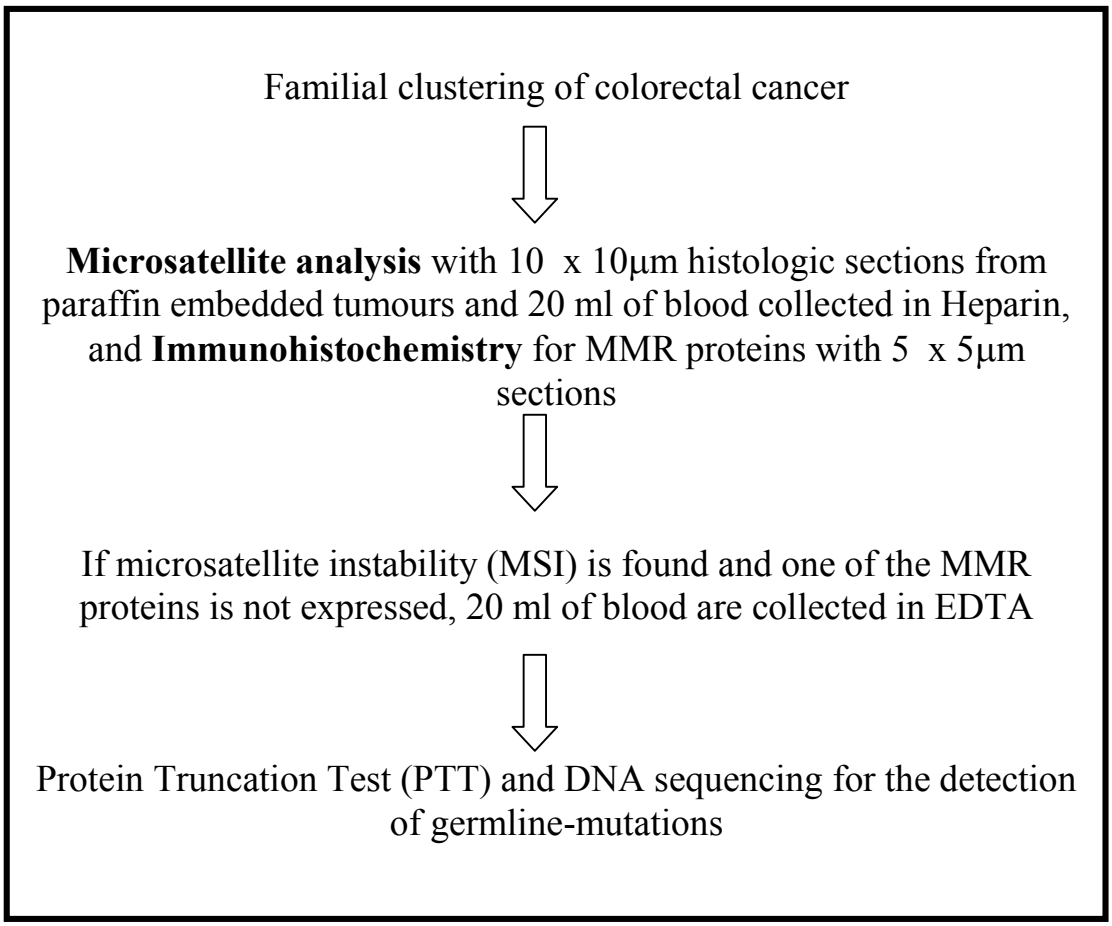
with *hPMS2* mutations, but *hMSH6* mutations are more often associated with a low degree MSI phenotype (Nicolaides et al., 1994; Miyaki et al., 1997).

Gene	Phenotypic features of HNPCC
hMLH1	Primarily typical HNPCC. ca30% of mutations are the missense type. Varying phenotype
hMSH2	Primarily typical HNPCC. Extracolonic manifestations occurring more frequently than in MLH1 mutation carriers
hMSH6	Typical or atypical HNPCC. Late CRC onset, frequently affected endometrium, distal location of CRC, MSI-Low tumours
hPMS2	Typical or atypical HNPCC.
hMSH3	Primarily atypical HNPCC. Distally located and MSI-Low tumours

Table 1: clinical features associated with germline mutations in the MMR genes associated with a predisposition to HNPCC

HNPCC Screening:

Given that colorectal cancer incidence in Switzerland is approximately 90 new cases per100'000 inhabitants each year, and that 1-5% of these are attributed to HNPCC, it is estimated that between 60 and 300 individuals in this country develop HNPCC colon cancer each year. Using the screening program outlined below, it is our aim to identify these individuals and to characterise the germline mutations in their MMR genes.



Microsatellite analysis is a valuable assessment of instability in repetitive regions of DNA and highlights those individuals that should be screened further for germline mutations in MMR genes. Matched tumour and normal DNA are extracted from the histologic sections and blood, respectively, and are analysed for differences in the lengths of a subset of microsatellite motifs (figure 3). Any differences indicate an unstable sequence in the tumour tissue and the case is referred for further screening.

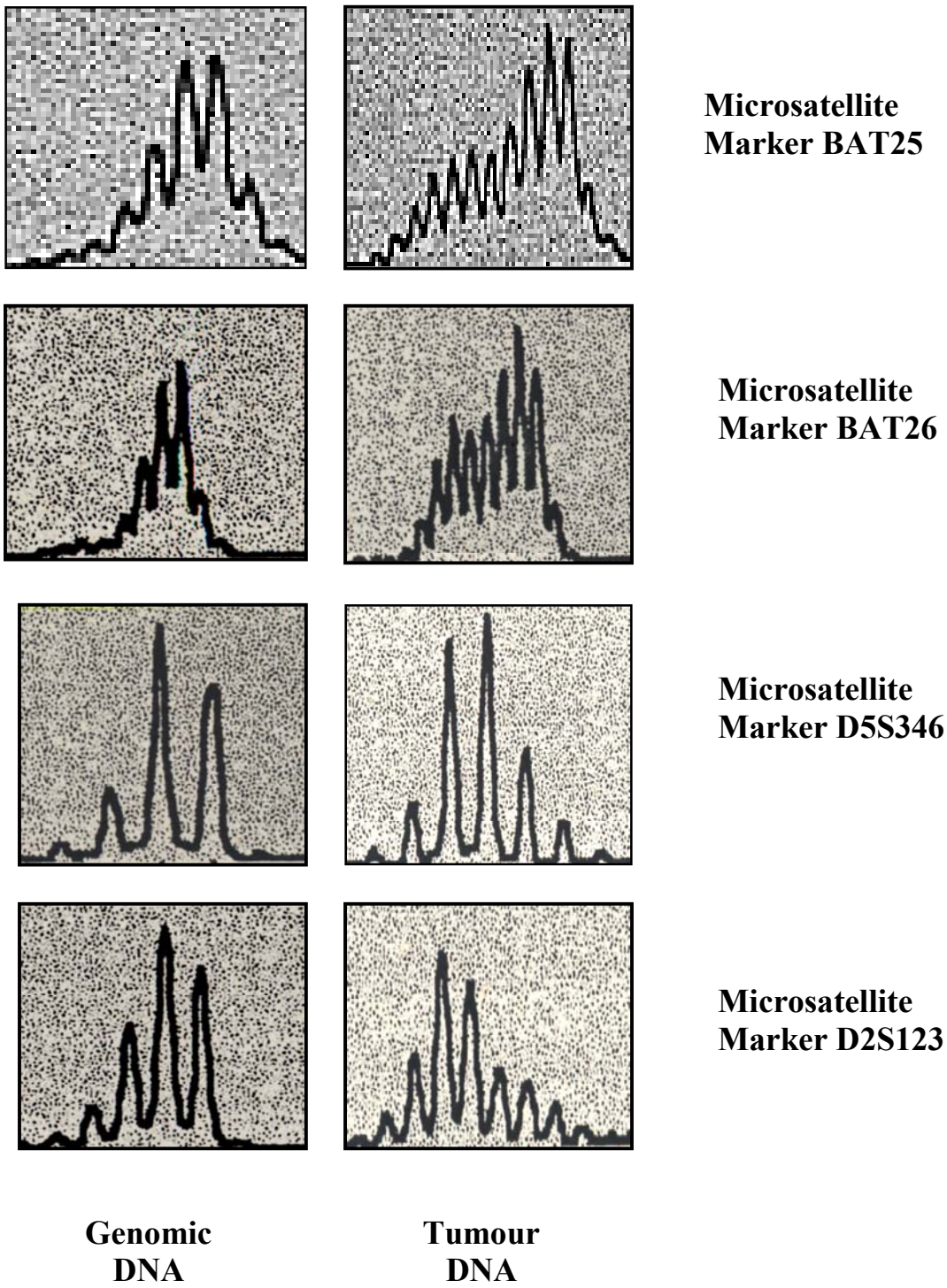


Figure 3. Microsatellite analysis. The comparison between DNA extracted from the normal tissue and from the tumour tissue is made for the microsatellite markers BAT25, BAT26, D5S346 and D2s123. Additional peaks in the tumour DNA are indicative of microsatellite instability (MSI)

Immunohistochemistry is a simple and effective method for determining the loss of MMR proteins from the tumour as a result of two events: the inherited germline mutation on one allele of the MMR gene and a second somatic event (ie. in the colonocytes) on the wild-type allele (mutation of loss of heterozygosity). Normal mucosa and tumour tissue are analysed in the same histologic section for the expression of hMSH2, hMSH6, hMLH1 and hPMS2 proteins. The loss of expression of one of these proteins suggests which MMR gene should be screened for the germline mutation.

The *protein truncation test (PTT)* is employed specifically for establishing truncating mutations and large insertions or deletions in MMR genes. In these cases, shorter gene products are detected on a denaturing gel.

With indications from both IHC and PTT, the search for mutated MMR genes is narrowed down. Subsequently, direct genomic ***DNA sequencing*** is employed to screen the genes for point mutations exon by exon. To date, there are more than 400 HNPCC mutations described in the databanks (http://www.nfdh.nl/database/mdbchoice.htm), with ~60% being in *hMLH1* and ~35% in *hMSH2*. In three years of screening, we have identified more than 100 Swiss HNPCC families carrying mutations in the *hMSH2* or *hMLH1* loci.

Relatives of the index patient are easily screened for the presence of the 'familiar mutation', the only requirement being 10 ml of blood in EDTA. Non-carriers can be excluded from the screening program, since cancer incidence in these individuals is comparable to that found in the general population. The mutation carriers are invited for regular (2-yearly) consultations, including endoscopic surveillance, which has proved itself to be extremely effective in colon cancer

prevention. In addition, these individuals are encouraged to enrol in genetic counselling programs, in order to gain the support they may require for dealing with the psychological burden of living with HNPCC.

The HNPCC screening information detailed here was initially written with clinicians and patients in mind and was published in the Schweizer Krebs Bulletin (No. 4. Dec 2001).

Familial Adenomatous Polyposis (FAP):

FAP is an autosomal dominant syndrome, accounting for ca.1% of all colorectal cancers, those results from germline mutations in the adenomatous polyposis coli (*APC*) gene. It is estimated to occur at a frequency of 1 in 8300 to 1 in 14,025 and affects both sexes equally (Bisgaard et al., 1994). Characteristically, teenaged patients develop multiple (>100) adenomatous polyps diffusely throughout the colon and rectum. Approximately 50% of FAP patients develop adenomas by 15 years of age and 95% by the age of 35 (Bulow et al., 1995). The average age at diagnosis ranges from 34.5 to 43 years. Colorectal cancer is inevitable in FAP patients if colectomy is not performed (Lynch et al., 2003).

FAP patients frequently develop a variety of benign extracolonic manifestations in addition to polyposis coli. These may include extracolonic adenomas (adenomas of the small intestine and stomach, fundic gland retention polyps of he stomach), cutaneous lesions (lipomas, fibromas, sebaceous, and epidermoid cysts), desmoid tumours, osteomas, dental abnormalities and pigmented ocular fundic lesions (congenital hypertrophy of the retinal pigment epithelium). Furthermore, extracolonic malignancies that can develop in FAP patients include hepatoblastoma, upper gastrointestinal tract malignancies, thyroid gland, biliary tree, pancreas and brain (Knudsen et al., 2000; Giardiello et al., 2001).

Almost all cases of FAP are attributed to germline mutations of the *APC* gene located on chromosome 5q21 (Bodmer et al., 1987; Groden et al., 1991). The *APC* gene is a tumour suppressor or "gatekeeper" gene with 15 exons encoding a protein considered essential in cell adhesion, signal transduction and transcriptional activation, with C-myc and β-catenin having been established as downstream targets (Fearnhead et al., 2001). An excess of 300 different *APC* mutations have been described to date, the majority being insertions, deletions and nonsense mutations that subsequently lead to frameshifts or premature stop codons, resulting in the truncation of the *APC* gene product (http://www.umd.necker.fr:2008). Such a truncated protein lacks all axin/conductin binding motifs and a variable number of the 20 amino acid repeats that are associated with the down regulation of intracellular β-catenin levels. In FAP, germline mutations are found throughout the 5' region of the *APC* gene. However, somatic mutations are found grouped between codons 1286 and 1513 in the so-called "Mutation Cluster Region" (MCR). The most commonly occurring APC mutation, detected in 10% of FAP patients, is a deletion of AAAAG in codon 1309 (Miyoshi et al., 1992).

Studies involving genotype-phenotype correlations have revealed that *APC* gene mutations between codons 169-1393 result in classical FAP (Fearnhead et al., 2001), whilst 3' and 5' mutation predispose to attenuated FAP (Su et al., 2001), and mutation I1307K increases colorectal cancer risk in Ashkenazi Jews (Zauber et al., 2003). Other observations include profuse colorectal polyposis between codons 1250 and 1464, predilection for extraintestinal manifestations at codons 1465, 1546 and 2621, and occurrence of retinal lesions with mutations located within codons 463 to 1444. However, it is well established that intra- and interfamilial phenotypic variability can occur even in patients with identical gene alterations (Laurent-Puig et al., 1998).

Attenuated Familial Adenomatous Polyposis A(FAP):

Attenuated familial adenomatous polyposis (AFAP) is a clinical variant of FAP and is characterised by less than 100 polyps and presents mutations in the extreme 5' or 3' region of the *APC* gene or in the alternatively spliced region of exon 9 (Fearnhead et al., 2001). Tumour development in at least some AFAP patients appears to require somatic second and third hits of the wild-type and attenuated *APC* alleles (Spirio et al., 1998; Su et al., 2000). Extracolonic manifestations commonly observed in AFAP include fundic gastric polyps and duodenal polyps, whereas less frequently detected are congenital hyperpigmentation of the retina (CHRPE) and desmoid tumours. The onset of colorectal cancer is 15 years later than in classical FAP, the average age being 55 years compared to 39 years, respectively. The disease manifestation of AFAP patients can phenotypically overlap with that of the HNPCC syndrome (Knudsen et al., 2003). However, *MYH* associated polyposis, arising from deficient base excision repair (BER), was initially reported in a single Caucasian family. Al Tassan et al. connected multiple adenomas and carcinomas to a previously undescribed autosomal recessive condition involving germline mutations of the base excision repair gene *MYH* (Al-Tassan et al., 2002).

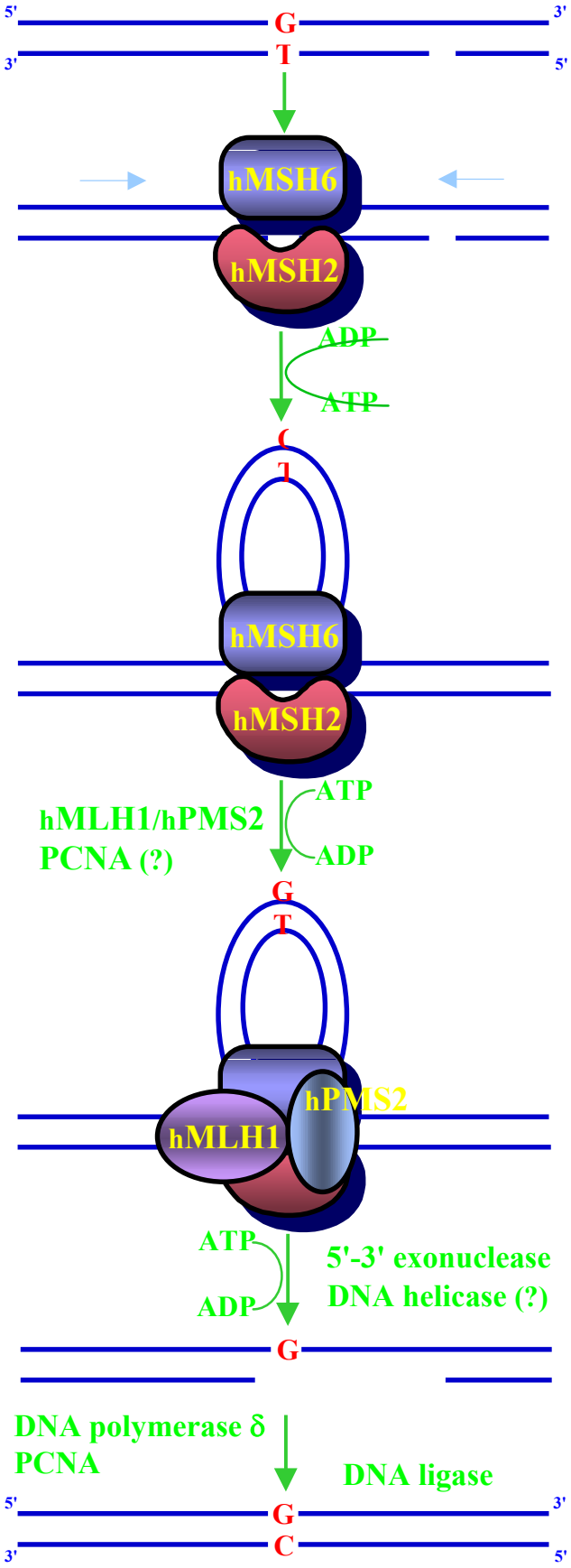
Mismatch Repair (MMR):

Mismatch repair operates to maintain genome stability by correcting mismatches and small insertion or deletion loops (IDLs) introduced through errors made by DNA polymerases during DNA replication. In addition, MMR counteracts recombination between homologous but diverged DNA sequences. Throughout the evolution of eukaryotes, the initial steps of MMR have been conserved. However, it appears that the mechanisms of the strand-discrimination signal and the downstream steps in mammalian MMR are mostly exclusive (Peltomaki and Vasen, 1997).

In eukaryotes, the heterodimeric MutS homologue MSH2/MSH6 (MutSα) functions in the repair of mismatches and short IDLs, whilst the MSH2/MSH3 (MutSβ) heterodimer repairs the longer IDLs. In addition, the MutL homologues, MLH1/PMS2, form a heterodimeric complex (MutLα) and aid the repair mechanism by recruiting a number of different proteins eg. helicases, exonucleases for excising abnormally based pairs (Jiricny and Nyström-Lahti, 2000).

Predisposition to colon cancer in HNPCC results from a germline-mutation (ie. inherited in all cells of the body) in one of several DNA mismatch repair (MMR) genes described so far (Peltomaki and Vasen, 1997). Gene mutations in either *hMSH2* (on chromosome 2) or *hMLH1* (on chromosome 3) have been found in the majority of HNPCC families, whilst only a few atypical kindred carry mutations in the gene encoding hMSH6 (on chromosome 2).

Figure 4 . Putative model of human MMR. (i) The mismatched <T< introduced into the newly synthesised strand by the replication complex, is recognised by the hMSH2/hMSH6 heterodimer. (ii) ATP drives the bi-directional threading of DNA which makes a loop and (iii) recruits other essential members of the MMR complex, such as the hMLH1/hPMS2 heterodimer and PCNA. (iv) Exonucleolytic degradation of the T-containing strand is initiated by an as yet unidentified helicase(s) and exonuclease(s). (v) DNA synthesis is re-initiated by the replication complex and a <C> is normally paired with <G>.


5'
3'
G
T
3'
5'
hMSH6
hMSH2
ADP
ATP
C
T
hMSH6
hMSH2
hMLH1/hPMS2
PCNA (?)
ATP
ADP
G
T
hPMS2
hMLH1
ATP
ADP
5'-3' exonuclease
DNA helicase (?)
G
DNA polymerase δ
PCNA
DNA ligase
5'
3'
G
C
3'
5'

Base Excision Repair (BER):

Germline mutations in the BER *MYH* gene may contribute to individuals with a multiple colorectal adenoma phenotype (Sieber et al., 2003).

Reactive oxygen species (ROS), for example hydrogen peroxide, superoxide and hydroxyl radicals, are the mutagenic by-products of normal aerobic cellular metabolism. Elevated levels of ROS can result in DNA damage and have been related to several degenerate diseases: cancer, immune system decline, cataracts, cardiovascular disease, ageing and brain dysfunction (Ames et al., 1991). One of the most stable products of oxidative DNA damage and also the most deleterious due to its mispairing capacity with adenine, is 7,8-dihydro-8-oxo-guanine (8-oxoG). 8-oxoG has been connected with spontaneous G:C→T:A transversion mutations in BER defective bacteria and yeast (Michaels et al., 1992; Thomas et al., 1997).

In the prevention of 8-oxoG induced mutagenesis, proteins from 3 genes of the BER pathway, *hMTH1, hOGG1* and *hMYH*, interact together both within the nucleus and the mitochondria. hMTH1, with its nucleoside triphosphatase activity, is responsible for the hydrolysis of 8-oxo-dGTP, hence preventing the inclusion of the oxidised nucleotide during DNA replication. hOGG1 establishes and eliminates ring-opened purine lesions and mutagenic 8-oxoG adducts, whilst hMYH, an adenine specific DNA gycosylase, removes adenines mismatched with 8-oxoG or guanines during DNA replication errors (Lindhal et al., 1993).

This thesis concentrates on two colorectal cancer causing diseases with clear identities, HNPCC and FAP, and aims to:

i) Study the mechanisms leading to hereditary colorectal cancer (Chapter I *parts i-iv*)

ii) Identify causing mutations (Chapter II)

iii) Assess the phenotypical consequences of established germline gene mutations (Chapters III, IV and V).

Thesis Chapters:

Chapter I part i details further correlations made between *MYH* germline mutations and *APC*-negative individuals in what was an attempt to establish genotype-phenotype correlations in a Swiss study cohort in order to aid in the optimisation of clinical screening and future prevention strategies. This chapter has been prepared for publication as a scientific paper and is currently under review by the participating authors.

Chapter I part ii, a collaboration with Petr Cejka already published in the EMBO Journal Vol. 22, No. 9, pg2245-2254, 2003, highlights the phenotypic consequences correlated to reduced levels of MMR proteins, as demonstrated by a new cell line, epithelial in origin, in which the expression of *hMLH1* could be strictly regulated by doxycycline (Dox).

Chapter I part iii, a collaboration with Luigi Lhagi that has been prepared as a scientific paper for publication***,*** investigated the prevalence of frameshift mutations in secondary mutator genes and in other target genes in a series of MSI-high CRCs with *hMLH1* and *hMSH2* deficiency, from both hereditary and sporadic cases in different pathological stages***.***

Chapter I part iv, a collaboration with Giancarlo Marra which will lead to the eventual publication of a scientific paper, reports further on the value of microsatellite instability testing and immunohistochemical analysis in the identification of MMR gene mutations.

Chapter I part v, a collaboration, with Giancarlo Marra as part of an ongoing study, branches onto new ground with the investigation into *hMSH6* mutations in HCT116+chr 3 clones, after treatment with the DNA methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

Chapter II reports on one of the largest FAP kindreds ever published. Although all affected family members harbour the same germline mutation of the *APC* gene, they display marked phenotypic variability. Through linkage analysis the 1p33-36 region was excluded as a modifier locus, and *MYH* as a modifier gene, for extracolonic disease in this FAP kindred. This paper has already been published in the European Journal of Human Genetics Vol.12 pg 365-371, 2004.

Chapter III of this thesis, a draft of a scientific paper prepared for publication, documents results cleaved from a study of 222 Swiss patients, where phenotypic and molecular differences between patients belonging to different HNPCC referral criteria groups were investigated. In addition, through the assessment of the diagnostic accuracy of different screening procedures, the most reliable algorithm for the identification of mismatch repair gene mutation carriers, has been defined.

Chapter IV, also a scientific paper draft, goes on to define the phenotypic differences between the MMR gene mutation positive and the mutation negative individuals in an attempt to highlight characteristics which may aid in the detection of HNPCC individuals and MMR gene mutation carriers.

The planned addition of data collected by Pierre Hutter, Institut Central des Hospitaux Valaisans, Sion, Switzerland, will enable us to publish chapters III and IV as Swiss national studies.

Chapter V has been submitted as a scientific paper to the Gastroenterology journal. It reports on a study that assessed the occurrence of genetic anticipation in HNPCC ie. the earlier age at diagnosis of colorectal cancer in successive generations. It appears to be a phenomenon that occurs in HNPCC kindreds with identified mismatch repair gene mutations. These results may have important implications for genetic counseling and clinical management of HNPCC families.

Chapter I part i

Prevalence of *MYH* germline mutations in Swiss *APC* mutation-negative polyposis patients

This chapter has been prepared for publication as a scientific paper and is currently under review by the participating authors.

Abstract

In 10-30% of patients with classical familial adenomatous polyposis (FAP) and up to 90% of those with attenuated (<100 colorectal adenomas; AFAP) polyposis no pathogenic germline mutation in the adenomatous polyposis coli (*APC)* gene can be identified (*APC* mutation-negative). Recently, biallelic mutations in the base excision repair gene *MYH* have been shown to predispose to a multiple adenoma and carcinoma phenotype. This study aimed to i) assess the *MYH* mutation carrier frequency among Swiss *APC* mutation-negative patients and ii) identify phenotypic differences between *MYH* mutation carriers and *APC/MYH* mutation-negative polyposis patients. Sixty-five unrelated *APC* mutation-negative Swiss patients with either classical (n=18) or attenuated (n=47) polyposis were screened for germline *MYH* mutations by dHPLC and direct genomic DNA sequencing. Eleven tumours from 4 biallelic mutation carriers were further investigated for microsatellite instability, loss of heterozygosity (LOH) at the *APC* locus and for somatic mutations in the mutation cluster region (MCR) of *APC* as well as in exon 1 of *KRAS*. Phenotype comparisons were statistically assessed using the Chi square, Fisher's exact and Student's t-test. Overall, 13 (20%) individuals were found to harbour *MYH* germline mutations (7 bi- and 6 monoallelic mutation carriers). Among patients with a family history compatible with autosomal recessive inheritance (n=33), 2 (22%) out of 9 classical polyposis and 5 (21%) out of 24 attenuated polyposis patients carried biallelic *MYH* alterations, 3 of which represent novel gene variants (R168H, R171Q and

R231H). Despite the absence of somatic mutations in *APC*'s MCR, LOH at the *APC* locus and the G12C mutation in *KRAS* were detected in adenocarcinomas from 2 biallelic *MYH* mutation carriers. Colorectal cancer was significantly ($p<0.01$) more frequent in biallelic mutation carriers (71%) compared to monoallelic and *MYH* mutation-negative polyposis patients (15 and 18%, respectively). In this nation-wide survey, 1 in 5 Swiss *APC* mutation-negative polyposis patients with a family history compatible with autosomal recessive inheritance was found to harbour biallelic *MYH* germline mutations. *MYH* mutation screening should be offered if the following criteria are fulfilled: i) absence of pathogenic *APC* mutation, ii) presence of classical or attenuated polyposis and iii) family history compatible with an autosomal recessive mode of inheritance.

Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited colorectal cancer (CRC) predisposition caused by germline mutations in the adenomatous polyposis coli (*APC*) gene and characterised by the development of hundreds to thousands of adenomatous polyps throughout the intestinal tract[1]. Attenuated FAP (AFAP) represents a clinical variant of classical FAP, associated with multiple (<100) colorectal adenomas and caused by mutations in the most 5' or 3' regions of *APC* or in the alternatively spliced region of exon 9[2-4]. With routine screening techniques failing to detect pathogenic *APC* germline mutations in 10 to 30% of classical FAP patients and in up to 90% of AFAP patients[5], investigations about the role of other polyposis predisposition genes are topical.

Recently, Al Tassan *et al.* demonstrated that biallelic germline mutations in the human homologue of the base excision repair gene *MutY* (*MYH)* cause a phenotype of multiple colorectal adenomas and carcinomas, thus describing for

the first time an autosomal recessively inherited CRC predisposition[6,7]. The DNA glycosylase MYH removes adenines from mispairs with 8-oxoguanine that occur during replication of oxidized DNA. Failure to correct these mispairs consequently leads to G:C→T:A transversion mutations, a typical "footprint" of oxidative DNA damage[19]. The observation of an excess of transversion mutations in tumours eventually led to the discovery of *MYH*-associated polyposis (MAP). A number of studies have already attempted initiated and conducted in attempts to establish the extent to which germline mutations in the *MYH* gene may contribute to individuals with an AFAP phenotype[6,7,8,9]. As a result, biallelic *MYH* germline mutations have been attributed to approximately 1-3% of all unselected CRC patients[6,7]. This nation-wide study aimed to i) assess the frequency of *MYH* mutation carriers in 65 unrelated Swiss patients presenting with either classical or attenuated polyposis and in whom no pathogenic *APC* germline mutation could be identified and ii) to identify phenotypic differences between biallelic, monoallelic mutation carriers and *APC/MYH* mutation-negative patients.

Patients and Methods

This nation-wide study investigated 65 unrelated Swiss index patients referred between 1994 and 2002 to either the Research Group Human Genetics, Division of Medical Genetics, Basel, or the Unit of Genetics, Institut Central des Hôpitaux Valaisans, Sion, Switzerland, because of classical (>100 polyps, n=18) or multiple adenomas/attenuated (5-99 polyps) FAP (n=47). In all patients, no germline *APC* mutation could be established by means of the protein truncation test and/or direct DNA sequencing (patients thereafter referred to as *APC* mutation-negative). In addition, 100 healthy Swiss individuals were enrolled in order to establish the carrier frequency of previously reported *MYH* variants as well as novel mutations of unknown pathogenic significance in unaffected individuals,. Informed consent for the study was obtained from all individuals

investigated. Patients were considered as anonymous cases and the results of the various genetic analyses were independently assessed by at least two reviewers.

DNA Extraction

Genomic DNA was isolated from EDTA blood using methods previously described by Miller, 1998[10]. Tumor DNA was isolated from formalin fixed, paraffin embedded tissue using the QIAMP DNeasy Tissue kit and according to the suggested protocol of the manufacturer (Qiagen, Basel, Switzerland). After verification of the tumor cell content (>50%) of HE stained tumor specimen, ten 5 to 8μm thick tumor sections were cut from each paraffin block. Lysis of the tissue was carried out overnight with Qiagen buffer, Proteinase K at an incubation temperature of 55°C. The samples were then washed twice with Qiagen wash buffer and the DNA finally eluted in 30 μl elution buffer provided.

***MYH* mutation analysis**

Exon specific primer pairs were used to amplify the 16 exons of *MYH*, including the respective exon-intron boundaries (GenBank accession number NM012222; primer sequences and PCR conditions available from the authors upon request). Twenty-five □l of PCR reaction mixture contained 50 ng of genomic DNA, 10 pmol of each primer and a PCR mastermix at 1.5 mM $MgCl_2$ according to the manufacturer's instructions (Invitrogen, Switzerland). All PCR reactions were done on a Hybaid OmnE thermocycler (Catalys AG, Wallisellen, Switzerland).

As a prescreening mean to detect DNA sequence changes denaturing high performance liquid chromatography (dHPLC) was performed using the 3500HT WAVE nucleic acid fragment analysis system (Transgenomic, Crewe, UK).

Melting temperatures for dHPLC were predicted by the Wavemaker software version 4.1.42 (Transgenomic) (dHPLC melting temperatures available from the authors upon request). Where different elution profiles were observed, in comparison to control samples run in parallel, direct DNA sequencing was performed in order to establish the nature of the sequence alteration.

For DNA sequencing, PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Basel, Switzerland). The sequencing reaction was performed using the Big Dye Teminator Cycle Sequencing kit (Applied Biosystems, Rotkreuz, Switzerland), according to the manufactures' guidelines. After purification using the DyeEx 2.0 Spin Kit (Qiagen, Basel, Switzerland) sequencing products were analysed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Germline mutations identified in *MYH* were confirmed in both forward and reverse directions, and from at least 2 independent PCR products. Exons 2, 5, 7, 8, 12 and 13 were routinely sequenced regardless of the dHPLC elution profile. Germline mutations Y165C and G382D were further confirmed by restriction enzyme digest, using *Ila*I and *Bgl*II, respectively.

Screening for Somatic *KRAS* and *APC* Mutations in Colorectal Tumors

Tumor tissue encompassing 2 colorectal adenocarcinomas and 9 adenomas was available from 4 patients harboring biallelic *MYH* mutations (no. 1775, 1828, 2013 and 2073). These tumours were investigated for mutations commonly found in exon 1 of the *KRAS* gene (primers and methods from Lipton, L., 2003)[11] and the mutation cluster region (MCR, codons 653-1513)[12] of *APC*. KRAS PCR products were subsequently sequenced, as described before. The 12 PCR fragments covering the MCR were first screened by dHPLC analysis. Whenever different elution profiles were observed, in comparison to control samples run in parallel, direct DNA sequencing was performed.

Loss of heterozygosity analysis at the *APC* gene locus

Loss of heterozygosity (LOH; allelic loss) analysis at the microsatellite loci MCB, D5S346 and D5S299 was performed according to the standard protocol (Applied Biosystems) with the use of fluorescently labeled oligonucleotides and analysed on an ABI PRISM 310 Genetic Analyser. Informative samples were defined as having allelic loss if the amount of one allele in the tumor was at least 50% lower than that of the other allele, after correction for the relative peak areas of the alleles found in leukocyte-derived DNA of the same patient[8].

Assessment of microsatellite instability (MSI)

Microsatellite instability was assessed using the monomorphic mononucleotide repeat BAT26. PCRs were carried out in a total volume of 25 □l containing 50 to 100 ng of leukocyte-derived and tumour DNA, respectively. PCR products were diluted 1:4 and 0.5 □l was added to 10 □l deionized formamide, denatured at 95°C for 5 min, chilled on ice and loaded on a ABI PRISM 310 genetic analyser. MSI was defined as the occurrence of novel alleles that differed by 3 nucleotides from the constitutional DNA (Loukola et al., 2001)[26].

Statistical Analysis

Statistical comparison of patients' features, encompassing phenotypic characteristics (gender, age at diagnosis, polyp number, extracolonic manifestations, family history), and mutational status, was performed using the Chi-square and Fisher's exact test for categorical variables, or Student's t-test for continuous variables, with all of the probabilities reported as two-tailed ps, considering a p value of <0.05 to be statistically significant.

Results

Sixty-five unrelated *APC* mutation-negative Swiss polyposis patients were taken from the Basel (n=44) and Sion (n=21) medical genetic centres and investigated for the presence of *MYH* germline alterations. Twenty-eight percent of the individuals were referred because of suspected classical FAP (n=18), whilst the majority exhibited an attenuated or multiple adenoma phenotype (n=47).

MYH mutation analysis

The complete coding sequence of the *MYH* gene was investigated in all 65 index patients. Thirteen (20%) individuals, 4 (22%) out of 18 FAP and 9 (18%) out of 47 AFAP patients, were identified either as biallelic (n=7) or monoallelic (n=6) *MYH* mutation carriers. If only individuals with a family history compatible with autosomal recessive inheritance were considered (n=33), 22% (2/9) of patients with classical polyposis and 21% (5/24) of AFAP patients harboured biallelic *MYH* germline mutations (Table 1).

Besides the homozygous mutations Y165C and G382D, each of which accounted for 29% of mutant alleles in the biallelic patients, a novel mutation R168H (Figure 1a) was present on both alleles in one AFAP patient. Additional novel mutations were detected in 2 AFAP patients compound heterozygote for Y165C/R171Q and Y165C/R231H (Figures 1b and 1c). In addition, one FAP patient was found to be a compound heterozygote with a 252delG/137insIW mutation previously reported by Sieber *et al*[8]. The healthy parents of this individual were available for investigation and were found to be heterozygous carriers of the 137insIW or the 252delG alteration, respectively. Although the pathogenicity of R168H, R171Q and R231H remains to be established by functional studies, such gene alterations were not observed in 200 chromosomes from healthy Swiss individuals. Furthermore, the 3 amino acid positions are known to be evolutionary highly conserved across species (*E.coli, S. pombe*, mouse, rat, and human).

Figure 1: Sequencing chromatograms displaying the three novel *MYH* germline variants: 1a) patient 2107 (T7 Forward Sequence), R168H (homozygous), 1b) patient 2073, (T7 Forward Sequence), R171Q (heterozygous) and 1c) patient 2185 (SP^ Reverse Sequence), R231H (heterozygous).

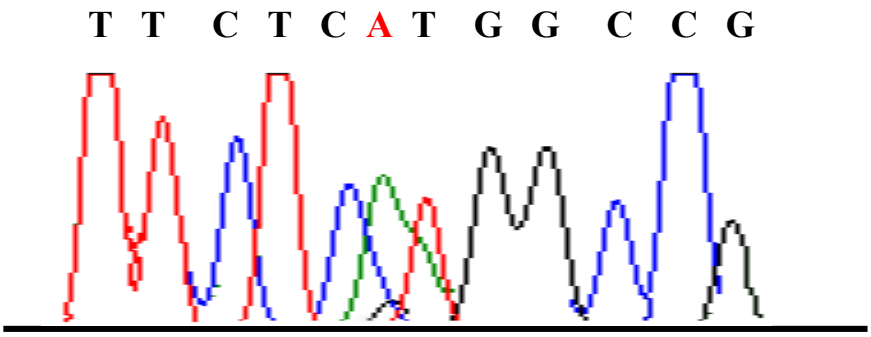


Figure 1a)

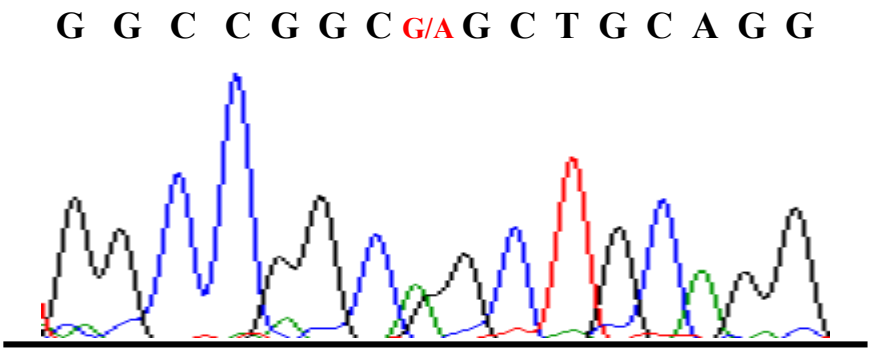


Figure 1b)

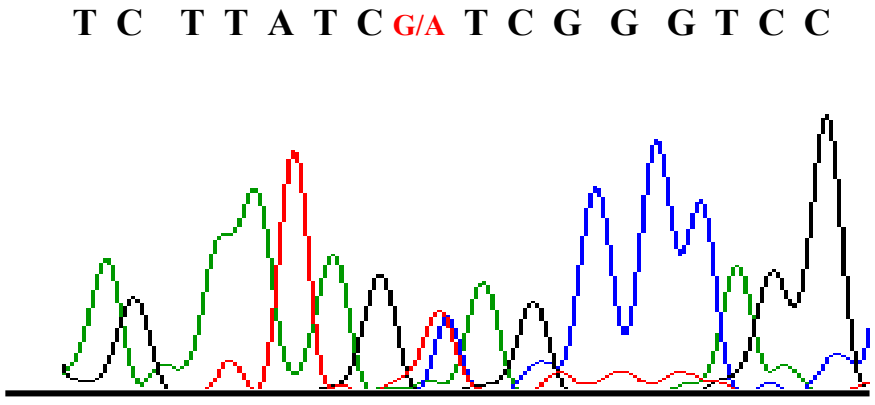


Figure 1c)

Table 1: Phenotypic features and germline mutations identified in *MYH* mutation carriers. CRC, colorectal cancer; FAP, familial adenomatous polyposis; AFAP, attenuated FAP.

Patient ID	Sex	Age	Polyp No.	Clinical classification	CRC	Extracolonic disease	MYH	
							1st Mutation	2nd Mutation
Biallelic *MYH* mutation carriers								
1775/01	M	38	>100	FAP	Yes	Yes	252del G	137ins IW
1828/01	F	42	<100	AFAP	Yes	No	Y165C	Y165C
2013/01	M	50	<100	AFAP	Yes	No	G382D	G382D
2073/01	F	60	>50	AFAP	No	No	Y165C	R171Q
2107/01	M	35	30	AFAP	Yes	No	R168H	R168H
2184/01	M	48	>100	FAP	No	No	G382D	G382D
2185/01	M	48	74	AFAP	Yes	No	Y165C	R231H
Monoallelic *MYH* mutation carriers								
1384/01	F	20	multiple	AFAP	Yes	No	G382D	wild-type
1665/01	F	54	>100	FAP	No	No	I209V	wild-type
DFAP 17	F	34	20	AFAP	No	Yes	G382D	wild-type
DFAP 82	M	58	100	FAP	No	No	G382D	wild-type
DFAP 99	F	63	43	AFAP	No	No	G382D	wild-type
SA 453	M	41	5	AFAP	No	No	G382D	wild-type

Six patients were identified as monoallelic *MYH* mutation carriers with the G382D mutation present in 5 (83%) of them (Table 1). In the remaining 52 (80%) patients, no pathogenic *MYH* mutations could be identified. The previously described polymorphisms in exon 2 (G64A; V22M) and exon 12 (G972C; Q324H) were detected with allele frequencies of 4% and 14%, respectively, similar to that of a Swiss control sample population (200 chromosomes) assessed in parallel (2% V22M and 12% Q324H).

Genotype-phenotype comparisons

The phenotypic features of the 7 biallelic *MYH* mutation carriers (5 males, 2 females) are depicted in Table 2, where two of them display classical FAP. In 5 (71%) patients colorectal carcinomas had been diagnosed at a median age of 38 years, with 3 of them located proximal to the splenic flexure. The family history in all biallelic mutation carriers corresponded to an autosomal recessive mode of inheritance. Remarkably, in 3 out of 11 siblings of patient 2073/01 (Y165C/R171Q) a CRC had been diagnosed at a median age of 51 years (range 49 to 54). Except for patient 1775, in whom duodenal adenomas had been detected, no apparent extracolonic disease manifestations were present in the other biallelic mutation carriers.

Among the 6 monoallelic *MYH* mutation carriers, AFAP patient 1384/01 had developed a CRC of the sigmoid colon at the age of 20 years (Tables 1 and 2). Three patients (no. 1384/01, DFAP17 and DFAP 82) had siblings with either CRC or polyps reported. A facial lipoma was present in patient DFAP 17.

Twenty (38%) out of 52 *MYH* mutation-negative patients had family histories on CRC and/or polyposis compatible with an autosomal recessive mode of inheritance and could therefore be included in the genotype-phenotype analysis (Table 2). Comparing the phenotypic properties of biallelic, monoallelic *MYH* mutation carriers and *APC/MYH* mutation-negative polyposis patients, colorectal

cancer was found to occur significantly more frequent in biallelic mutation carriers than in the other subgroups (71% vs 18% and 15%, respectively; Fisher's exact test $p<0.01$). No further statistically significant phenotypic differences with respect to polyp number, age at diagnosis or extracolonic disease were observed.

Table 2: Phenotypic characteristics of biallelic, monoallelic *MYH* mutation carriers and *APC*/*MYH* mutation-negative patients with a family history compatible with autosomal recessive inheritance.

	Biallelic *MYH* mutation carriers n=7	**Monoallelic *MYH* mutation carriers** n=6	***MYH* mutation-negative patients** n=20
Sex			
Male	5 (71%)	2 (33%)	12 (60%)
Female	2 (29%)	4 (67%)	8 (40%)
Clinical classification			
FAP (≥100 polyps)	2(29%)	2 (33%)	5 (25%)
AFAP (<100 polyps)	5 (71%)	4 (67%)	15 (75%)
Age at diagnosis (years)			
Median	48	47.5	46
IQR	10.5	24.0	16.0
Range	35-60	20-63	22-77
Colorectal cancer			
Present	5 (71%)	1 (17%)	3 (15%)
Absent	2 (29%)	5 (83%)	17 (85%)
Extracolonic disease			
Present	0	1 (17%)	2 (10%)
Absent	7 (100%)	5 (83%)	18 (90%)
***MYH* Polymorphisms:**			
Q324H	0	1 (17%)	5 (25%)
V22M	1 (14%)	0	2 (10%)

Molecular analysis of tumours from biallelic mutation carriers

We further investigated the presence of somatic mutations typical of *MYH* (base excision repair) deficiency *ie*. G to T transversions, in the mutation cluster region (MCR) of the *APC* gene, spanning codons 653-1513, a renown mutational hotspot. In total, 11 tumours, 9 colorectal adenomas and 2 adenocarcinomas, from 4 biallelic *MYH* mutation carriers (no. 1775, 1828, 2013 and 2073) could be investigated. No somatic *APC* mutations could be identified within the MCR region. Consistent LOH (at the MBC and the D5S346 loci) was only observed in colorectal adenocarcinomas of patients 1828 and 2013, respectively. Additionally, these cancers as well as a tubular adenoma of patient 2013 were found to harbour the *KRAS* target gene mutation 34G>T (G12C). All tumours investigated were microsatellite stable as judged by the BAT26 amplification profile.

Discussion

In this nation-wide survey on Swiss *APC* mutation-negative polyposis patients, about 20% were found to harbour either biallelic (n=7) or monoallelic (n=6) germline mutations in the base excision repair gene *MYH*. Considering only patients with a family history compatible with autosomal recessive inheritance, biallelic and monoallelic mutation carriers accounted for 22% of patients with classical as well as 21% of those with attenuated polyposis, respectively. No *MYH* alterations were identified in patients exhibiting a family history suggestive of an autosomal dominant inheritance pattern.

In addition to the most frequent pathogenic missense mutations, Y165C and G382D[6-8,13], three novel alterations in the *MYH* gene, R168H, R171Q and R231H, were detected. Two hundred control chromosomes, assessed in parallel, did not harbour these missense changes, which proved to be target amino acids highly conserved across 5 species. Furthermore, whilst R168 and R171 constitute part of a 6 helix barrel domain which contains the Helix-Hairpin-Helix motif, R231 lies within the alpha-8 helix making up the cluster domain[14]. Together

they form part of a DNA binding complex where 9 lysines and 5 arginines form an electrostatically positive DNA interaction surface. Functional studies are needed to ascertain the pathogenicity of these mutations. Moreover, since the parents of the individuals harbouring these gene alterations were not available for screening, we cannot exclude the possibility that the mutations in the compound heterozygotes may lie on the same allele.

In our study population, the overall allele frequency of the missense variants Y165C and G382D amounted to 0.03 (3 from 130) and 0.07 (9 from 130), respectively. In contrast, these alterations were not present in Swiss control samples (0/100), similar to reports on Finnish blood donors (0/424) and healthy British controls (2/100) [6,13]. This further substantiates the view that the frequency of the Y165C and G382D mutations in the general population is too low to justify large-scale mutation screening[19].

Biallelic and monoallelic mutation carriers were evenly classified by the classical FAP (11% and 11%, respectively) and the AFAP (11% and 8%, respectively) phenotypes. These values are comparable to previously reported data by Sieber et al.[8]. Five (71%) out of 7 biallelic *MYH* mutation carriers were found to have less than 100 polyps at the time of diagnosis, four of which also presented with colorectal cancer. Thus, in contrast to initial studies reporting classical disease (>100 adenomas) in all biallelic mutation carriers[7], the *MYH* associated-polyposis phenotype in our patients is predominantly an attenuated one, which is in accordance with recent investigations by Enholm et al. who investigated a population-based series of Finnish CRC patients[13].

Loss of heterozygosity (LOH) at the *APC* locus was only present in colorectal carcinomas, available from two biallelic mutation carriers. Mutation screening of the mutation cluster region (MCR) of the *APC* gene did not reveal any pathogenic somatic mutation, in particular G>T changes, in the colorectal tumor specimens. This could in part be due to technical problems and/or background contamination

with normal tissue. Alternatively, the somatic mutations may lie outside of the region screened. Similar to a recent report by Lipton et al.[11], who detected *KRAS* oncogene mutations in 60% of carcinomas and 30% of colorectal adenomas, we identified the *KRAS* hotspot mutation K12C in both adenocarcinomas as well as in 1 out of 9 colorectal adenomas. All tumours investigated were microsatellite stable confirming the negative association reported by Lipton et al.[11].

Based on clinicopathological features, it is virtually impossible to separate biallelic from monoallelic *MYH* mutation carriers and *MYH* mutation-negative polyposis patients who have a family history compatible with autosomal-recessive inheritance. In all groups, median age at diagnosis did not differ significantly and family history as well as occurrence of extracolonic disease were similar. Colorectal adenocarcinomas were significantly ($p<0.01$) more frequent among biallelic as compared to monallelic *MYH* mutation carriers and *MYH* mutation-negative polyposis patients, but due to the small number of CRC patients in the latter group (n=4) no meaningful statistical evaluations could be performed.

In conclusion, biallelic *MYH* germline alterations were identified in about 20% of Swiss *APC* mutation-negative patients with a family history compatible with autosomal recessive inheritance and they occurred at similar frequencies in those with a classical as well as those with an attenuated polyposis phenotype. Colorectal cancer was significantly more frequent in biallelic as compared to monoallelic mutation carriers or those without *MYH* alterations. Based on our experience and earlier reports, we suggest that *MYH* mutation screening should be offered to individuals who fulfill all of the following criteria: a) presence of classical or attenuated polyposis, b) absence of an *APC* germline mutation and c) pedigree compatible with autosomal recessive mode of inheritance. It remains to be determined within the framework of international collaborative studies if monoallelic *MYH* mutation carriers, compared to the general population, may actually be at an increased risk for developing colorectal cancer.

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Chapter I part ii

Methylation-induced G2/M arrest requires a full complement of the mismatch repair protein hMLH1

A collaboration with Petr Cejka, University of Zürich, already published in the EMBO Journal Vol 22. No. 9, p 2245, 2003

Introduction

In approximately 50% of all hereditary nonpolyposis colon cancers (HNPCC) the mismatch repair (MMR) gene *hMLH1* is mutated, whilst in approximately 25% of sporadic tumors of the right colon *hMLH1* is transcriptionally silenced. Through the study of HNPCC tumor cells it has been demonstrated that repeated sequence elements (microsatellites) in the genomic DNA are commonly mutated[1]. Microsatellite instability (MSI) is now a distinctive feature of defective mismatch repair, having been demonstrated in all organisms tested to date, and has been proven to be present in all tumor cell lines having lost both alleles of *hMSH2* and *hMLH1*[2,3]. It is hence assumed that for a MMR defect to be apparent, both wild type alleles of the respective MMR gene in cells of HNPCC tumors have been lost or inactivated by mutation.

The cells' tendency to acquire mutations increases, especially in genes with microsatellite repeats, once both MMR gene alleles have been inactivated[4]. In cells where the mutated genes are involved in the control of cell proliferation, for example in the colonic epithelium, then uncontrolled dividing of the cells would give rise to adenomatous polyp formation. The acquisition of additional mutations upon subsequent cell divisions within this benign growth would lead to the rapid transformation of the adenoma into a carcinoma[5].

However, the theory behind cellular transformation and tumour progression still has to address one question; does the transformation process commence only following the inactivation of both MMR gene alleles. or does it already begin when only one allele is affected or when the expression of the MMR gene is only reduced, such as in cells where the *hMLH1* promoter is partially methylated.

In order to be able to study the phenotypic consequences correlated to reduced levels of MMR proteins, the Zurich group developed a new cell line, epithelial in origin, in which the expression of *hMLH1* could be strictly regulated by doxycycline (Dox) in conjunction with the TetOff system. Cells grown in the presence of 0.1, 0.2, 0.4, 0.8 and 1.5 ng/ml Dox contained steadily decreasing amounts of *hMLH1* and *hPMS2*, as compared with cells grown in the absence of the drug. My part in this study was to assess the MSI status of the BAT26 chromosomal locus of the 284 293T Lα cells expressing varying amounts of *hMLH1*.

MSI analysis

293T Lα cells grown with 50, 0.2 and 0 ng/ml Dox were subcloned, and grown independently for 35 generations. The chromosomal DNA was extracted using the TRI Reagent (Molecular Research Center, Lucerne, Switzerland). MSI was assessed for 284 clones at the mononucleotide repeat locus BAT26. PCRs were carried out in a total volume of 25 ml containing ~100 ng of genomic DNA, as described by Loukola et al. (2001). The PCR products were diluted 1:4 and 0.5 ml was added to 10 ml deionized formamide (including 0.5 ml GS size standard 400 ROX), denatured at 95°C for 5 min, chilled on ice and loaded on a 96-capillary ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems). MSI was defined as the occurrence of novel alleles that differed by ±3 nucleotides from the control[3].

Results

For the analysis of MSI, the BAT26 microsatellite marker, which contains a repeat of 26 deoxyadenosines, and which is considered to be a reliable indicator of MSI, was employed. Since the 293T Lα cells are hypotriploid, and because the cell line was MMR deficient for many generations prior to this investigation, the BAT26 locus was found to be highly heterogeneous. The product of PCR amplification had on average 8 peaks (Tables 1-3) and hence the HNPCC criteria of MSI were applied[3] whereby only PCR products that differed by 3 or more peaks at this locus were considered to be a sign of MSI. Following these criteria, the BAT26 instability in the cells propagated for 35 generations in 0 or 0.2 ng/ml Dox was approximately 1%, whereas cells grown with 50 ng/ml Dox displayed MSI that was approximately 5 fold higher (Table 4). Closer inspection of the data however, revealed that cells propagated in 0 or 0.2 ng/ml Dox displayed no alleles (0/211) that differed by more than 4bp from the median. In contrast, 2 such alleles (2 out of 73: 2.7%) were found in the cells grown with 50 ng/ml Dox (Table 1, numbers in brackets). This suggests that MSI at the BAT26 locus in the 293T Lα cells is substantially greater than in cells expressing *hMLH1*, and thus that expression of even low amounts of hMutLα are sufficient to correct MMR defect in these cells.

Table 1: Instability of the BAT26 chromosomal locus in 293T Lα cells expressing varying amounts of *hMLH1*

Dox (ng/ml)	*MSI+ve / total*	*% MSI +ve*
0	2 (0) / 131	1.5
0.2	1 (0) / 80	1.3
50	4 (2) / 73	5.5 (2.7)

MSI+ve clones were defined as those displaying more than 3 extra peaks in the sequence of the PCR product. Numbers in brackets refer to clones with more than 4 extra peaks.

Discussion

It was observed in this study that *hMLH1* expression in 293T Lα cells corrected the MMR effect *in vitro* and *in vivo*. The 293T Lα$^+$ cells also proved to be >100 fold more sensitive to killing by MNNG than the isogenic cells lacking *hMLH1*. MMR proficient cells treated with MNNG were arrested in the G_2/M phase of the cell cycle which was a reaction found to be solely and entirely dependent on the function of *hMLH1*.

It was also apparent that the consistency of hMLH1/hPMS2 heterodimer levels, essential for MMR proficiency and DNA damage repair, were significantly different[6]. Previously proposed was the theory that the constant loading of hMutSα sliding clamps at ^{Me}G/T mispairs was responsible for the transmission of the DNA damage signal to the checkpoint machinery[7], and that this process becomes less efficient in cells expressing only low amounts of the mismatch binding factor hMutSα. However, in this study, the levels of hMutSα in 293T Lα$^+$ and 293T Lα$^-$ cells were equal, and in similar quantities to those found in MMR proficient cells. Therefore, the results cleaved here, add to the above hypothesis and suggest that the signal transduction process also needs the hMLH1/hPMS2 heterodimer, which is involved in downstream damage recognition. Interestingly, MMR proficiency was restored even at low hMLH1 concentrations, while checkpoint activation required a full complement of hMLH1.

Furthermore, this investigation illustrated that cells with lower than wild-type amounts of MMR proteins are not phenotypically normal, despite being MMR proficient. The observed abnormal DNA damage signalling may affect cellular transformation and tumour progression, especially in epithelial cells that are proliferating quickly and that may be exposed to stress or carcinogens. Upon epithelial cell damage, the cell should undergo apoptosis and prevent the production of mutant progeny. However, cells with defective DNA damage signalling, such as those with suboptimal levels of MMR proteins, fail to activate cell cycle checkpoints and apoptosis and hence cells acquire mutations that

enable them to uncontrollably proliferate and progress eventually into an adenoma. In the MMR proficient cells, activation of the MNNG-induced G_2/M checkpoint was accompanied by phosphorylation of p53, but the cell death pathway was p53 independent, as the latter polpeptide is functionally inactivated in these cells by SV40 large T antigen.

This study has shown that the activation of exogenous *hMLH1* transcription reverses the MMR defect carried by the 293T cells and reactivates their responsiveness to methylating agents, only when MMR protein levels are sufficiently high enough to initiate the DNA damage induced checkpoint. The fully isogenic system used in this study should be employed further for the research into other DNA metabolism pathways that involve MMR mechanisms. Furthermore, 293T Lα cells would be valuable for the screening of substances that preferentially kill MMR deficient cells, hence proving crucial in the treatment of tumours displaying aberrant MMR.

Table 1: Samples 1-136, 0-DOX. MSI present in %

Sample No.	Peak size 1	Peak size 2	Total allele no.	MSI (≥ 11 alleles)
1	112.38	115.35	8	stable
2	112.38	115.4	8	stable
3	112.4		7	stable
4	112.43	115.45	8	stable
5	112.44	116.4	9	stable
6	112.36	116.33	9	stable
7	112.27	116.35	9	stable
8	112.37	115.36	8	stable
9	LOW			
10	111.33	115.43	10	stable
11	112.4	115.3	7	stable
12	111.27	115.37	8	stable
13	113.43		6	stable
14	112.37	115.41	9	stable
15	112.45	116.4	9	stable
16	112.39	115.33	9	stable
17	111.41	115.38	10	stable
18	112.29	115.27	10	stable
19	112.37	115.31	9	stable
20	114.32		8	stable
21	112.43		8	stable
22	112.41	115.5	8	stable
23	112.37	116.33	9	stable
24	112.38	116.37	9	stable
25	112.42		8	stable
26	112.46		7	stable
27	112.3	116.42	9	stable
28	112.43		8	stable
29	112.38	115.33	8	stable
30	112.36	115.38	9	stable
31	111.4	114.32	9	stable
32	112.35	115.27	8	stable
33	112.29	116.29	9	stable
34	112.3	115.24	10	stable
35	113.37	116.36	8	stable
36	111.4	115.37	9	stable
37	112.42		8	stable
38	112.4		7	stable
39	112.46		8	stable
40	112.41	116.41	9	stable
41	112.44	115.37	8	stable
42	112.35	116.29	9	stable
43	112.33	116.34	9	stable
44	LOW			
45	112.3	116.27	9	stable
46	112.32	116.31	9	stable
47	112.52	115.32	9	stable
48	112.41	115.4	**11**	unstable
49	112.44	116.43	9	stable
50	111.45	115.4	8	stable
51	112.41	115.41	8	stable
52	112.37	115.34	7	stable
53	112.39	115.33	7	stable
54	112.37	116.3	9	stable
55	112.41	114.37	8	stable
56	111.34	115.3	9	stable
57	112.35	116.26	9	stable
58	112.34	116.32	9	stable
59	112.5		8	stable
60	111.43	115.39	9	stable
61	112.29	115.4	8	stable
62	112.38	115.41	8	stable
63	112.41	115.45	8	stable
64	112.41	115.45	9	stable
65	112.32	116.37	9	stable
66	112.36	116.3	9	stable
67	112.37	115.39	10	stable
68	112.39	116.35	9	stable

69	112.55		9	stable
70	112.36	116.33	8	stable
71	112.48	115.34	8	stable
72	111.51	114.35	9	stable
73	112.31		8	stable
74	112.42	115.4	9	stable
75	112.37	115.35	8	stable
76	112.39	116.34	9	stable
77	112.38	116.34	9	stable
78	112.32		9	stable
79	112.31	115.35	9	stable
80	112.39	116.28	9	stable
81	112.37	115.31	8	stable
82	112.31		9	stable
83	112.41		9	stable
84	112.26	115.31	8	stable
85	112.28		9	stable
86	111.49	115.38	9	stable
87	111.42		9	stable
88	112.41	116.41	9	stable
89	112.35	116.36	9	stable
90	112.35	116.35	9	stable
91	111.39	115.4	9	stable
92	112.35	116.38	9	stable
93	112.35		8	stable
94	112.32	115.28	9	stable
95	112.36	115.34	9	stable
96	112.39		8	stable
97	111.33	115.36	9	stable
98	112.35	115.35	8	stable
99	112.33	116.31	10	stable
100	112.38	115.41	8	stable
101	112.39	116.34	9	stable
102	112.43	115.36	8	stable
103	112.47		8	stable
104	110.45	115.36	**11**	unstable
105	112.37		8	stable
106	110.39	112.35	9	stable
107	112.35	116.29	10	stable
108	112.35	115.28	8	stable
109	112.44		8	stable
110	112.48		7	stable
111	112.39	115.37	9	stable
112	111.42	115.38	9	stable
113	112.44	116.36	9	stable
114	112.42	115.32	8	stable
115	112.35	115.33	8	stable
116	112.36	115.38	8	stable
117	LOW			
118	112.33	116.34	9	stable
119	112.32	115.25	8	stable
120	112.35	115.33	8	stable
121	112.43	115.38	8	stable
122	LOW			
123	112.27	115.36	9	stable
124	112.51	114.44	8	stable
125	112.37	115.35	9	stable
126	112.43	115.38	9	stable
127	112.43	115.38	9	stable
128	112.32	115.32	9	stable
129	112.48	115.39	9	stable
KO				
130	112.33		8	stable
131	112.38		8	stable
132	111.36		7	stable
133	112.41		8	stable
134	112.41	116.35	9	stable
135	LOW			
136	112.35	115.44	8	stable

Table 2: Samples 1-84, 50-DOX. MSI present in %

Sample No.	Peak size 1	Peak size 2	Total allele no.	MSI (≥ 11 alleles)
1	111.43		8	stable
2	112.4		8	stable
3	112.43	114.34	8	stable
4	112.33		9	stable
5	LOW			
6	LOW			
7	112.43	115.42	8	stable
8	112.39	115.32	9	stable
9	111.55		8	stable
10	112.45		8	stable
11	112.41	115.4	9	stable
12				
13	112.44	115.45	9	stable
14	112.49	115.43	9	stable
15	112.3		8	stable
16	110.48	115.38	**11**	unstable
17	112.47	115.48	9	stable
18	112.45	115.42	9	stable
19	112.41	115.46	9	stable
20	111.47	115.5	8	stable
21	115.41		8	stable
22	112.4		9	stable
23	112.35	115.4	9	stable
24	112.42		8	stable
25	111.42	115.38	10	stable
26				
27	112.44		5	stable
28	112.41	115.41	9	stable
29	111.39	115.39	9	stable
30	112.44	115.44	9	stable
31	112.42	114.41	8	stable
32	112.4		9	stable
33	111.45		9	stable
34	112.45		8	stable
35	112.44		9	stable
36	112.37		8	stable
37	112.45		8	stable
38	112.41		9	stable
39	112.41	115.41	8	stable
40	111.39	115.4	8	stable
41	112.39	115.38	9	stable
42	112.37	115.31	9	stable
43	111.38	115.39	9	stable
44	112.38	115.36	9	stable
45	109.47	115.4	13	unstable
46	111.34	113.32	8	stable
47	112.42	115.39	9	stable
48	112.37		9	stable
49	112.38		9	stable
50	112.31	116.3	9	stable
51	112.35	115.33	10	stable
52	112.36	115.34	10	stable
53	112.37	114.35	9	stable
54	LOW			
55	112.28		9	stable
56	112.34	115.36	9	stable
57	112.45		8	stable
58				
59	112.31	115.35	9	stable
60	111.38	115.38	10	stable
61	112.35		9	stable
62	112.52	115.5	9	stable
63	112.39		8	stable
64	112.32	115.32	9	stable
65	LOW			
66	112.31	115.29	9	stable
67	112.42	115.42	9	stable
68	LOW			

69	LOW			
70	111.37	115.4	8	stable
71	112.33	115.32	9	stable
72	LOW			
73	112.34	115.27	9	stable
74	112.29	115.28	10	stable
75	112.29	115.28	10	stable
76	112.36	115.31	10	stable
77	LOW			
78	112.31	115.32	8	stable
79	112.36		8	stable
80	110.35	114.29	**11**	unstable
81	112.34		8	stable
82	112.37	115.37	9	stable
83	112.33		6	stable
84	112.32		**13**	unstable

Table 3: Samples 1-86, 0.2-DOX. MSI present in %

Sample No.	**Peak size 1**	**Peak size 2**	**Total allele no.**	**MSI (≥ 11 alleles)**
1	112.54	115.37	9	stable
2	112.29	115.27	8	stable
3	112.4	115.4	8	stable
4	112.42	115.34	7	stable
5	112.55	115.38	9	stable
6	112.38	116.37	9	stable
7	112.46	115.46	7	stable
8	112.36	115.4	9	stable
9	112.4	115.38	9	stable
10	112.42	116.29	9	stable
11	112.41		8	stable
12	LOW			
13	112.36	116.36	9	stable
14	112.35	116.32	9	stable
15	112.4	115.37	8	stable
16	112.43	115.45	9	stable
17	112.39	115.33	9	stable
18	112.38	116.4	9	stable
19	112.32	115.17	8	stable
20	112.47	115.4	9	stable
21	112.42	115.4	9	stable
22	112.47	115.47	8	stable
23	112.37	116.36	9	stable
24	112.35	116.35	9	stable
25	112.37	116.33	9	stable
26	LOW			
27	110.43	114.34	9	stable
28	111.45	115.37	10	stable
29	112.33	116.31	9	stable
30	112.37	116.33	9	stable

31	112.4	115.4	8	stable
32	LOW			
33	112.4	116.34	10	stable
34	112.41	115.34	9	stable
35	112.36	115.35	8	stable
36	112.43		7	stable
37	112.35	116.35	9	stable
38	112.34	116.32	9	stable
39	112.34		6	stable
40	112.39	115.29	9	stable
41	112.39	115.34	8	stable
42	112.32	116.37	9	stable
43	112.38	115.27	8	stable
44	112.36	115.34	8	stable
45	111.31	115.28	9	stable
46	111.38	114.35	9	stable
47	112.38	115.41	8	stable
48	112.32	115.29	9	stable
49	111.27	115.27	9	stable
50	112.33	115.26	9	stable
51	112.38		9	stable
52	112.33	116.28	10	stable
53	112.45	115.41	9	stable
54	112.33	116.34	9	stable
55	112.29	115.27	9	stable
56	112.31	115.29	9	stable
57	112.34	116.35	9	stable
58	LOW			
59	112.32		9	stable
60	112.32	115.33	9	stable
61	112.34	116.35	9	stable
62	112.3	116.33	10	stable
63	112.33	115.26	10	stable
64	112.29	116.29	10	stable
65	112.32	115.25	9	stable
66	112.27	115.26	10	stable
67	112.35	116.32	10	stable
68	112.34	115.27	9	stable
69	LOW			
70	112.36	115.34	9	stable
71	112.28	114.32	8	stable
72	112.34	115.29	9	stable
73	112.32	115.32	8	stable
74	LOW			
75	112.37	115.37	8	stable
76	112.29	115.3	9	stable
77	110.34	112.28	**11**	unstable
78	111.4	114.34	9	stable
79	112.3	116.27	10	stable
80	112.29	116.26	10	stable
81	112.28	115.3	9	stable
82	112.31		7	stable
83	112.29	115.3	10	stable
84	112.33	116.44	9	stable
85	112.32	116.34	10	stable
86	111.39	115.33	10	stable

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Chapter I part iii

Mismatch repair haploinsufficiency and accumulation of target gene mutations in colorectal cancer with microsatellite instability

A collaboration with Luigi Lhagi, Istituto Clinico Humanitas, Rozzano, that has been prepared as a scientific paper for publication

Introduction

It is now well known that cancers with defects in the mismatch repair system display characteristic changes at repetitive DNA sequences[1], termed as microsatellite instability[2]. MSI has been observed in both hereditary cancer syndromes (Hereditary Non Polyposis Colorectal Cancer, HNPCC) and those arising sporadically[3]. On acquiring a germline mutation in one of the mismatch repair genes *hMLH1, hMSH2* or *hMSH6*, the gene becomes predisposed to inactivation[4]. In sporadic MSI cancers, the inactivation of hMLH1 is often a result of epigenetic silencing through promoter hypermethylation[5].

MMR proficiency is reliant on multiple protein interactions which form functional DNA repair complexes. Hence, the inactivation of one gene causes unbalanced protein equilibrium and results in deficient repair complexes. In a fully functional MMR system, the hMSH2-hMSH6 (MutS-α) preferentially recognises mispaired bases and single repeat frameshifts due to insertion/deletion loops, whilst the larger loops are delt with by the hMSH2-hMSH3 heterodimers (MutS-β). The hMLH1-hPMS2 (MutL) complex functions to excise replication errors[6]. Therefore, despite sharing the common characteristic of MSI, carcinomas harbouring a MMR defect differ with regards to the specific mutation type[7,8] depending on the

MutS-α/β or MutL activity and also in relation to the presence of hMSH3 and hMSH6[9]. It has further been shown that MSI cancers accumulate inactivating frameshift mutations in secondary mutators as well as in other cancer related genes eg. target genes TGFβRII, BAX, CASP-5, TCF4 and MBD4. The joint existence of primary and secondary mutations can reduce further the efficiency of repair at mutated sequences, and hence increase the risk of accumulating replication errors[10]. It therefore follows that the accumulation of several MMR gene mutations may result in haploinsufficiency of the MMR, which would subsequently be reflected in the degree of the mutator phenotype and hence, if the mutational spectrum of MSI cancer cells reflect the type and the extent of MMR deficiency, the prevalence of frameshift mutations in target genes may be related to the deficiency of the primary mutator as well as to the presence of genetic alterations in secondary mutators. Although research with cell lines support this hypothesis, additional data from MSI tumour series are required to define this theory further.

Mutations in the *hMSH3* and *hMSH6* genes may affect the haplotype of the repair complexes MutSα and MutSβ, respectively, and hence the variability of frameshift mutations, and the degree to which secondary mutator frameshift mutations affect these, aswell as the effect on the pathological invasive behaviour of the MSI CRC caused by such genetic damage, may differ between MSI CRCs. It was therefore the aim of this study to investigate the prevalence of frameshift mutations in secondary mutator genes and in other target genes in a series of MSI-high CRCs with *hMLH1* and *hMSH2* deficiency, from both hereditary and sporadic cases in different pathological stages.

We established 18 CRCs to be microsatellite unstable, through BAT26 analysis, and arranged to have them included in the Milan mismatch repair haploinsufficiency research project. My part in this study was to confirm MSI status and extract DNA from the 18 CRC samples proving to be unstable. I also

conducted the DNA sequence analysis for 2 individuals suspected of harbouring an *hMSH2* gene mutation, as determined through immunohistochemistry testing.

Methods

DNA Extraction

Tumor DNA was isolated from formalin fixed, paraffin embedded tissue using the QIAMP DNeasy Tissue kit and according to the suggested protocol of the manufacturer (Qiagen, Switzerland). After verification of the tumor cell content (>70%) of HE stained tumor specimen, 10x 5-8μm thick tumor sections were cut from each paraffin block. Lysis of the tissue was completed overnight with Qiagen buffer, Proteinase K and an incubation temperature of 55°C. The samples were then washed twice with Qiagen wash buffer and the DNA finally eluted in 100 μl elution buffer provided.

Microsatellite Instability Analysis

Microsatellite instability was confirmed for a total of 18 tumors at the mononucleotide repeat locus BAT26. PCRs were carried out in a total volume of 25 ml containing ~50 ng of tumor DNA. The PCR products were diluted 1:4 and 0.5 ml was added to 10 ml deionized formamide (including 0.5 ml GS size standard 400 ROX), denatured at 95°C for 5 min, chilled on ice and loaded on a 96-capillary ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems). MSI was defined as the occurrence of novel alleles that differed by ±3 nucleotides from the median, 8 peaks (Loukola et al., 2001).

Direct DNA Sequencing of *hMSH2*

Exon specific primer pairs (sequences as reported by Kolodner) were used to amplify the 16 exons of *hMSH2*, including the respective exon-intron boundaries,

from genomic DNA. Fifty microlitres of PCR reaction mixture contained 100ng of genomic DNA, 0.5μM each primer, 2.5μM each dNTP, 5mM $MgCl_2$, 10x reaction buffer, and 0.2U Taq polymerase (Qiagen, Switzerland). The reaction parameters were; 94°C-3 mins for 1 cycle, 94°C-30 secs, 53°C-30 secs and 72°C-45 secs for 35 cycles, and 72°C-3 mins for 1 cycle, for a Hybaid OmnE Thermocycler (Catalys AG, Wallisellen, CH). The sequencing reaction was completed using the Thermosequenase Sequencing Kit (Amersham Pharmacia, Switzerland). PCR amplicons were diluted 1:3 and enzymatically purified with shrimp alkaline phosphatase and exonuclease I (Amersham Pharmacia, Switzerland). The purified PCR products were run through a cycle sequencing reaction with primers labeled with an infrared dye; T7-IRD800 and SP6-IRD-800 for forward and reverse sequencing, respectively. Cycle sequencing parameters were 95°C-3min. for 1 cycle, 95°C-30 sec. 55°C-30 sec. and 72°C-1min. for 30 cycles. The resulting products were loaded onto a 6% denaturing polyacrylamide gel and analysed on a LiCor 4000L automated DNA Sequencer (LiCor, Lincoln, NE).

Results

We involved 18 MSI CRC samples from the cohort of patients enrolled in the HNPCC screening program, of the Human Genetics Department, Basel. These together with 43 identified from the patients undergoing surgery for colorectal cancer at the Istituto Clinico Humanitas from 1997 to 2002, comprised a total of 61 MSI CRC specimens. All cancers shared *BAT26* instability; hMLH1 and hMSH2 protein loss (either by *in vitro* test or by immunoistochemistry) was determined in every instance, and mutational status was assessed in 25 cases. Seven *hMLH1* and 6 *hMSH2* germline mutations were detectable (Table 1 for my contribution). Amsterdam criteria for HNPCC were fulfilled in 29 cases. Among the investigated cancers, 38 were of A or B Dukes' stage, and 19 were of C or D stages, and in 4 cases it was not possible to properly ascertain the pathological stage.

Considering the 7 studied targets, the medians of frameshift mutations were similar in *hMLH1* and in *hMSH2* deficient cancers (4 and 5, respectively), and inheritance did not correlate with the severity of the mutator phenotype. However, the *hMSH2* deficient and the *hMLH1* deficient cancers with frameshifted *hMSH3* and/or *hMSH6* harboured a median of 4 frameshifts in the other investigated genes, while the hMLH1 deficient cancers with wild-type secondary mutators had a median of only 2 frameshifts ($p<0.0023$). Non-metastic and metastatic MSI CRC also showed similar frameshift medians (5 and 4, respectively), but the prevalence of cancers with alterations of both MutSα and MutSβ haplotypes was higher in non-metastatic (23 out of 29, 79%) than in metastatic (15 out of 28, 53%) cases ($p=0.04$).

hMSH2 Gene Sequence Analysis Results

Family ID	Gene	Exon	Codon	DNA Change	Mutation Consequence	MSI/ RER	Sex	Age at Diagnosis	Primary Cancer
BItalia 1	MSH2	10	526	1576del A	Frameshift	MSI-High	F	36	Rectum
BItalia 9	MSH2	16	882	2646del A	Frameshift	MSI-High	M	76	Sigmoid

Table 1: The 2 Basel samples I sequenced, for the specific identification of *hMSH2* mutations

Discussion

In microsatellite unstable colorectal tumours (MSI CRCs), instability at target genes varies. The individual mutational frequency and also the distribution of frameshift mutations differs among colorectal cancers with a mismatch repair deficiency[11,12]. Laghi *et al.* discovered that in the tumours they investigated in this study, carcinomas with MutS haplotype deficiencies displayed a higher number of frameshift mutations than the MutL deficient cancers. Such a finding fits the

progressive model of mutator mutations initially suggested by Malkhosyan et al[13], which notes the importance of secondary monoallelic mutator mutations in the context of a cumulative haploinsufficiency model[14]. Laghi also observed that a higher number of frameshifts occurred in those tumours with MutS haplotype deficiencies as compared to those with MutL deficiency only, and in addition, any MutS haplotype deficiency can lead to an increased number of frameshifts of the investigated targets in MutL deficient tumours. Previous studies report that an inherited modality of MMR defects can affect the extent of the microsatellite mutator phenotype[2]. However, Laghi observed in this study that an increased number of frameshifts in tumours with an *hMLH1* loss correlates with MutS deficiencies, as opposed to correlating with the inheritance of a CRC predisposition[15,16].

In this study, the *hMSH2* deficient tumours, with and without secondary mutator alterations, had a median of four target gene frameshifts at the investigated targets. This suggests that the MutS deficient tumours share a similar unrepaired instability of short repeats once their mutator phenotype is established. They suggest further investigation into the role of *hMSH3* and *hMSH6* mutations in *hMSH2* deficient cancers in order to assess the relevance of secondary mutator mutations in *hMLH1* deficient cell lines[11,12].

With the exception of the early *TGFβRII* mutations[17,18] the genotype-phenotype correlations of CRCs during carcinogenesis of the microsatellite pathway are not well documented. In addition, it is still a question whether some target gene mutations (eg *TGFβRII, BAX*) can influence MSI tumour progression[19,20,21]. Recent reports suggest that the frequency of somatic mutations of *TGFβRII, BAX, hMSH3, hMSH6, TCF4* and *IGFRII* is not different in the metastatic tumours as compared to the primary ones[22]. In the tumours Laghi investigated, target gene frameshifts were as equally common in non metastatic MSI CRCs as they were in metastatic ones. Hence, the accumulation of frameshifts at the

targets observed does not appear to be related to the stage of tumour progression. Laghi suggests that it is rather a multistep mutator damage pathway that affects MMR proficiency and hence unrepaired target gene mutations accumulate during MSI carcinogenesis. In addition, since defects in both MutS haplotypes was observed to be significantly associated with non metastatic disease in Laghi's study, importantly, a high degree of instability can potentially be a positive prognostic factor.

In conclusion, this study has been able to expand the notion that MMR haploinsufficiency in CRCs of the microsatellite mutator phenotype vary to an extent, by illustrating that frameshift mutations inactivating the alleles of target genes are more prevalent in tumours with MutS deficiencies than in tumours with MutL deficiencies only.

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Chapter I part iv

The investigation into the loss of MMR genes in a consecutive series of 1048 colorectal tumors from patients with familial colorectal carcinomas

A collaboration with Giancarlo Marra, University of Zürich, which will lead to the eventual publication of a scientific paper.

Introduction

In 60-70% of HNPCC kindreds the disease is caused by germline mutations in one of the DNA mismatch repair (MMR) genes, *hMSH2, hMLH1,hMSH6, hPMS1* or *hPMS2*[1]. Two of these genes, *hMLH1* and *hMSH2*,account for almost 90 percent of all identified mutations known to date. *hMSH6* accounts for almost 10 percent, but its role in the typical as opposed atypical hereditary nonpolyposis colorectal cancers remains to be fully established[2,3]. Additionally, rarer germline mutations have been reported in *hPMS2*[4,5].

Gene	*No. of mutations identified to date*	No. of missense mutations *(% of total)*	*No. of polymorphisms*
hMLH1	164	47 (29)	20
hMSH2	121	19 (16)	24
hMSH6	31	12 (39)	43
hPMS2	1	0	0
hPMS1	5	1 (20)	5

Table 1: Total of mutations and polymorphisms established to date in patients suspected of harboring HNPCC. Data source: the International Collaborative Group on HNPCC, http://www.nfdht.nl

When inactivated they lead to genetic instability and thus, by increasing the genome-wide mutation rate, indirectly promote tumour growth[6]. The evolutionarily highly conserved MMR genes function as "guardians of the genome." They detect and initiate the repair of both base:base mispairs and insertion/deletion mispairs which occur during replication and in addition prevent the recombination of divergent sequences (for review see Jirincy, 1999)[7]. Inactivation of the MMR system through mutation of one of its components consequently leads to genomic instability, as illustrated by microsatellite instability (MSI). MSI can be observed in 75 to almost 100% of tumours stemming from HNPCC patients[8,9]. Approximately 11-38% of sporadic CRCs also display genomic instability in conjunction with a somatic mismatch repair defect, mainly due to promoter hypermethylation of *hMLH1*[10].

The immunohistochemical technique has been proven to be the most sensitive and specific method for the identification of *hMLH1* and *hMSH2* gene alterations in our studies (data not published 2003) and hence, along with the conclusions of previous reports[11,12] this method can be said to be a rapid and efficient means of detecting colorectal carcinomas associated with the HNPCC syndrome.

The Zurich group investigated, via IHC screening, 1048 consecutive colorectal cancers in a prospective study of patients suspected of having an HNPCC syndrome. They found that 13.2% of these individuals lacked the expression of one of the mismatch repair proteins. They felt it necessary to confirm the IHC data with MSI analysis. They sent the tumour DNA from 187 patients to our department for me to conduct BAT26 MSI testing.

In addition, the IHC screening identified 15 patients with tumours depicting a loss of the *hPMS2* protein in the presence of *hMLH1*. The hPMS2 gene encodes a homolg of the bacterial MutL and the yeast PMS1 MMR proteins. It functions in a heterodimer with MLH1, which may bind to a complex of hMSH2 and

mismatched DNA[12]. Subsequently, the mismatched bases are excised and replaced with the appropriate nucleotides[12]. The identification of germline mutations in hPMS2 in patients affected with HNPCC has highlighted its role in cancer predisposition[4,5,13]. To assess the true nature of this *hPMS2* IHC negative staining, three routes of investigation were proposed i) *hPMS2* LOH analysis, ii) *hPMS2* methlation status analysis and iii) *hPMS2* direct DNA sequencing. I conducted the *hPMS2* LOH analysis.

Methods

MSI analysis

MSI was assessed for a total of 187 tumors from patients with suspected HNPCC, at the mononucleotide repeat locus BAT26. DNA was extracted by the Zürich research group and sent to Basel. DNA concentrations varied from 12 ng/μl (microdissected tumor) to 99 ng/μl. PCRs were carried out in a total volume of 25 ml containing ~100 ng of tumour DNA, 50ng of DNA for the microdissected sample. The PCR products were diluted 1:4 and 0.5 ml was added to 10 ml deionized formamide (including 0.5 ml GS size standard 400 ROX), denatured at 95°C for 5 min, chilled on ice and loaded on a 96-capillary ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems). MSI was defined as the occurrence of novel alleles that differed by ±3 nucleotides from the median, 8 peaks[14].

PMS2 LOH Analysis

Loss of heterozygosity (allelic loss) analysis at the microsatellite loci D7S517, D7S518 and D7S666 (for *hPMS2*) was performed according to the standard protocol with the use of 6-FAM, HEX and TET-labeled oligonucleotides. Twenty-five microlitres of PCR reaction mixture contained approximately 50ng of genomic DNA, 0.5μM each primer, 2.5μM each dNTP, 5mM $MgCl_2$, 10x reaction buffer, and 0.2U Taq polymerase (Invitrogen, Switzerland). The reaction parameters were; 94°C-2 mins for 1 cycle, 94°C-1 min, 55°C-1 min and 72°C-1

min for 30 cycles, and 72°C-6 mins for 1 cycle, for a Hybaid OmnE Thermocycler (Catalys AG, Wallisellen, CH). Samples were analysed on an ABI Prism 310 Genetic Analyser (Applied Biosystems) and classified as having allelic loss if the dose of one allele in the tumor was at least 50% lower than that of the other allele.

Results

For the analysis of MSI, the BAT26 microsatellite marker, which contains a repeat of 26 deoxyadenosines, and which is considered to be a reliable indicator of MSI, was employed. The product of PCR amplification had on average 8 peaks and hence the HNPCC criteria of MSI were applied[14] whereby only PCR products that differed by 3 or more peaks at this locus were considered to be a sign of MSI. Following these criteria, the BAT26 instability in the tumors samples investigated was equal to 75% (141 from 187 unstable, Table 2). These unstable tumors proved to be 100% consistent with those lacking the expression of either *hMLH1* or *hMSH2 (data not shown)*.

Table 2: The MSI Status of the 187 tumors investigated as determined by microsatellite analysis with BAT26.

Sample No.	Hospital	Tumour No.	Year	DNA Conc. ng/ul	BAT26 MSI Status
1	Aarau	22529	2000	100	unstable
2	Aarau	23416	2000	60.5	stable
3	Aarau	22104	2000	57	stable
4	Aarau	20758	2000	91.5	unstable
5	Aarau	6579 J	2000	100	unstable
6	Aarau	6579 K1	2000	61	unstable
7	Aarau	9465	2000	86	stable
8	Aarau	10570	2000	88	unstable
9	Aarau	15659	2000	48	unstable
10	Aarau	25013	2000	100	unstable
11	Aarau	6110	2001	42	unstable
12	Aarau	7869	2001	100	stable
13	Aarau	15581	2001	63	unstable
14	Aarau	1398	2004	100	unstable
15	Aarau	B00.16655	2000	100	unstable
16	Aarau	B01.5194	2001	100	unstable
17	Aarau	B01.6831	2001	100	unstable
18	Aarau	B01.7467	2001	100	unstable
19	Aarau	B01.14685	2001	100	unstable
20	Aarau	5160	2002	95	unstable
21	Aarau	10335	2001	100	unstable
22	Aarau	B01.7467	2001	100	unstable
23	Luzern	B 1081	2000	100	unstable
24	Luzern	B 55121	2000	35	unstable
25	Luzern	B 53430	2000	100	unstable

26	Luzern	B65453	2000	93	unstable
27	Luzern	B63887	2000	100	unstable
28	Luzern	B58560	2000	38	unstable
29	Luzern	B56727	2000	96.5	unstable
30	Luzern	B56601	2000	100	stable
31	Luzern	B63850	2000	100	unstable
32	Luzern	B69999	2000	100	unstable
33	Luzern	B67543	2000	46	unstable
34	Luzern	B50005	2001	93.5	unstable
35	Luzern	B53460	2001	100	unstable
36	Luzern	B9549	2001	40.5	unstable
37	Luzern	B63.585	2001	100	unstable
38	Luzern	B60661	2001	60	unstable
39	Luzern	B67255	2001	46	unstable
40	Luzern	B70511	2001	100	unstable
41	Luzern	B20244	2001	71.5	stable
42	Luzern	B52458	2002	100	unstable
43	Luzern	B50263	2002	64.5	unstable
44	Luzern	B52201	2002	100	unstable
45	Luzern	50716	2002	100	stable
46	Luzern	55998	2000	100	unstable
47	Luzern	307	2001	100	unstable
48	Luzern	23.348	2001	100	unstable
49	Luzern	55843	2002	100	unstable
50	Luzern	54631	2002	96	unstable
51	Luzern	B6852	2001	46	stable
52	Luzern	B60404	2002	100	unstable
53	Luzern	B61104	2002	100	unstable
54	Luzern	B64041	2002	100	unstable
55	Luzern	B65509	2002	100	unstable
56	Luzern	B66221 14X	2002	100	unstable
57	Luzern	B66221 5X	2002	100	unstable
58	Luzern	B66221 6X	2002	100	unstable
59	Luzern	B66062	2002	100	stable
60	Luzern	B64501	2000	63	unstable
61	Luzern	B69770	2000	100	unstable
62	Luzern	B53072	2001	100	unstable
63	Luzern	B61263	2001	83	unstable
64	Luzern	B59255	2001	100	unstable
65	Luzern	B66543	2001	100	unstable
66	Luzern	B66686	2001	100	stable
67	Luzern	B66732	2001	100	unstable
68	Luzern	B69101	2001	100	unstable
69	Luzern	B52557	2002	51	stable
70	Luzern	B54832	2002	100	unstable

71	Luzern	B53989	100	unstable
72	Luzern	B60244	100	stable
73	Luzern	B61162	73	unstable
74	Luzern	B65950	100	unstable
75	Luzern	B17676	100	stable
76	Luzern	B50612	79	stable
77	Luzern	B51692	100	stable
78	Luzern	B52086	64	stable
79	Luzern	B50.316	15	stable
80	Luzern	B61282	100	unstable
81	Luzern	B51385	100	unstable
82	Luzern	B2248	100	stable
83	Luzern	B51527	100	stable
84	Luzern	B52187	100	unstable
85	Luzern	B50.316 BIS	100	stable
86	Luzern	B52557 BIS	92	stable
87	Luzern	B52013	100	unstable
88	Luzern	B51.470	100	unstable
89	Luzern	B52285	100	unstable
90	Luzern	B67035	100	stable
91	Luzern	B50633	100	unstable
92	Luzern	B54013	100	stable
93	Luzern	B59519	100	unstable
94	St Gallen	B00/218 D	99	unstable
95	St Gallen	B00/2019 F	100	unstable
96	St Gallen	B00/2503 H	100	unstable
97	St Gallen	B00/12306 C	48	unstable
98	St Gallen	B00/17296 D	75	unstable
99	St Gallen	B00/17520 F	52	unstable
100	St Gallen	B00/18573 I	67	unstable
101	St Gallen	B00/19353 D	82	unstable
102	St Gallen	B00/7366 H	60	unstable
103	St Gallen	B00/21492 D	100	unstable
104	St Gallen	B00/22391	45	unstable
105	St Gallen	B00/18102 BIS	100	unstable
106	St Gallen	B00/27498 BIS	100	unstable
107	St Gallen	B00/31381 BIS	100	stable
108	St Gallen	B00/17879	56	unstable
109	St Gallen	B00/27300 G	100	stable
110	St Gallen	B00/31381 D	62	stable
111	St Gallen	B00/34746 E	100	unstable
112	St Gallen	B00/32757 F	100	unstable
113	St Gallen	B00/32707 F	100	unstable
114	St Gallen	B00/35096 I	100	unstable
115	St Gallen	B00/37919 J	42	unstable

116	Triemli	9521	45	unstable
117	Triemli	10024	100	stable
118	Triemli	7713	100	stable
119	Triemli	8771	97	stable
120	Triemli	12167/00	100	stable
121	Triemli	13458T/00	100	unstable
122	Triemli	662/02	100	unstable
123	Triemli	14016/02	59	unstable
124	Triemli	00/11628	100	unstable
125	Triemli	13297/00	100	unstable
126	Triemli	7784/00	100	stable
127	Triemli	2421/00	100	unstable
128	Triemli	6534/00	100	unstable
129	Triemli	6459/00	99	unstable
130	Triemli	655/00	75	unstable
131	Triemli	B01.20140	90	unstable
132	Triemli	3950/02	100	stable
133	Triemli	12886/02	100	unstable
134	Triemli	14503/02	100	unstable
135	Triemli	00/20719T	100	stable
136	Triemli	00/19372T	100	unstable
137	Triemli	00/17325	100	unstable
138	Triemli	00/16165	100	unstable
139	Triemli	5941/00	43	unstable
140	Triemli	00/20079T	100	unstable
141	Triemli	14895/00	100	unstable
142	Triemli	22857 T4/00	100	unstable
143	Triemli	22643/00	100	unstable
144	Triemli	B01.19698	100	unstable
145	Triemli	B01.12251	76	stable
146	Triemli	B01.1648	100	unstable
147	Triemli	B01.11768	100	unstable
148	Triemli	B01.13452	100	unstable
149	Triemli	B01.13674	100	unstable
150	Triemli	14230 T/00	100	unstable
151	Triemli	20719T BIS	100	stable
152	Triemli	12287 I/01	100	unstable
153	Triemli	12406 T2/01	100	stable
154	Triemli	11656 T3/01	100	unstable
155	Triemli	11779 T1/01	100	unstable
156	Triemli	14124 T1/01	100	unstable
157	Triemli	15600 T2/01	100	unstable
158	Triemli	17291 T3/01	100	unstable
159	Triemli	19604 T1/01	100	unstable
160	Triemli	4734 T3/01	100	unstable

161	Triemli	22752 T1/01	100	unstable
162	Triemli	22666 T4/01	100	unstable
163	Triemli	23697/01	91.5	stable
164	Triemli	700/01	100	unstable
165	Triemli	9658 T1/01	100	unstable
166	Triemli	11318 T1/01	100	unstable
167	Triemli	13617 T1/01	90.5	unstable
168	Triemli	21532 T1/01	100	stable
169	Triemli	22577 T2/01	100	stable
170	Triemli	B01.21269	100	unstable
171	Triemli	B00.11752	100	stable
172	Triemli	B00.7429	100	unstable
173	Triemli	B00.20498T	100	unstable
174	Triemli	B00.13894T	30	stable
175	Triemli	B00.4556	87	stable
176	Triemli	B00.12167 BIS	100	stable
177	Triemli	B02.22058 T3	37	unstable
178	Triemli	B02.22058 M	82	unstable
179	Triemli	B02.25698	146	unstable
180	Triemli	B02.25898	38	unstable
181	Triemli	B02.24854	126	unstable
182	Triemli	B03.1953	87	unstable
183	Triemli	B03.2243	35	unstable
184	UNIspital	B01.20938	90	stable
185	UNIspital	B01.20938 BIS	89	unstable
186	UNIspital	B01.27499	63	stable
187	UNIspital	B01.27499	12 (microdissected)	stable

The 187 tumour samples investigated for BAT26 MSI. Microsatellite unstable tumours were defined as those displaying more than 3 extra peaks in the sequence of the PCR product. The hospital which made the referral for analysis is listed.

Further analysis involved the investigation of 15 tumour samples, depicting loss of the hPMS2 protein in the presence of hMLH1 as demonstrated via IHC testing, for PMS2 LOH. Three microsatellite markers were employed, D7S517, D7S518 and D7S666. Non of the samples showed LOH for any of the hPMS2 markers. However, MSI was detected through the use of these markers in 13/15 (87%) of the tumours (Table 3). The microsatellite marker D7S517 identified 11/15 (73%)

of the unstable tumours, whilst marker D7S666 highlighted instability in 8/15 (53%) and marker D7S518 only 2/15 (13%). Overall, marker D7S517 was the most informative giving a result for each tumour sample analysed. Markers D7S666 and D7S518 produced results after repeated runs for 9/15 (60%) and 5/15 (33%) of the samples, respectively.

Sample No.	Hospital	Tumour No.	D7S517	D7S518	D7S666
1	Aarau	5194	MSI	Non informative	MSI
2	Aarau	16655	MSI	MSI	MSI
3	Triemli	11318	MSI	0.85	MSI
4	Triemli	20498	1.02	Non informative	MSI
5	Luzern	53072	0.64	0.74	MSI
6	Luzern	66543	MSI	Non informative	Non informative
7	Luzern	66732	MSI	Non informative	Non informative
8	Luzern	52557	1.22	Non informative	Non informative
9	Luzern	54832	MSI	MSI	1.40
10	Luzern	59519	MSI	Non informative	MSI
11	Luzern	64501	MSI	Non informative	Non informative
12	Luzern	61263	1.03	Non informative	Non informative
13	Luzern	53989	MSI	1.05	Non informative
14	Luzern	61162	MSI	Non informative	MSI
15	Luzern	65950	MSI	Non informative	MSI

Table 3: The 15 tumour samples investigated for LOH at the D7S517, D7S518 and D7S666 hPMS2 loci. Non depicted LOH although 87% showed MSI.

Discussion

The concordance between BAT26 instability testing and IHC negative staining is 100% accurate and proves not only the sensitivity and specificity of both methods in identifying tumors of an HNPCC nature, but also highlights their efficiency as prescreening methods for the identification of mismatch repair (MMR) gene mutations.

BAT26, has before been proven to be the most sensitive and the most specific microsatellite marker for the identification of aberrant mononucleotide repeats[15] and certainly proves its value here. However, despite its high sensitivity and specificity in this study, and other recent reports that use BAT26 as the sole studied microsatellite marker, caution needs to be applied as MSI-High tumors have been described that do not display instability at the BAT26 locus[16]. Caution should also be taken when employing immunohistochemistry as the sole screening method for the identification of MMR gene alterations. Optimal screening, and hence the best rate of mutation detection, is possibly only achieved through a combined approach, incorporating immunohistochemical analysis as well as a DNA and an mRNA-based method. This is a suggestion based on two facts i) different screening methods have their own failures: IHC and PTT fail to detect missense mutations whilst direct DNA sequencing fails to identify large, exon spanning deletions/insertions aswell as hypermethylation of the *hMLH1* promoter ii) MMR genes have a diverse mutational spectra and lack regions of “hot spots”.

The microsatellite marker D7S517 proved to be the most effective and reliable LOH/MSI marker, giving a result for each tumor sample analysed and depicting *hPMS2* microsatellite instability in 73% of the investigated tumor samples. However, further investigations, ie. DNA methylation and sequence analyses, into the cause of the *hPMS2* protein loss have yet to be conducted and the results cleaved. Before these projects are completed, the full extent to the

success of IHC in the screening of colorectal cancer patients cannot be concluded and phenotypic/geneotypic correlations concerning the *hPMS2* IHC negative staining cases cannot be made.

When the Zürich group have completed all lab based investigations and their collection of clinical data on the consecutive series of the 1048 patients enrolled in this study, the results cleaved from the analyses correlating phenotypical and genotypic characteristics will be ultimately important in aiding the future identification of MMR gene mutations in HNPCC patients.

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Chapter I part v

Frequency of MSH6 mutations in HCT116 clones on treatment with the DNA methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

A collaboration, with Giancarlo Marra, University of Zürich, as part of an ongoing study.

Introduction

Only a limited number of germline mutations in *hMSH6* and *hMSH3* have been reported in HNPCC patients suggesting that inherited mutations in these mismatch repair genes do not play a crucial role in the predisposition to hereditary colon cancers[1-5]. The proteins of these two genes independently form complexes with hMSH2[6-8]. The hMSH2-hMSH6 complex recognises single-base mispairs and small (ie. single-base) insertion/deletion loops[9,10], whilst the hMSH2-hMSH3 complex focuses on small and large deletion loops but does not appear to have the ability to identify single-base substitution mispairs[6,7,9,11,12].

Germline mutations of *hMSH6* found to be associated with HNPCC have been reported in limited numbers[13-17]. Since *hMSH6* gene alterations result in the high accumulation of base substitution mutations there are two possible explanations for the rarity of *hMSH6* mutations in HNPCC families[11,18]. In the first instance, all investigated HNPCC families were microsatellite unstable (MSI) at dinucleotide repeat loci, a phenotype not caused by the loss of MSH6 function, hence disqualifying potential *hMSH6* mutant families from further study[4,11,15]. Secondly, the great majority of target tumor suppressor genes inactivated in HNPCC are done so by frameshift mutations in mononucleotide repeats[11,19,20]. Since the loss of functional MSH6 proteins does not cause the rate of frameshift mutations to

increase[11] these target genes may not be hypermutable when *hMSH6* is mutated.

Since the very first reports on *hMSH6* mutations were made[13,14], an atypical clinical phenotype of families with *hMSH6* germline mutations has begun to formulate. An excess of endometrial cancers[17], and late onset[17,21] have characterised the individual, whilst a low degree of microsatellite instablity[16] and/or preferential involvement of mononucleotide repeats have proposed to characterise the tumors from patients carrying *hMSH6* mutations[15,22]. However, typical Amsterdam-I HNPCC families have also been documented as *hMSH6* mutation positive[17], with the prevalence of endometrial cancers reportedly low[21, 22]. In addition, *hMSH6* mutations are occasionally related to the early onset of cancer[15] and a high degree of tumor microsatellite instability[13,17]. In families not harboring *hMSH2* or *hMLH1* germline mutations, the frequency of *hMSH6* germline mutations vary from 0% among Amsterdam I families with MSI-high tumours[4] to 22% among families with suspected HNPCC and MSI-low tumours[16]. In families with *hMSH2* and *hMLH1* germline mutations excluded, *hMSH6* germline mutations have been reported to occur in 5-10%[17,21].

The Zürich research group I was working with in collaboration on this project, observed that on treatment with the DNA methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), HCT116+chr.3 clones did not express MSH6 proteins. It appears to be a response exclusive to the HCT116+chr.3 cell line since they treated other cell lines similarly but did not achieve the same response. It was my responsibility to conduct the *hMSH6* mutation analysis on the DNA from these clones in order to establish any *hMSH6* germline mutations. I established an optimised protocol for the rapid and sensitive mutation analysis of h*MSH6* via high performance liquid chromatography (DHPLC) and subsequent direct DNA sequencing.

Methods

MSH6 PCR Amplification

Exon specific primer pairs (sequences as reported by Kolodner *et al*. 1999)[21] were used to amplify the 10 exons of *MSH6,* including the respective exon-intron boundaries. Twenty-five microlitres of PCR reaction mixture contained 50ng of genomic DNA, 0.5μM each primer, 2.5μM each dNTP, 5mM $MgCl_2$, 10x reaction buffer, and 0.2U Taq polymerase (Invitrogen, Switzerland). The reaction parameters were set in three different programs; ① 96°C-4 mins for 1 cycle, 96°C-20 sec, 70°C-20 sec (-1°C/cycle) and 68°C-20 sec for 15 cycles; 96°C-20 sec, 55°C-20 sec and 68°C-20 sec for 25 cycles, and 68°C-7 mins for 1 cycle, ② 95°C-10 mins for 1 cycle, 95°C-40 sec, 65°C-20 sec (-1°C/cycle) and 72°C-20 sec for 10 cycles; 94°C-20 sec, 55°C-20 sec and 72°C-20 sec for 25 cycles, and 72°C-7 mins for 1 cycle, ③ same as program 2 although the time for incubation at 72°C was increased to 40 sec, all for a Hybaid OmnE Thermocycler (Catalys AG, Wallisellen, CH).

Denaturing High Performance Liquid Chromatography (dHPLC)

dHPLC was performed using the 3500HT WAVE nucleic acid fragment analysis system (Transgenomic, Crewe, UK). Melting temperatures for dHPLC were predicted by the Wavemaker software version 4.1.42 (Transgenomic) (dHPLC melting temperatures available from author upon request). Where different elution profiles were observed, in comparison to control samples run in parallel, direct DNA sequencing was performed in order to establish the nature of the sequence alteration.

MSH6 Mutational Analysis

PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Basel, Switzerland). The sequencing reaction was performed using the Big Dye Teminator Cycle Sequencing kit (Applied Biosystems, Rotkreuz, Switzerland), according to the manufactures' guidelines. Subsequently, sequencing products were purified using the DyeEx 2.0 Spin Kit (Qiagen, Basel, Switzerland) and

analysed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Germline mutations established in *MSH6* were confirmed in both forward and reverse directions, from at least 2 independent PCR products.

Results

In table 1 are listed the sequencing results from the *hMSH6* mutation analysis screening. All clones harboured the same *hMSH6* gene alteration in exon 5, an inserted C at position 2631. Clones 202G, 202T and 202U displayed a frameshift mutation which resulted from the deleted G at position 2804-2805. In clone 202 a splice donor site mutation was established. This was the only clone to display a G>A substitution. This latter mutation is the only mutation to be known and documneted as a result of MNNG treatment.

Exon	BVEC D9	BVEC E2	BVEC F7	202 A	202 C	202 E	202 G	202 Q	202 T	202 U	202 X	HCT116 +3
1	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
2	OK	OK	OK	OK	OK	OK	OK	SD(+1) G>A	OK	OK	OK	OK
3	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
4A	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
4B	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
4C	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
4D	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
4E	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
4F	OK	OK	OK	OK	OK	OK	2804-5delG	OK	2804-5delG	2804-5delG	OK	OK
5	326 insC	326 insC	326 insC	326 insC	326 insC	326 insC	326 insC	326 insC	326 insC	326 insC	326 insC	326 insC
6	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
7	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
8	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
9	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
10	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK

Table 1: The results of the *hMSH6* mutation analysis involving the direct DNA sequencing of HCT116+chr.3 clones. *hMSH6* germline mutations are highlighted in green.

Discussion

MNNG causes methylation in the O^6 position of guanine and the resulting O^6-methylguanine (O^6-MeG) pairs with thymine instead of cytosine, leading to GC to AT transition mutations[23,24,25]. O^6-MeG paired with thymine is subject to repair by the mismatch repair system[26,27]. O^6-MeG-generating agents are powerful mutagens and carcinogens but a lack of mismatch repair confers resistance to cytotoxicity and hence raises the level of mutagenic response in cells[28,29,30]. This suggests that genotoxic and cytotoxic effects of O^6-MeG are mediated by mismatch repair. Such an erroneous mismatch repair response may be related to the repeated misincorporation of thymine opposite O^6-MeG or by the signalling for apoptotic functions due to faulty mismatch repair mechanisms.

The repair of O^6-methylguanine-thymine base pairs by mismatch repair is correlated to the binding of the MSH2-MSH6 protein complex[31,32]. Although functional studies have been conducted on the individual mismatch repair proteins, little has been reported on the regulation of mismatch repair as a whole. It has previously been shown that MSH2 is cell cycle dependent, since MSH2 is higher in abundance in proliferating rather than resting cells[33]. Whether MSH2 and/or other mismatch repair proteins are controlled by exogenous stimuli, especially mutagenic treatments, has yet to be established.

One group[34] reported an observed increase in MutSα and GT binding activity in the nucleus on treatment with O^6-methylguanine generating mutagens, indicating a novel type of genotoxic stress response. They suggest that the regulation of mismatch repair upon DNA damage occurs primarily at the level of post-translational modification (including nuclear transportation) as opposed to at the level of gene activation. The early translocation of mismatch repair proteins into the nucleus is intended to increase mismatch repair capacity in the nucleus. This would be highly important with regard to the O^6-MeG/C lesions forming on replication of the mutagenic GT mismatches.

Mismatch repair defects are associated with various hereditary cancers[35,32,36] and have also been shown to increase immensely the resistance of cells to O^6-MeG generating agents[23,24,30,37,38]. Mismatch repair defects hence have a strong involvement with the mutagenic and carcinogenic response of cells to alkalyting agents.

The Zürich group are in the process of planning further projects in relation to this study. Future studies may involve the transfection of MSH3 into this cell line, which is currently *hMSH3* mutated, to establish any correlation between MSH3 being present and functional, and the presence of MSH6.

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Chapter II

Exclusion of a modifier gene locus on chromosome 1p33-36 in a large Swiss familial adenomatous polyposis kindred

This paper has already been published in the European Journal of Cancer.

Abstract

Familial adenomatous polyposis (FAP), an autosomal dominantly inherited colorectal cancer predisposition syndrome, displays considerable inter- and intra-familial phenotypic heterogeneity, which represents a major problem in genetic counselling of *APC* mutation carriers. The *Min* mouse model indicated a putative disease modifier locus on chromosome 4, which is syntenic to human chromosome 1p35-36. This finding was subsequently supported by parametric and non-parametric linkage analyses in FAP families, however, without identifying functional variants in candidate genes. Recently, germline mutations in the base-excision repair gene *MYH*, which maps to the 1p33-34 region, have been described in patients with multiple adenomas, pointing to a possible role as disease modifier in FAP. Here, we present critical re-assessment of one of the largest FAP kindreds published, which was previously used in linkage mapping of 1p35-36. In this family all affected members harbour the same germline mutation (5945delA) at codon 1982 of the *APC* gene but display marked phenotypic variability, in particular regarding the occurrence of extracolonic disease which segregates in several branches of the family tree.

Using up-dated clinical information, additional mutation carriers and polymorphic markers, fine-mapping of the critical region as well as mutation analysis of the *MYH* gene were performed. These investigations allowed us to (i) significantly exclude the 1p33-36 region as a modifier locus and (ii) *MYH* as a modifier gene

for extracolonic disease in this FAP kindred. The results indicate that linkage analysis of further putative candidate regions is necessary to identify a disease modifier locus in FAP.

Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited predisposition to colorectal cancer caused by germline mutations in the *APC* (adenomatous polyposis coli) gene. Patients develop hundreds to thousands of adenomas throughout the large intestine some of which, unless prophylactic colectomy is performed, eventually progress to colorectal cancer before the age of 40 [1]. Phenotypically, it is a heterogenous disease in which patients may also present with a number of extracolonic disease manifestations such as congenital hypertrophy of the retinal pigment epithelium (CHRPE), osteomas, and soft tissue tumors (epidermoid cysts, lipomas, fibromas, desmoid tumors), as well as upper gastrointestinal (GI) polyposis [2,3]. Desmoid tumours and duodenal cancer represent the major cause of mortality in FAP patients who have undergone colectomy [4]. The inability to predict disease severity in the individual FAP patient (*APC* mutation carrier) represents a major difficulty in genetic counselling and in defining optimal clinical screening and prevention strategies. Part of the inter- and intra-familial phenotypical differences can be explained by the position of the germline *APC* mutations. However, despite established genotype-phenotype correlations, many phenotypic differences can not be completely explained by the site and type of the germline *APC* mutation [5], and other genetic factors (modifier genes) are expected to play important roles in disease development.

In support of this, two loci that modify polyp multiplicity in the FAP phenotype have been identified in the *Min* (multiple intestinal neoplasia) mouse model of FAP. The *Mom1* locus (Modifier of Min 1) has been assigned to the mouse chromosome 4 [6], which has synteny to human chromosome 1p35-36, and where

the secretory phospholipase A2 (*Pla2g2a*) gene has been identified as a strong candidate for suppression of the Min phenotype [7,8]. The second locus, *Mom2* (Modifier of Min 2), has recently been mapped to mouse chromosome 18 which has synteny to the human chromosome 18q21 and 18q23 [9,10]. Both loci, *Mom1* and *Mom2*, were found to reduce the number of polyps in Min mice and their synteny regions on the human chromosomes 1 and 18 are known to be frequently deleted/lost in a variety of human cancers, including colon tumors [11-14].

So far, by means of mutation analysis, no functional variants of *Pla2G2A* have been detected in humans [15-17], and the possibility that another locus close to *Pla2G2A* actually represents *Mom1* lead our group and others to further investigations of the 1p35-36 region by means of parametric- and non-parametric linkage analysis. However, these studies neither significantly excluded nor confirmed a human FAP modifier locus in 1p35-36 [18,19]. Furthermore, germline mutations in the base-excision gene *MYH*, which maps to the 1p33-34 region, have recently been described in patients with multiple adenomas, in some of which extracolonic disease (desmoids) are also present [20,21]. This may implicate *MYH* as a possible FAP modifier, as *MYH* mutations/variants in combination with germline *APC* mutations could be expected to enhance the FAP disease phenotype.

To assess the role of the 1p32-36 region as a candidate modifier locus, we re-investigated a large Swiss FAP kindred (No. 1460) part of which was previously used in linkage analysis of this region, and where a lod score of 2.08 was found for an autosomal recessive model [19]. In the 7 years since this analysis has been performed, 13 additional members of family 1460 were identified and up-dated clinical information on the known mutation carriers gathered, which enabled us to perform an extended linkage analysis of the 1p32-36 region as well as a mutation analysis of the new candidate modifier gene in this region, the *MYH* gene.

Methods

Patient data

The large Swiss FAP kindred comprises over 200 family members, whereof all affected members (n=63) share the same *APC* germline mutation in exon 15n, 5945delA, leading to a frameshift starting from codon 1982 and a premature stop codon at position 2044. In 50 members (Figure 1; Table 1) belonging to the pedigree branches with extracolonic manifestations, histopathological data and reports from colonoscopies, gastro-duodenal endoscopies, computer tomographies, surgery, autopsies, as well as information from regular dental examinations, were collected and re-evaluated for the present study. Only patients with verified data from clinical and histopathological reports were used for linkage analysis. Written informed consent was obtained from all individuals.

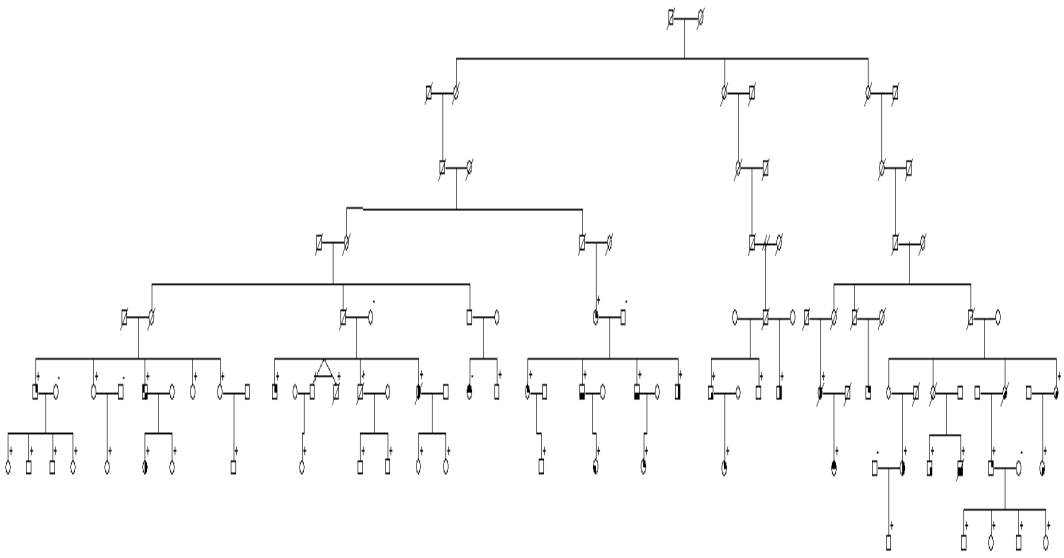

Figure 1 Extract from FAP kindred no.1460 displaying branches with extracolonic disease manifestations. Symbol description: (a) upper right quadrant: presence of desmoids and fibromas, (b) lower right quadrant: upper gastrointestinal polyps, (c) lower left quadrant: osteomas, (d) upper left quadrant: other extracolonic manifestations.

Genotyping of polymorphic markers

Genotyping was performed using fluorescently-labelled primers from the ABI Prism Linkage mapping Set-MD10 (PE Applied Biosystems; 22-24) and by means of custom primers of the markers from the 1p32-36 region. These markers were selected according to their map location and their heterozygosity status, using the following internet websites: http://www.ucsc.genome.org [25], ftp://bioinformatics.weizmann.ac.il/pub/databases/genethon/Gmap/Nature1995/data/ [24]. Primers were FAM and HEX fluorescently-labelled, and PCR reactions done according to the manufactures' protocol, using True Allele PCR Premix (Applied Biosystems), and a Gradient Mastercycler (Eppendorf). PCR products were pooled according to their size, subsequently combined with ROX400-HD size standard (Applied Biosystems) and electrophoresed on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems). Genotype determinations were automated using GeneScan and Genotyper softwares (Applied Biosystems). Genotyping was only performed in *APC* mutation carriers and their married-in members.

Linkage analysis

Microsatellite data was checked for genotyping errors using the PEDCHECK program [26]. Two-point parametric LOD score linkage analysis was performed using the MLINK program from the LINKAGE package [27]. Lod scores were calculated for both autosomal dominant (disease allele frequency 0.0781) and autosomal recessive (disease allele frequency 0.1000) models. Marker allele frequencies were set to be equal. Disease allele frequencies for the dominant model were calculated using the Hardy-Weinberg equilibrium formula ($p^2+2pq+q^2=1$), assuming a 10% frequency of extracolonic disease manifestations in FAP patients. Penetrance of 1.00 and 0.95 was used for the recessive model, and of 0.90 and 0.85 for the dominant model. Furthermore, age dependent penetrance for extracolonic manifestation was estimated from our

pedigree and followingly 6 different liability classes were used in the dominant model: 0.157 (≤20 years), 0.368 (≤30 years), 0.684 (≤40 years), 0.895 (≤50 years), 0.947 (≤60 years), 0.999 (≤70 years). Penetrance of phenocopies was set to be 0.001. Only *APC* mutation carriers (and their married-in members) were included in the analysis, as only in these members extracolonic manifestations are expected to result from both *APC* and modifier gene mutations. Polyposis patients with colonic disease only were classified as having an "unaffected" affection status, married-in members as having an "unknown" phenotype. Patients presenting with extracolonic disease manifestation(s) were evaluated applying two different sets of criteria: (a) stringent criteria: only patients displaying at least adenomatous polyps in the upper GI tract and/or desmoids were classified as `affected` with the others being classified as `unknown`; (b) loose criteria: all patients with confirmed extracolonic manifestation(s) were scored as `affected` (Table 1). One patient with bronchial carcinoma was classified as `unknown` in all analyses.

MYH Mutational Analysis

Exon specific primer pairs were used to amplify the 16 exons of *MYH*, including the respective exon-intron boundaries[20]. Twenty-five microlitres of PCR reaction mixture contained 100ng of genomic DNA, 0.5μmol/l each primer, 2.5μmol/l each dNTP, 1.5mmol/l $MgCl_2$, 10x reaction buffer, and 0.2U Taq polymerase (Invitrogen). The reaction parameters were; 95°C-5 mins for 1 cycle, 95°C-1 min, 60°C-1 min and 72°C-1 min for 35 cycles, and 72°C-10 mins for 1 cycle, for a Hybaid OmnE Thermocycler (Promega).

Subsequently, dHPLC was performed using the 3500HT WAVE nucleic acid fragment analysis system (Transgenomic). Melting temperatures for dHPLC were predicted by the Wavemaker software version 4.1.42 (Transgenomic). Where different elution profiles were observed, in comparison to control samples run in parallel, direct DNA sequencing was performed in order to establish the nature of the sequence alteration.

PCR products were purified using the QIAquick PCR Purification kit (Qiagen). The sequencing reaction was performed using the Big Dye Teminator Cycle Sequencing kit (Applied Biosystems), according to the manufactures' guidelines. Sequencing products were purified using the DyeEx 2.0 Spin Kit (Qiagen) and analysed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Germline mutations established in *MYH* were confirmed in both forward and reverse directions, from at least 2 independent PCR products.

Results

Clinical data

In previous studies [19,28] we reported a large FAP kindred (no. 1460), originating from the Poschiavo region in Switzerland, whose affected members present with a highly variable phenotype, on the level of both, colonic as well as extracolonic disease manifestations. Fifty family members of this kindred, belonging to sub-branches displaying extracolonic disease were clinically re-evaluated for this study (Table 1). In general, the polyposis phenotype among *APC* mutation carriers was found to be relatively mild, as could be expected by the site of the germline mutation, with 26 (65%) patients displaying attenuated polyposis (less than 100 polyps). However, the polyposis phenotype was very variable, ranging from severe forms with more than 1000 polyps (2 patients) to the very mild form, where no polyps (3 patients at age 22, 29, and 47, respectively) or less then 10 polyps (3 patients at age 32, 33, 47, respectively) were present.

In 26 patients, extracolonic tumours developed, the majority of these being desmoids (15/26; 57.7%) and upper gastrointestinal polyps (16/26; 61.5%). Adenomatous origin of the polyps was confirmed in 9 patients, other polyps were diagnosed as fundus gland polyps, which developed to a great extent in 3 patients (one of them without colon polyps at age 47). Apart from these frequent disease manifestations, others were also reported (see Table 1).

ID	Colorectal polyps	Stomach polyps	Duodenum polyps	Desmoids or Fibromas		Included in LA	AS
1460-1	<100					yes	1
1460-4	<100			yes		yes	2
1460-6	>100			yes		yes	2
1460-7	<100	yes		yes		yes	2
1460-8	unknown			yes		yes	2
1460-9	>100					yes	1
1460-10	<100	yes		yes		yes	2
1460-11	<100					yes	1
1460-16	>100	yes			Osteoma	yes	0/2
1460-19*	>100			yes		yes	2
1460-21	unknown				Osteoma	yes	0/2
1460-24	<100		yes		salivary gland adeno-carcinoma prostate tumour	yes	2
1460-26	>100	yes	yes	yes		yes	2
1460-28	<100					yes	1
1460-33	<100					yes	1
1460-42	>100					yes	1
1460-44	<100					yes	1
1460-46*	<100			yes		yes	2
1460-47	>100				Bronchial-Ca	yes	0
1460-48*	>100					yes	1
1460-55	>100					yes	1
1460-86	<100					yes	1
1460-88	<100					yes	1
1460-89	<100					yes	1
1460-91	<100	yes		yes		yes	2
1460-93	>100					yes	1
1460-12	<100					yes	1
1460-106	<100					yes	1
1469-1	<100	yes	yes	yes	Osteomas, Lipoma	yes	2
1469-4	<100			yes	Osteoma	no	0/2
1489-B*	>100			yes		yes	2
1489-E	unknown					yes	1
1501-1*	< 100	yes		yes	Osteomas	yes	2
1501-2	>100	yes	yes			yes	2
1501-4	<100			yes		yes	2
1501-5	unknown					yes	1
1747-1	<100					yes	1
1779-1*	>100	yes			Osteoma	yes	0/2
1489-C	unknown					yes	1
1489-D	unknown					yes	1
1489-F	unknown					yes	1
1460-112	<100	yes				no	0/2
1779-2	>100	yes				yes	0/2
1460-116	<100	yes	yes			no	2
1460-105	<100					yes	1
1460-122	unknown			yes	leukemia	no	2
1460-5	unknown					yes	1
1489-no	<100		yes	yes		no	2
1489-no	unknown		yes			yes	2
1624-4	<100	yes	yes	yes		no	2

Table 1: Phenotypic characteristics in 50 *APC* mutation carriers from FAP family no.1460.
LA = linkage analysis; AS = affection status used in linkage analysis;
* = patients included in *MYH* mutation screening

Linkage analysis

Simulation linkage analysis, previously performed in family no.1460 using the same diagnostic criteria and parameters as employed for linkage analysis (see methods), revealed a maximum expected lod score of 3.8 to 5.3 for autosomal dominant models, and 1.9 to 2.7 for autosomal recessive models.

Twenty-eight polymorphic markers spanning 58.7 cM and 50.2 Mb [25] of the 1p32-36 region (Figure 2), respectively, were used for two-point linkage analysis under an autosomal dominant model with age-dependent penetrance. No evidence for the existence of a dominant modifier locus for extracolonic FAP disease was found. Lod scores throughout the region 1p33-36 were below –2 (except for three markers, D1S3669, D1S255 and D1S2733, with lod scores of -1.6, -1.9 and –0.6, respectively), thus excluding this region as a possible modifier locus (Table 2). This region is known to include both the *Pla2G2A* and *MYH* genes. In the region 1p32.1-32.3, although most of the markers gave negative lod scores, only a portion of markers showed lod scores below –2, thus significantly excluding some loci. In one marker a slightly increased lod score (D1S417, maximum lod score of 0.7 at □=0) was found, which is not exclusive, and possibly results from low informativity of this particular marker in our family.

To exclude the possibility that our negative results were due to choosing the wrong mode of inheritance, the analysis was also performed using an autosomal recessive model at penetrance 0.95 and 1.00, as well as an autosomal dominant model at reduced penetrance of 0.90 and 0.85, respectively (data not shown). All analyses, under both stringent and loose diagnostic criteria, resulted in negative lod scores below –2, except for the above mentioned markers D1S3669 and D1S417, and marker D1S231 (lod scores below –1.5, 0.1, and 0.8, respectively) hence excluding the 1p32-36 region as a modifier locus of extracolonic disease in our FAP kindred.

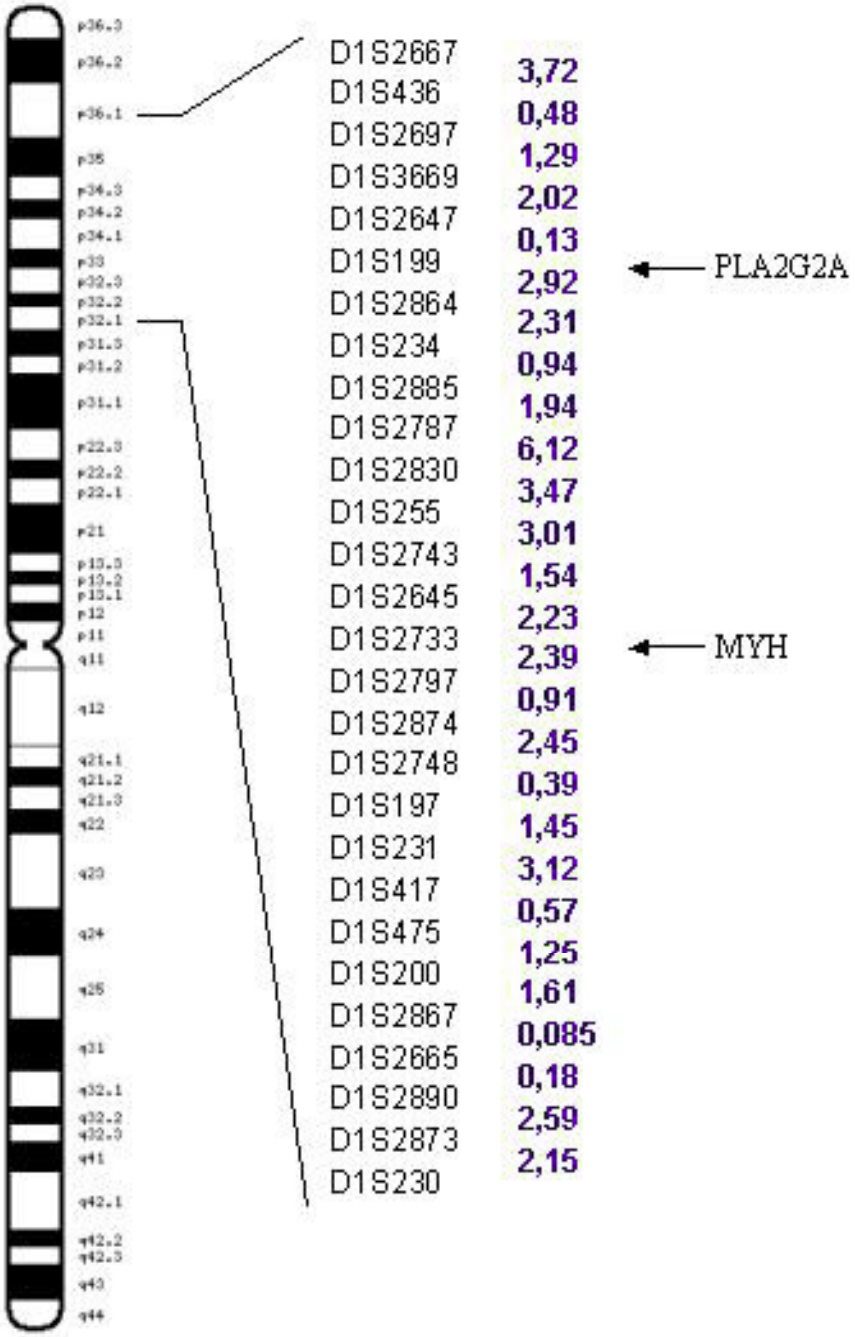

Figure 2 Physical map of the 1p32-36 region. Marker order and physical distance (Mb) were determined according to the UCSC genome bioinformatics site [25].

		Lod score at recombination fraction (θ)												
Marker	***Region***	*0*	*0.005*	*0.01*	*0.02*	*0.03*	*0.04*	*0.05*	*0.06*	*0.07*	*0.1*	*0.2*	*0.3*	*0.4*
D1S2667	1p36.22	-4.94	-2.67	-2.34	-1.99	-1.77	-1.60	-1.46	-1.35	-1.25	-1.01	-0.53	-0.25	-0.08
D1S436	1p36.13	-3.95	-1.72	-1.41	-1.11	-0.93	-0.81	-0.72	-0.65	-0.59	-0.47	-0.28	-0.18	-0.09
D1S2697	1p36.13	-3.45	-2.03	-1.70	-1.33	-1.11	-0.94	-0.81	-0.70	-0.61	-0.40	-0.10	-0.03	-0.01
D1S3669	1p36.13	-1.63	-1.55	-1.49	-1.38	-1.28	-1.19	-1.11	-1.04	-0.97	-0.79	-0.40	-0.17	-0.04
D1S2647	1p36.13	-2.37	-2.18	-2.03	-1.80	-1.62	-1.47	-1.35	-1.24	-1.15	-0.93	-0.49	-0.26	-0.11
D1S199	1p36.13	-5.86	-4.18	-3.79	-3.32	-3.01	-2.75	-2.54	-2.36	-2.20	-1.81	-0.97	-0.46	-0.15
D1S2864	1p36.12	-4.71	-4.07	-3.70	-3.19	-2.82	-2.53	-2.28	-2.06	-1.88	-1.42	-0.56	-0.18	-0.03
D1S234	1p36.11	-6.23	-5.10	-4.62	-4.01	-3.59	-3.25	-2.98	-2.74	-2.53	-2.03	-1.01	-0.46	-0.14
D1S2885	1p36.11	-4.59	-3.63	-3.15	-2.56	-2.16	-1.86	-1.62	-1.42	-1.25	-0.86	-0.24	-0.06	-0.03
D1S2787	1p35.3	-2.38	-2.26	-2.15	-1.97	-1.81	-1.68	-1.56	-1.45	-1.36	-1.11	-0.56	-0.25	-0.07
D1S2830	1p35.1	-2.87	-2.19	-1.88	-1.50	-1.24	-1.04	-0.88	-0.74	-0.62	-0.34	0.11	0.18	0.07
D1S255	1p34.3	-1.98	-1.48	-1.23	-0.92	-0.71	-0.56	-0.43	-0.33	-0.24	-0.04	0.23	0.24	0.12
D1S2743	1p34.2	-2.77	-1.86	-1.56	-1.22	-1.00	-0.83	-0.69	-0.58	-0.48	-0.26	0.10	0.17	0.10
D1S2645	1p34.2	-2.50	-2.14	-1.91	-1.57	-1.33	-1.14	-0.98	-0.84	-0.72	-0.44	0.02	0.13	0.08
D1S2733	1p34.1	-0.66	-0.63	-0.60	-0.55	-0.50	-0.45	-0.41	-0.37	-0.33	-0.23	-0.04	0.00	-0.01
D1S2797	1p33	-2.66	-1.76	-1.46	-1.12	-0.91	-0.75	-0.62	-0.51	-0.42	-0.22	0.07	0.08	0.01
D1S2874	1p33	-2.74	-2.41	-2.17	-1.82	-1.57	-1.37	-1.20	-1.06	-0.94	-0.65	-0.12	0.07	0.07
D1S2748	1p33	-3.99	-3.06	-2.72	-2.32	-2.04	-1.82	-1.63	-1.48	-1.34	-1.00	-0.37	-0.10	0.00
D1S197	1p33	-0.78	-0.75	-0.71	-0.65	-0.59	-0.54	-0.49	-0.45	-0.41	-0.31	-0.11	-0.04	-0.04
D1S231	1p32.3	-0.08	-0.07	-0.06	-0.05	-0.04	-0.03	-0.03	-0.02	-0.02	-0.01	-0.01	-0.01	-0.01
D1S417	1p32.3	0.77	0.76	0.74	0.72	0.70	0.67	0.65	0.63	0.61	0.54	0.36	0.22	0.10
D1S475	1p32.3	-1.94	-1.60	-1.39	-1.10	-0.91	-0.76	-0.63	-0.53	-0.44	-0.25	0.03	0.06	0.02

D1S200	1p32.3	-1.73	-1.25	-0.99	-0.68	-0.47	-0.31	-0.17	-0.06	0.03	0.24	0.51	0.46	0.24
D1S2867	1p32.2	-2.21	-1.78	-1.52	-1.15	-0.90	-0.71	-0.55	-0.42	-0.31	-0.07	0.25	0.22	0.06
D1S2665	1p32.2	-2.24	-1.92	-1.71	-1.43	-1.22	-1.06	-0.93	-0.82	-0.72	-0.48	-0.09	-0.01	-0.06
D1S2890	1p32.2	-2.14	-2.01	-1.89	-1.69	-1.51	-1.36	-1.22	-1.09	-0.98	-0.69	-0.13	0.07	0.06
D1S2873	1p32.1	-0.95	-0.74	-0.59	-0.37	-0.22	-0.10	-0.01	0.07	0.13	0.26	0.38	0.28	0.11
D1S230	1p31.3	-2.57	-2.37	-2.22	-1.98	-1.80	-1.65	-1.51	-1.39	-1.28	-1.00	-0.41	-0.13	-0.01

Table 2 Lod scores for autosomal dominant model with age dependent penetrance using markers from the 1p32-36.

MYH mutation analysis

Six patients from different pedigree branches and with different extracolonic manifestations were selected for *MYH* gene mutation analysis (Table 1): three patients, 1460-46, 1489-B and 1460-19, with desmoids coming from different pedigree branches (with <100, >100 and >1000 polyps, respectively); patient 1460-48 with more than 1000 colonic polyps; patient 1779-1 with osteomas, fundus gland polyps and >100 colonic polyps; patient 1501-1 with multiple desmoids, osteomas, stomach and duodenum adenomas, and only one colonic polyp. No DNA variants could be detected in all but one of them. Patient 1501-1 was found to harbour a heterozygous G64A alteration (exon 2), resulting in a substitution of valine to methionine at codon 22 (V22M) of the *MYH* gene. Subsequent segregation analysis of the V22M variant identified only one more patient (1460-16) and his non-affected father as carriers. The variant represents an already described polymorphism, which was previously reported at a population frequency of 9-10% [20,21].

Discussion

In the present study the 1p33-36 region can be excluded as a modifier gene locus for extracolonic disease in our large Swiss FAP kindred no.1460. The analysis was performed on updated family information, and investigating both more affected family members and more microsatellite markers. Since our initial investigation restricted to the 1p35-36 region in 1996, 13 additional patients either developed extracolonic tumours or were newly referred to our department. Out of these, 4 were classified as having an `affected` or `unknown` diagnosis, depending on the stringency of the affection criteria used. Four patients previously classified as `affected` were for the present analysis scored as `unknown`, because original data provided by the patient's record could not be confirmed from histopathological records. Furthermore, unlike the previous analysis, only *APC* mutation carriers and their spouses were used for linkage analysis. These differences may explain why the lod score for the autosomal recessive model dropped from a previously observed 2.08 (D1S211) to below -2 (instead of marker D1S211, markers D1S2645 and D1S2733 were used), and for the autosomal dominant model a decrease from 1.77 (D1S197) to below –0.7 (Table 2).

Although our linkage results for an autosomal recessive mode of inheritance resulted in significant exclusion of the 1p32-36 region, we put our emphasis on autosomal dominant models which seem to be more appropriate in our FAP kindred for several reasons. Firstly, the ratio of *APC* mutation carriers with compared to those without extracolonic disease varied between 0.42 and 0.52, depending on the affection criteria applied. Secondly, in some of the sub-branches of family no.1460, extracolonic manifestations are clearly transmitted through generations (Figure 1). When comparing 12 parent-child pairs with extracolonic disease present, transmission of extracolonic disease through the generations could be observed in 100% of informative pairs, suggesting an autosomal dominant mode of inheritance. Using the stringent phenotype criteria,

ie. only including patients with at least upper GI adenomatous polyps or desmoids, transmission was seen in 8 out of 9 pairs (88%).

Phenotype analysis revealed the same clinical heterogeneity as previously reported [28]. Furthermore, when comparing the group of patients with <100 and those with >100 colorectal polyps, no statistically significant relationship could be found between polyp number and the occurrence of extracolonic disease in general (χ^2=0.44, p=0.50). The same was true if only desmoids (χ^2=1.20, p=0.27) or only upper GI polyps were taken into account (χ^2=0.10, p=0.75). This indicates that the severity of colonic polyposis does not correlate with the presence of extracolonic disease manifestations, hence, polyp number and extracolonic disease may represent two genetically related but distinct entities.

Our linkage analysis data are in agreement with the results from the mutation screening in *PLA2G2A*[16] and *MYH*, where, except for the heterozygous V22M variant present in two patients belonging to different branches of family tree, no other DNA alterations could be identified in the coding region of the *MYH* gene. Although residues 6 to 32 of the MYH protein contain a conserved replication protein A (RPA)-binding motif [29], valine22 does not belong to the conserved amino acids. In view of these findings, the V22M variant is unlikely to contribute to extracolonic disease in this family.

In conclusion, our data on this large Swiss FAP kindred significantly exclude the 1p33-36 region as a modifier locus and *MYH* as a modifier gene for extracolonic disease. Since simulation linkage analysis revealed a maximum expected lod score of 3.8 to 5.3 for autosomal dominant and 1.9 to 2.7 for autosomal recessive models, future work will concentrate on performing a genome-wide linkage analysis in this FAP kindred which should help in the identification of a modifier locus in FAP.

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Chapter III

The phenotypic characterisation of hereditary nonpolyposis colorectal cancer patients in relation to mismatch repair gene mutation status

A draft of a scientific paper prepared for publication.

Abstract

Hereditary nonpolyposis colorectal cancer (HNPCC), an autosomal dominantly inherited syndrome, accounts for approximately 1-5% of all colorectal cancers and is hence one of the most commonly inherited cancer predispositions. HNPCC has a frequency of between 1:2000 and 1:200 but has a lower than expected detection rate, probably accountable for by the failure of the mutation detection techniques employed, to identify all occurring mismatch repair (MMR) gene mutations. This study aims to further characterise the phenotype of HNPCC patients by comparing MMR gene mutation carriers to gene alteration negative individuals in an attempt to ultimately aid the identification of HNPCC individuals and MMR gene mutation carriers. One hundred and twenty individuals suspected of HNPCC were assigned to either the Amsterdam Criteria I/II (AC), the restricted (ie. minus ACI/II) Bethesda Guidelines (rBG) or the Neither Criteria (NC) group. Forty-six individuals were assigned a MMR gene mutation positive status, a further 84 individuals were established as mutation negative, as determined via microsatellite marker analysis, immunohistochemistry, direct DNA sequencing and multiplex PCR of short fluorescent fragments for the detection of large genomic deletions. Statistical evaluation of genotype-phenotype correlations involved the Chi-square, Fischer exact or Student's t-test, with all probabilities reported as two-tailed Ps, considering a P of <0.05 to be statistically significant. Ninety-four percent (n=43) of the mutation positive patients were classified by

either the Amsterdam Criteria (AC) or the restricted Bethesda Guidelines (rBG) in comparison to only 76%, of the mutation negative individuals ($p<0.0005$). Mutation positive patients were on average 3 years younger than mutation negative individuals at the time of their CRC diagnosis. Whilst the sex ratio divided the overall study population evenly, the investigated females were significantly more frequently found to be mutation negative than mutation positive (73%vs27%, $p<0.0005$). Sixty-three percent of the mutation positive patients had CRCs located in the proximal region of the colon compared to 21% in the mutation negative individuals ($p<0.0001$). In addition, a higher prevalence of extra colonic manifestations was observed and more syn-/metachronous CRCs were found, in mutation positive compared to mutation negative patients ($p<0.03$ and $p<0.05$, respectively). Using the HNPCC referral criteria as a basis, and subsequently phenotypic differences such as those established in this study, namely age at CRC diagnosis, CRC location, the occurrence of syn-/metachronous cancers, and the presence of extracolonic manifestations, a possible distinction between mutation positive and mutation negative individuals could be made by clinicians and be used as a means to prioritise patients for genetic surveillance, mutation screening and genetic counselling.

Introduction

One of the most crucial stages in the diagnosis of a hereditary cancer syndrome is the compilation of an indepth family pedigree that highlights cancer development[1-3]. Hereditary nonpolyposis colorectal cancer (HNPCC) is the most frequently occurring form of hereditary colorectal cancer[4] and affects multiple generations with carcinomas at an early age.

Syndromes that possess distinguishing phenotypes are more simple to diagnose than hereditary disorders that lack clear phenotypic characteristics. Where HNPCC is concerned, there are 5 cardinal features that help in the identification of affected families. Primarily, there is the earlier than average age of cancer

onset compared to the general population ie. the average of onset of hereditary nonpolyposis colorectal cancer is 45 years[5] compared to 63 years in the general population. Secondly, there is a specific spectrum of primary cancers segregating within the pedigree, such as colonic and endometrial cancers[5,6]. There is also an excess of synchronous colorectal cancer (multiple colorectal cancers at or within six months after surgical resection for colorectal cancer) and metachronous colorectal cancer (colorectal cancer occurring more than six months after surgery)[5]. Furthermore, there is an excess of extracolonic manifestations eg. carcinoma of the ovary (second only to colorectal cancer in frequency), ovary, stomach (especially in Asian countries such as Japan and Korea)[7], small bowel, pancreas, hepatobiliary tract, brain and upper uroepithelial tract[6,8]. Thirdly is the survival rate that differs from the norm for the particular cancer[9-12].Forthly is distinguishing pathological features[13] and finally, there is the identification of a germline mutation in affected members of the family[4]. As far as the colorectal tumours are concerned, those stemming from patients with HNPCC are more commonly proximally located (approximately 70% are proximal to the splenic flexure), more likely to have diploid DNA, possess microsatellite instability, harbour mutations in the mismatch repair genes, and behave less aggressively than other carcinomas[5]. They also appear more often to be poorly differentiated, with an excess of mucoid and signet-cell features and possess infiltrating lymphocytes within the tumour[14-17]. Patients with HNPCC may also present sebaceous adenomas, sebaceous carcinomas, and multiple keratocanthomas[5,18].

Since microsatellite instability (MSI) is established in almost all hereditary nonpolyposis colorectal carcinomas[19] it is perhaps unnecessary to investigate for mismatch repair (MMR) gene germline mutations (ie, *hMSH2* and *hMLH1*) in patients whose tumours do not display MSI. However, the exception to this rule may be in families with *hMSH6* mutations, in which MSI may or may not be present[20,21].

Germline mutations in MMR genes have previously been established in between 40 and 80 percent of the families fulfilling the Amsterdam Criteria I and between 5 and 50 percent of families fulfilling the Amsterdam Criteria II[22,23]. However, some investigated families, despite a family history indicative of HNPCC, are established as mismatch repair gene mutation negative and appear not to harbour a MMR gene alteration. This phenomenon may correlate to as yet undiscovered gene mutations being responsible for the presence of the syndrome or that the aggregation of cancers may be attributed to environmental factors or be due to chance[14].

Estimates made to date describing the frequency of HNPCC occurrence, are more than likely low. The majority of mutational studies have not included the investigation for *hMSH6* mutations, which undoubtedly account for a proportion of HNPCC cases or predispose to an atypical and more benign form of this syndrome[20]. In addition, some of the more conventional techniques for mutation detection cannot highlight mutations that are only obvious when the two alleles are studied separately[24] eg, mainly mutations in control regions or introns that affect transcription or splicing[25]. Furthermore, large deletions in the *hMSH2* gene are more common than previously thought and can be detected through Southern hybridisation[26] or multiplex PCR of short fluorescent fragments[27].

Due to the low detection of HNPCC individuals and MMR gene alterations, it would be useful to further characterise the phenotype of MMR gene mutation carriers in order to help in the identification of affected individuals and hence, quickly organise the necessary treatment, surveillance and genetic counselling required.

Patients and Methods

This study investigated 130 unrelated Swiss patients who were referred to the Medical Genetics department by the consulting physician due to an observed

familial clustering of colorectal cancer (CRC) or young age at diagnosis of CRC. Informed consent was obtained from all individuals studied and following assessment of a detailed personal and familial history, patients were assigned to one of the following referral criteria groups: the Amsterdam Criteria I (ACI) which are defined as follows; 1) three or more relatives with histologically verified CRC one of whom is a first-degree relative of the other two; 2) CRC involving at least two generations; 3) one or more CRC cases diagnosed before the age of 50 years; and 4) familial adenomatous polyposis must be excluded (*14*). The Amsterdam Criteria II (ACII) differs from the ACI only in that they encompass a defined spectrum of additional HNPCC associated cancers (cancer of the endometrium, small bowel, ureter or renal pelvis) (*12*).

The Bethesda Guidelines (BG) are fulfilled if any of the following criteria are met: 1) individuals with cancer in families that fulfill the Amsterdam Criteria; 2) individuals with at least 2 HNPCC related cancers, including synchronous and metachronous CRC (endometrial, ovarian, gastric, hepatobiliary or small-bowel cancer or transitional cell carcinoma of the renal pelvis or ureter); 3) individuals with CRC and a first degree relative with CRC and/or HNPCC related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age <45 years, and the adenoma diagnosed at <40 years; 4) individuals with CRC or endometrial cancer diagnosed at <45 years; 5) individuals with right-sided CRC with an undifferentiated pattern on histopathology diagnosed at age <45 years; 6) individuals with signet-ring CRC diagnosed at <45 years; 7) individuals with adenomas diagnosed at age <40 years (*15*). In order to clearly distinguish the referral groups and avoid double classification of patients, only patients that did not fulfill the ACI or ACII but otherwise complied with the BG were included in the so-called restricted Bethesda Group (rBG). Patients fulfilling neither the AC nor the rBG constituted the Neither Criteria (NC) group.

All patients were investigated as anonymous cases and the results of the various analyses were assessed by at least two reviewers independently.

DNA Extraction

Genomic DNA was isolated from EDTA blood using the methods previously described by Miller (*16*). In short, 10ml blood were mixed with 30ml EL buffer (55mM NH_4Cl, 10mM $KHCO_3$, 1mM EDTA, pH7.4) and left on ice for 15 minutes. The lysate was centrifuged at 2000rpm for 10 minutes and washed twice with EL buffer. The resulting pellet of intact lymphocytes was resuspended in NL buffer (10mM Tris.HCl, pH8.2, 400mM NaCl, 2mM Na_2EDTA, 1% SDS and 200μg/ml protein K) and subsequently incubated at 37°C overnight. The following day, 1ml of 6M NaCl was added, the mix was vigorously shaken and then centrifuged in order to remove cellular proteins. The supernatant containing DNA was transferred to a fresh tube and the DNA precipitated with ethanol. The final DNA pellet was washed with 70% ethanol, dried briefly and resuspended in 1ml of TE buffer (10mM Tris.HCl, pH 7.5, 0.7m EDTA).

Tumor DNA was isolated from formalin fixed, paraffin embedded tissue using the QIAMP DNeasy Tissue kit and according to the suggested protocol of the manufacturer (Qiagen, Switzerland). After verification of the tumor cell content (>70%) of HE stained tumor specimen, 10x 5-8μm thick tumor sections were cut from each paraffin block. Lysis of the tissue was completed overnight with Qiagen buffer, Proteinase K and an incubation temperature of 55°C. The samples were then washed twice with Qiagen wash buffer and the DNA finally eluted in 200 μl elution buffer provided.

MSI Analysis

For MSI analysis, matched normal (ie. leukocyte-extracted) and tumor DNA were investigated using a panel of 14 microsatellite markers in two stages. Initial screening consisted of microsatellite markers BAT 25, BAT 26, D10S197, D18S58, D2S123, D5S346 and MFD15. In cases where none or only one of the markers was unstable anadditional set of markers were employed to detect low degree instability: BAT 40, D18S69, D19S210, D22S257, D3S1265, D4S243,

and MYCL1. PCR amplifications were performed with approximately 100ng of genomic DNA and 200ng of tumor DNA, in a total volume of 50μl, using a Hybaid Omn-E Thermocycler (Catalys AG, Wallisellen, CH); 94°C-3mins for 1 cycle, 94°C-20 secs, 56°C-30 secs, and 72°C-45secs for 35 cycles, and 72°C-5mins for 1 cycle. Subsequently, PCR products were loaded onto an ABI PRISM 310 Genetic Analyser using the POP4 polymer (PE Applied Biosystems, USA), a HEX, TET, FAM and TAMRA matrix, and the GENESCAN software for analysis. Although experiments were repeated several times, PCR amplification was not possible in 9 tumor specimens. These patients were therefore omitted from further study. In addition, another 2 patients, with an MS-Stable status, were eliminated from the study since their tumor content was below 70% and thus too low for reliable assessment of MSI status. MSI was allocated with respect to the number of microsatellite markers displaying allelic expansions or contractions. Assessment was based on the recommendations of the NCI workshop on microsatellite instability (*17*): >30% of the investigated loci unstable were classified as being MSI-High (MSI-H), >0% and <30% unstable loci MSI-Low (MSI-L) and no unstable microsatellite loci defined MSI-Stable (MSS). Loss of heterozygosity (LOH) was defined as a $\geq$50% reduction in relative intensity of one allele compared to the other.

IHC

Four micrometer serial sections from paraffin blocks were mounted on silanized slides, deparaffinized and rehydrated. Antigen retrieval was obtained by heating the sections in a pressure cooker at 120°C for 2 min in 10mM citrated-buffered solution (pH 6.0). DAKO peroxidase blocking reagent and goat serum were sequencially used to suppress nonspecific staining due to endogenous peroxidase activity and nonspecific binding of antibodies, respectively. Incubations with primary monoclonal antibodies were performed as follows: anti-hMSH2: 24 hours at 4C with Ab NA26 (Oncogene Research), 1μg/ml; anti-hMSH6: 2 hours at RT with Ab G70220 (Transduction Laboratories), 4μg/ml; anti-

hMLH1: 1 hour at RT with Ab 13271A (PharMingen), 1.2 µg/ml; anti-hPMS2: 24 hours at 4C with Ab 65861A (PharMingen), 3 µg/ml. After washing, anti-mouse secondary antibodies conjugated to peroxidase labelled polymer (DAKO EnVision+kit) were applied for 30 min at RT, and the peroxidase activity was developed by incubation with 3,3,diaminobenzidine (DAB) chromogen solution (DAKO). Sections were then counterstained slightly with hematoxylin.

Sequence Analysis

Exon specific primer pairs (sequences as reported by Kolodner (*19,20*) were used to amplify the 16 exons of *hMSH2* and the 19 exons of *hMLH1,* including the respective exon-intron boundaries, from genomic DNA. Fifty microlitres of PCR reaction mixture contained 100ng of genomic DNA, 0.5μM each primer, 2.5μM each dNTP, 5mM $MgCl_2$, 10x reaction buffer, and 0.2U Taq polymerase (Qiagen, Switzerland). The reaction parameters were; 94°C-3 mins for 1 cycle, 94°C-30 secs, 53°C-30 secs and 72°C-45 secs for 35 cycles, and 72°C-3 mins for 1 cycle, for a Hybaid OmnE Thermocycler (Catalys AG, Wallisellen, CH). The sequencing reaction was completed using the Thermosequenase Sequencing Kit (Amersham Pharmacia, Switzerland). PCR amplicons were diluted 1:3 and enzymatically purified with shrimp alkaline phosphatase and exonuclease I (Amersham Pharmacia, Switzerland). The purified PCR products were run through a cycle sequencing reaction with primers labeled with an infrared dye; T7-IRD800 and SP6-IRD-800 for forward and reverse sequencing, respectively. Cycle sequencing parameters were 95°C-3min. for 1 cycle, 95°C-30 sec. 55°C-30 sec. and 72°C-1min. for 30 cycles. The resulting products were loaded onto a 6% denaturing polyacrylamide gel and analysed on a LiCor 4000L automated DNA Sequencer (LiCor, Lincoln, NE).

Multiplex PCR of short fluorescent fragments

Short exon fragments corresponding to the 19 *hMLH1* exons and the 16 *hMSH2* exons were amplified via PCR from approximately 50 ng of genomic DNA, using

6-FAM labeled primers (Charbonnier, F., 2000). Exons 1-10 and exons 10-19 of *hMLH1* and exons 2, 3, 5, 8-10, 12, 14 and 15 and exons 1, 4, 6, 7, 8, 11, 13 and 16 of *hMSH2* were PCR amplified in four separate tubes. PCR was performed in a final volume of 50μl containing between 0.2 and 1μM of each pair of primers and 1 unit of Taq DNA polymerase (Invitrogen, Switzerland). After a 3 min denaturation at 95^0C, the PCR consisted of (a) nine cycles of 10 secs at 94^0C, 10 secs at 60^0C (with a decrease of 1^0C/cycle), and 10 secs at 72^0C; (b) 12 cycles of 10 secs at 94^0C, 10 secs at 48^0C, and 10 secs at 72^0C; and (c) a final 7 min extension at 72^0C. Subsequently, PCR products were loaded onto an ABI PRISM 310 Genetic Analyser using the POP4 polymer (PE Applied Biosystems, USA), a HEX, TET, FAM and TAMRA matrix, and the GENESCAN software for analysis.

Statistical Analysis

Statistical comparison of patients' features, encompassing referral criteria, phenotypic characteristics (sex, age at diagnosis of CRC, tumor location, extracolonic cancers and degree of differentiation), MSI and mutational status, was performed using the Chi-square and Fisher exact test for categorical variables, or Student's t-test for continuous variables, with all of the probabilities reported as two-tailed Ps, considering a P of <0.05 to be statistically significant. To assess the value of the referral criteria and the screening methods employed calculations for sensitivity, specificity, false positive and negative rates as well as diagnostic accuracy were performed according to Jaeschke, Guyati and Sackett (*21*).

Results

This study aimed to further characterise the phenotype of HNPCC patients by comparing MMR gene mutation carriers to gene alteration negative individuals in an attempt to aid the identification of HNPCC individuals and MMR gene mutation carriers. In total, 130 Swiss HNPCC individuals were investigated. All

were screened for mutations via microsatellite (MSI) analysis, immunohistochemistry (IHC) and for those subsequently displaying a MMR protein loss, direct DNA sequencing was performed. Forty-six (35%) individuals has a confirmed MMR gene mutation (Table 1), the remaining 84 (65%) individuals were classified as MMR mutation negative.

Of the mutation positive patients, 54% (n=25) were found to harbour a mutation in the MMR gene *hMLH1*, whilst 41% (n=19) had mutations in *hMSH2* established. The most common overall gene alteration was a frameshift mutation (1bp – 3 exons long deleted), making up 41% (n=22) of the total observed. Other mutations found were base substitutions, 27% (n=12) and splice site mutations, 23% (n=10). Of the *hMLH1* mutations, frameshifts were the most common, accounting for 48% (n=12) of the total, followed by base substitutions (28%, n=7) and splice site mutations (24%, n=6). From the *hMSH2* mutations, frameshifts were observed in 53% (n=10), base substitutions in 26% (n=5) and splice site mutations in 21% (n=4).

GENE/ EXON	DNA CHANGE	AMINO ACID CHANGE	REFERRAL CRITERIA	FAMILY NUMBER
hMLH 1				
2	1995 G>A	G67R	AC	1652
2	184 C>T	Q61X	rBG	1900
3	292 G>C	G98R	AC	2047
4	341 C>G	T117R	AC	434

4	1050 C>T	T350M	AC	1936
5	IVS4-2 A>G	splice acceptor site	AC	1500
7-9	Exons 7-9 del	frameshift	AC	1806
10	811-815delTCCTT	frameshift	AC	1805
10	IVS9-4/791-5delTTAGATCGT	frameshift	AC	1834
13	1490 insC	frameshift	AC	1754
13	1490 insC	frameshift	rBG	1902
13	1490 insC	frameshift	NC	1906
13	1410-1413 Del AAAG	frameshift	BG	1917
15	1731 G>A	splice donor site	AC	1801
15	1690-1693delCTCA	frameshift	rBG	1808
16	1896 Del G	frameshift	AC	1033
16	1946-1848 Del AAG	frameshift	AC	2151
16	1946-1848 Del AAG	frameshift	AC	1848
16	1846-1848delAAG	frameshift	rBG	1760

16	1852 A>T	K618X	BG	1956
16	1896+1 G>T	splice donor site	AC	2048
17	1976 G>C	A658P	AC	1921
18	IVS18+1 G>T	splice donor site	AC	1813
19	IVS18-2A>T	splice acceptor site	AC	1121
19	IVS18+1 G>T	splice donor site	AC	1831
hMSH 2				
2	261-262delTT	frameshift	rBG	1820
3	388-389 Del TC	frameshift	AC	1097
5	942+3 A>T	splice donor site	BG	2170
5	942+3 A>T	splice donor site	BG	1893
7	1148 C>T	R383X	AC	1587
7	1165 C>T	STOP	AC	2025
7-8	Exons 7-8 del	frameshift	AC	1817
7-8	Exons 7-8 del	frameshift	AC	1835
10	1576 Del A	frameshift	AC	1846
10	1576 delA	frameshift	NC	BItalia1
11	1740 G>T	E580X	AC	1642

11	IVS11+2 T>C	splice donor site	AC	1807
12	1787-1789 AAT Del	frameshift	AC	1383
12	1853delC	frameshift	rBG	1886
12	1760-1 G>A	splice acceptor site	AC	1989
14	2261delC	frameshift	AC	1827
15	2503 A>G	N835D	AC	1991
16	2740 G>T	E914X	AC	1841
16	2646 del A	frameshift	NC	BItalia9

Table 1: Mismatch repair gene mutation carriers and the confirmed mutations to date.

No statistically significant phenotypic differences were observed between mutation positive patients harbouring *hMLH1* mutations and those patients carrying *hMSH2* mutations. This enabled all subsequent phenotypic comparisons between mutation positive and mutation negative individuals to be done directly, regardless of the MMR gene affected.

Ninety-four percent (n=43) of the mutation positive patients were classified by either the Amsterdam Criteria (AC) or the restricted Bethesda Guidelines (rBG) (32 AC and 11 rBG, Table 2). In comparison a smaller proportion, 76% (n=54), of the mutation negative individuals fulfilled the referral criteria (27 AC and 44 rBG). Interestingly, whilst 70% of the mutation positive patients satisfied the AC, only 32% of the mutation negative individuals were classified by these criteria. More

mutation negative patients fulfilled the rBG as compared to the mutation positive patients, 44% vs 24%, respectively ($p<0.0005$) (Figure 1).

		Total	Mutation Positive Individuals	Mutation Negative Individuals
REFERRAL CRITERIA:	Amsterdam Criteria (I/II)	59	32	27
	rBethesda Guidelines	48	11	37
	Neither Criteria	23	3	20
AGE AT DIAGNOSIS:	Average (yrs) SD (yrs) Range (yrs)		45.5 11.94 27-76	48.1 13.48 22-90
SEX:	Male	52	25	27
	Female	21	41	57
TUMOUR LOCATION :	Proximal	46	28	18
	Distal	84	18	66
ADDITIONAL CANCERS:	Syn-/ meta-chronous CRC	11	8	3
	Extracolonic Cancer	16	9	7
TOTAL:		130	46	84

Table 2: Overall phenotypic characteristics of all patients investigated and divided according to mutational status.

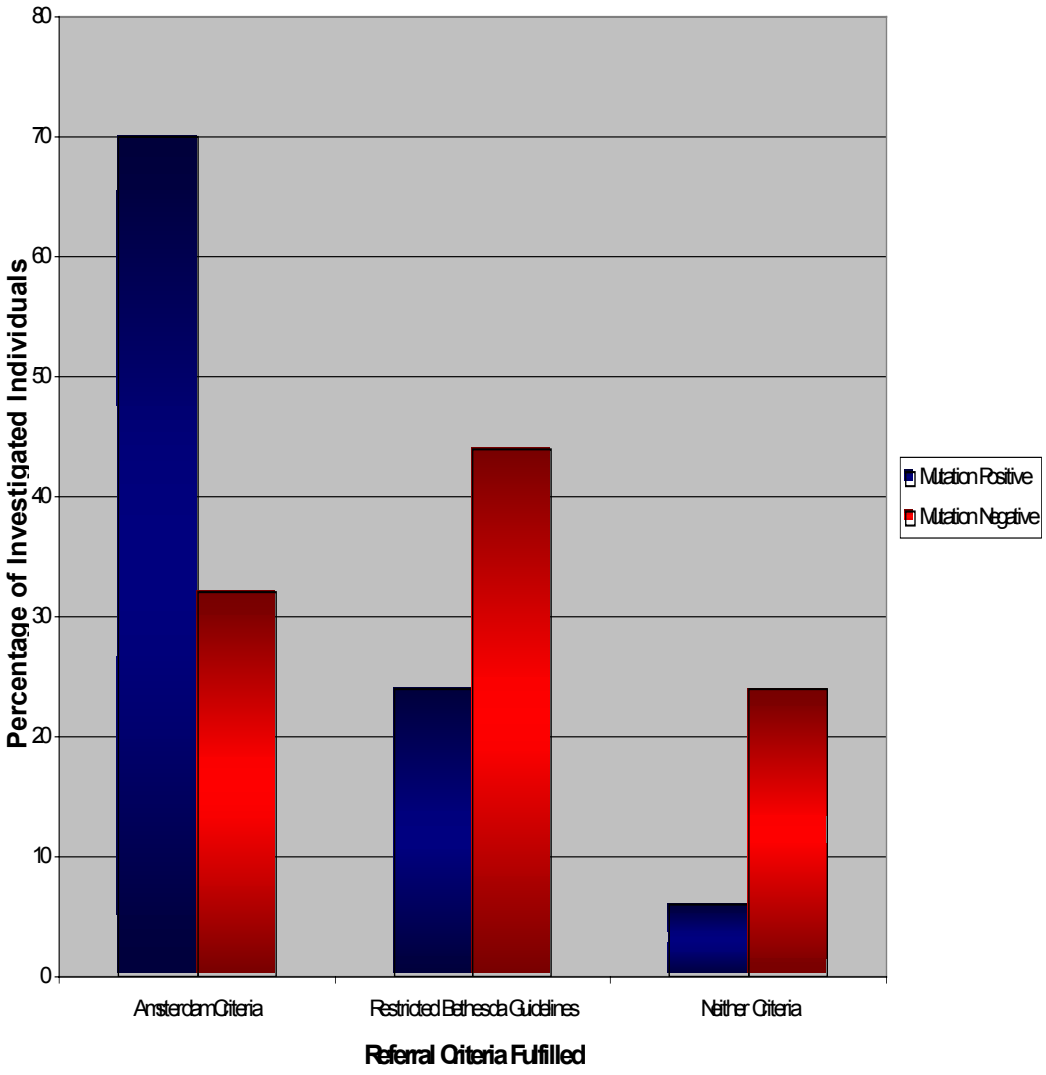

Figure 1: The 46 mutation positive and the 84 mutation negative individuals divided according to the referral criteria they fulfil.

In the 30-39 and the 40-49 age groups, mutation positive patients dominated, whilst in all of the other age categories, it is the mutation negative individuals that make up the greater proportion (Figure 2). The oldest mutation positive patient at

76 years, is 14 years younger than the oldest mutation negative patient at 90 years.

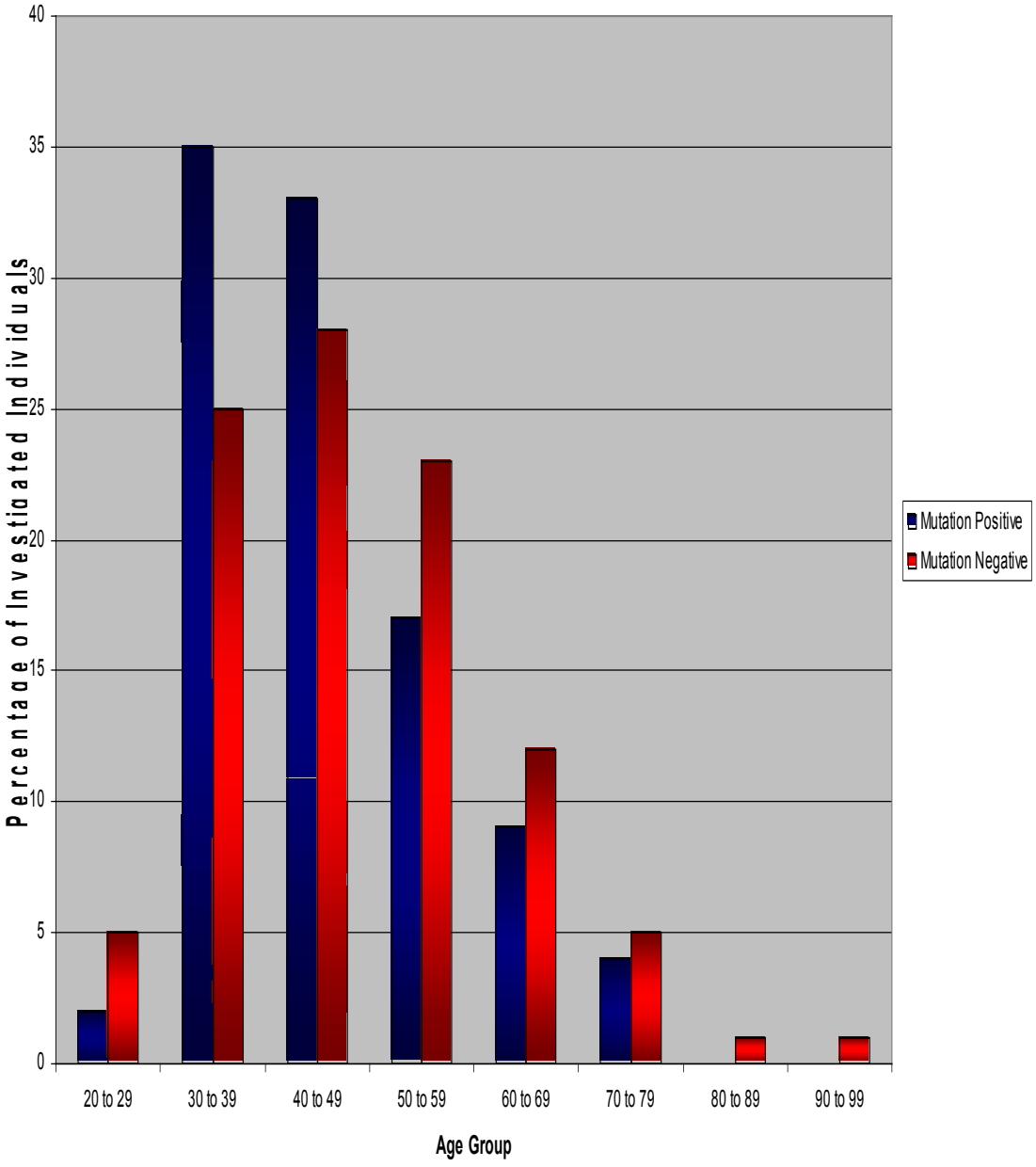

Figure 2: The age categories that the mutation positive and mutation negative patients fall into.

The average age at diagnosis of a colorectal cancer in mutation positive patients it 45 years (SD ± 11.94, range 27-76). This is on average 3 years younger than the mutation negative individuals who have an average age at diagnosis of 48 years (SD ± 13.48, range 22-90).

The overall sex ratio of the 130 investigated patients was 40%:60% (males:females). Whilst the mutation positive patients were evenly split by males:females, 54% to 46%, the mutation negative group was not so evenly divided, 32% to 78%. Females were significantly more frequently found to be mutation negative than mutation positive (73% vs 27%, respectively, $p<0.0005$, Table 2).

Another statistically significant finding was related to the location of the CRC. Sixty-three percent of the mutation positive patients had CRCs located in the proximal region of the colon compared to only 21% proximally located carcinomas in the mutation negative individuals ($p<0.0001$, Figure 3). Furthermore, almost half (45%) of the CRCs stemming from mutation negative patients were found located in the sigma ($p<0.0001$).

Extracolonic manifestations (ECMs) were observed in 20% of the mutation positive patients compared to only 8% of the mutation negative individuals ($p<0.03$). Of the ECMs observed, endometrium carcinomas were the overall most commonly occurring, with 38% of those patients having an ECM, possessing it in the endometrium. Furthermore, endometrium carcinomas were most frequently observed in the mutation positive patients with 67% of the ECM affected mutation positive patients having an endometrium carcinoma. Other ECMs observed in mutation positive patients were carcinomas of the skin (11%), ovaries (11%) and the adrenal glands (11%). Mutation negative individuals had ECMs of the stomach (30%), ovaries (14%), liver (14%), oesophagus (14%), breast (14%) and of the renal cells (14%) (Table 2).

In addition, mutation positive patients had a higher prevalence of synchronous and metachronous CRCs compared to mutation negative individuals, 17% vs 4%, respectively ($p<0.05$, Table 2).

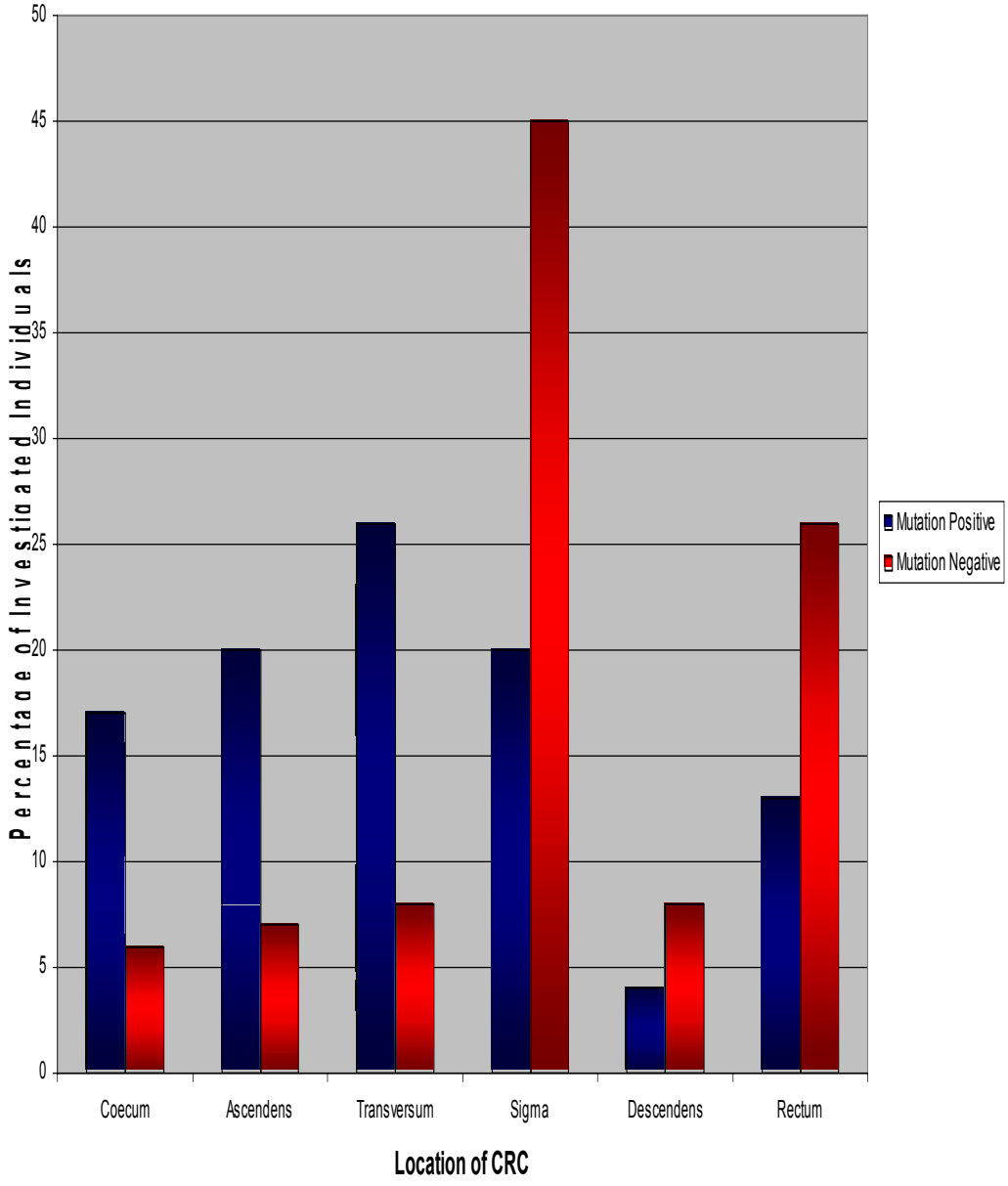

Figure 3: Location of colorectal cancers according to mutation positive and mutation negative status.

Discussion

HNPCC predisposition is correlated with germline mutations in five genes functional in mismatch repair (MMR); *MSH2, MLH1, MSH6, PMS1* and *PMS2.* To date, more than 300 different mutations in these genes (the majority in *MSH2* and *MLH1*) have been identified and are known to account for HNPCC in approximately 500 kindreds from various countries (28 and http://www.nfdht.nl). Although a great number of predisposing mutations have been found, the connections between genotype and phenotype are insufficiently understood. No statistically significant phenotypic differences were observed in this study between mutation positive patients harbouring *hMLH1* mutations and those patients carrying *hMSH2* mutations. However, previous studies have tentatively made some associations, for example, Vasen et al.[29] determined an increase risk of extracolonic manifestations in carriers of *MSH2*, compared to *MLH1*, mutations and Kruse et al.[30] found that *MSH2* mutations were significantly more frequently found than *MLH1* mutations in connection with the Muir-Torre variants of HNPCC. Furthermore, Jager et al.[31] found that one common mutation in *MLH1*, exon 4, dictated a milder phenotype, especially with less frequently observed extracolonic cancers. The severity vs mildness of the disease may in part be due to the ability[32] vs inability[31] of the defective proteins to produce a dominant negative effect on the overall action of the mismatch repair system. The majority of information available concerning the HNPCC phenotypes comes from retrospective studies on families already identified as HNPCC due to a observed family history of HNPCC associated cancers. These data suggest that the phenotype in HNPCC varies from individual to individual[33].

The age at diagnosis of colorectal cancer in mutation positive patients was 45 years, with a peak in the 4th decade. Mutation negative patients were on average 3 years older than mutation positive patients at the time of their diagnosis, and although also having a peak incidence occurring in the 4th decade, the mutation negative patients were more frequently observed in the 5th to the 7th, and only in the 8th and 9th, decades. The peak incidence occurring in the 4th decade differed

significantly ($p<0.001$) from the Swiss population (Swiss Cancer Registries' Association database, 2003) where the incidence peaks in the 7th and 8th decades. All studies previously conducted indicate that CRC risk is at its peak between the ages of 40 and 60, but that the risk before the age of 40 is considerable, and perhaps to begin colorectal screening at the age of 25 is justifiable[34].

Interestingly, females were significantly more frequently found to be mutation negative than mutation positive ($p<0.0005$). However, no other phenotypic characteristics appeared to separate the males from the females in both the mutation positive and mutation negative groups. Data cleaved to date show that male mutation carriers have a lifetime colorectal cancer risk of 74% or more, but whilst female mutation carriers have half this risk, it is still many times higher than in the general population and still warrants the same intensive screening as that suggested for males[33].

Sixty-three percent of the mutation positive patients had CRCs located in the proximal region of the colon compared to only 21% proximally located CRCs in the mutation negative individuals. These observations are consistent with those made by Lynch et al.[35] where 70% of the CRCs in HNPCC patients occurred proximal to the splenic flexure.

The frequency of extracolonic manifestations were similar to reports previously made[36,37], with endometrial cancer representing 38% of the extracolonic cancers observed. Lifetime endometrial cancer risk is 42% or more, with some evidence that risk is elevated in *MSH2*- compared to the *MLH1*-mutation carriers and with highest incidence between age 40 and 60[33]. Other ECMs observed were cancers of the stomach, ovary, breast, liver, oesophagus, skin, adrenal gland and renal cell cancer. There is much evidence that MMR mutation carriers are at elevated risk from ovarian, gastric, urologic tract, small bowel and hepatobiliary tract and for brain tumours[36,38]. For a number of ECM types it has been suggested that

significant inter-family variation in risk exists[31,36,29] and for some, intracultural and secular variations in cancer risk are observed[39].

As expected, in agreement with previous documentation, mutation positive patients had a significantly higher prevalence of synchronous and metachronous CRCs compared to mutation negative individuals ($p<0.05$)[11].

Such a study of genotype-phenotype correlations, involving one population, of Swiss individuals, has its plus and minus points. Variations possibly introduced via cultural and environmental differences[39] are minimised, although at the same time, only a limited proportion of all known mutations occurring in the disorder is represented. However, here we can conclude that the use of phenotypic features such as age of CRC diagnosis, CRC location, the presence of ECMs and the occurrence of syn-/metachronous CRCs, can be used to make a distinction between mutation positive and mutation negative individuals and hence aid clinicians in the prioritisation of patients for mismatch repair gene mutation screening and genetic counselling.

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Chapter IV

Evaluation of referral criteria and screening procedures in the identification of HNPCC patients

A draft of a scientific paper prepared for publication.

Abstract

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited cancer predisposition syndrome essentially caused by germline mutations in DNA mismatch repair (MMR) genes. This study aimed to (i) assess the phenotypic and molecular differences between patients belonging to different referral criteria groups, and (ii) determine the diagnostic accuracy of the criteria and screening procedures employed in identifying individuals with mismatch repair (MMR) gene alterations. A consecutive series of 222 unrelated Swiss patients, referred because of clinically suspected HNPCC, were assigned to either the Amsterdam Criteria I/II (AC), the restricted (ie. minus ACI/II) Bethesda Guidelines (rBG) or the Neither Criteria (NC) group. Individuals were screened for MMR gene alterations applying microsatellite marker analysis, immunohistochemistry, direct DNA sequencing and RT-PCR/protein truncation test. Statistical evaluation involved the Chi-square, Fischer exact or Student's t-test, with all probabilities reported as two-tailed Ps, considering a P of <0.05 to be statistically significant. Of the referred patients, 37% fulfilled the AC, 34% the rBG and 29% the NC group. MSI analysis established the stability status of 95% of the tumours; 41% MSI and 59% MSS. Fifty-seven percent of the MSI tumours stemmed from the AC, 24% from the rBG and 18% from the NC group ($p<0.01$). Eighty-three percent of the unstable tumors had gene alterations whilst in comparison 95% of the stable tumors were gene alteration negative ($p<0.01$). Gene alteration positive tumors were more frequently unstable than gene

alteration negative tumors ($p<0.001$), and were more often proximally located ($p<0.02$). Of the screening methods employed, immunohistochemistry proved to be the most sensitive and specific of all screening procedures with sensitivity and specificity values equal to 1 for both *hMLH1* and *hMSH2* gene alterations. The BG were of superior sensitivity and diagnostic accuracy compared to AC I/II alone, in identifying patients with MMR gene alterations. Notably, individuals belonging to the NC group displayed a later age at diagnosis of colorectal cancer, although still occurring significantly earlier than in the general population. In addition their tumors were predominantly MSS, pointing to a genetic predisposition unrelated to mismatch repair deficiency. Based on the evaluation of the different screening techniques employed in this study, the following diagnostic approach should allow optimal identification of individuals with MMR gene alterations: (1) Testing for MSI combined with immunohistochemical loss of MMR proteins as initial screening methods and (2) subsequent mutational analysis of the positively scored individuals encompassing both a DNA and a mRNA-based technique.

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited cancer predisposition syndrome which leads mainly to the development of colorectal carcinoma (CRC) at an average age of 45 years (*1-3*). It is believed to account for 2 to 5% of the total CRC burden (*4*). Colorectal tumors from HNPCC patients are predominantly located proximal to the splenic flexure, often occur syn- and metachronously, and patients display enhanced survival from CRC in comparison to matched controls (*5-7*). In addition, a defined spectrum of extracolonic tumors is associated with the disease, primarily endometrial carcinoma (*8*).

In 60-70% of HNPCC kindreds the disease is caused by germline mutations in one of the DNA mismatch repair (MMR) genes, *hMSH2, hMLH1, hMSH6, hPMS1*

or *hPMS2* which function as “guardians of the genome.” Inactivation of the MMR system through mutation of one of its components consequently leads to genomic instability, as evidenced by microsatellite instability (MSI). MSI can be observed in 75 to almost 100% of CRCs stemming from HNPCC patients (*9*). Approximately 11-38% of sporadic CRC also display genomic instability in conjunction with a somatic mismatch repair (MMR) defect, mainly due to promoter hypermethylation of *hMLH1* (*10*).

Originally drawn up to identify the genes responsible for HNPCC, and exclusively focusing on a family history of CRC, the so-called Amsterdam criteria (ACI) were recently extended to also include endometrial, small bowel and upper renal tract cancers (Amsterdam criteria II, ACII) (*11,12*). In parallel, the Bethesda guidelines (BG) were set up to define which colorectal tumors should be tested for MSI in order to identify HNPCC patients in the general population (*13*).

Only limited data are available however, which assess the value of the different referral criteria in combination with MSI testing and the various mutation screening methods applied. By studying a consecutive series of 222 unrelated patients, referred to the Medical Genetics department because of clinically suspected HNPCC, this survey aimed to (i) assess the phenotypic and molecular differences between patients belonging to different referral criteria groups, and (ii) determine the diagnostic accuracy of the criteria and screening procedures employed in identifying individuals with mismatch repair (MMR) gene alterations.

Patients and Methods

This study investigated 98 unrelated Swiss patients who were referred to the Medical Genetics department by the consulting physician due to an observed familial clustering of colorectal cancer (CRC) or young age at diagnosis of CRC. Informed consent was obtained from all individuals studied and following assessment of a detailed personal and familial history, patients were assigned to

one of the following referral criteria groups: the Amsterdam Criteria I (ACI) which are defined as follows; 1) three or more relatives with histologically verified CRC one of whom is a first-degree relative of the other two; 2) CRC involving at least two generations; 3) one or more CRC cases diagnosed before the age of 50 years; and 4) familial adenomatous polyposis must be excluded (*14*). The Amsterdam Criteria II (ACII) differ from the ACI only in that they encompass a defined spectrum of additional HNPCC associated cancers (cancer of the endometrium, small bowel, ureter or renal pelvis) (*12*).

The Bethesda Guidelines (BG) are fulfilled if any of the following criteria are met: 1) individuals with cancer in families that fulfill the Amsterdam Criteria; 2) individuals with at least 2 HNPCC related cancers, including synchronous and metachronous CRC (endometrial, ovarian, gastric, hepatobiliary or small-bowel cancer or transitional cell carcinoma of the renal pelvis or ureter); 3) individuals with CRC and a first degree relative with CRC and/or HNPCC related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age <45 years, and the adenoma diagnosed at <40 years; 4) individuals with CRC or endometrial cancer diagnosed at <45 years; 5) individuals with right-sided CRC with an undifferentiated pattern on histopathology diagnosed at age <45 years; 6) individuals with signet-ring CRC diagnosed at <45 years; 7) individuals with adenomas diagnosed at age <40 years (*15*). In order to clearly distinguish the referral groups and avoid double classification of patients, only patients that did not fulfill the ACI or ACII but otherwise complied with the BG were included in the so-called restricted Bethesda Group (rBG). Patients fulfilling neither the AC nor the rBG constituted the Neither Criteria (NC) group.

All patients were investigated as anonymous cases and the results of the various analyses were assessed by at least two reviewers independently.

DNA Extraction

Genomic DNA was isolated from EDTA blood using the methods previously described by Miller (*16*). In short, 10ml blood were mixed with 30ml EL buffer (55mM NH_4Cl, 10mM $KHCO_3$, 1mM EDTA, pH7.4) and left on ice for 15 minutes. The lysate was centrifuged at 2000rpm for 10 minutes and washed twice with EL buffer. The resulting pellet of intact lymphocytes was resuspended in NL buffer (10mM Tris.HCl, pH8.2, 400mM NaCl, 2mM Na_2EDTA, 1% SDS and 200μg/ml protein K) and subsequently incubated at 37°C overnight. The following day, 1ml of 6M NaCl was added, the mix was vigorously shaken and then centrifuged in order to remove cellular proteins. The supernatant containing DNA was transferred to a fresh tube and the DNA precipitated with ethanol. The final DNA pellet was washed with 70% ethanol, dried briefly and resuspended in 1ml of TE buffer (10mM Tris.HCl, pH 7.5, 0.7m EDTA).

Tumor DNA was isolated from formalin fixed, paraffin embedded tissue using the QIAMP DNeasy Tissue kit and according to the suggested protocol of the manufacturer (Qiagen, Switzerland). After verification of the tumor cell content (>70%) of HE stained tumor specimen, 10x 5-8μm thick tumor sections were cut from each paraffin block. Lysis of the tissue was completed overnight with Qiagen buffer, Proteinase K and an incubation temperature of 55°C. The samples were then washed twice with Qiagen wash buffer and the DNA finally eluted in 200 μl elution buffer provided.

RNA Extraction:

RNA was isolated from EDTA blood using a Qiagen RNeasy Mini Kit and the protocol supplied by the manufacturer (QIAGEN, Switzerland). A maximum of $1x10^7$ cells were disrupted in 350μl lysis buffer and homogenized, in order to shear genomic DNA and reduce viscosity of the lysate. 250μl 100% ethanol was added to the mixture before the sample was applied to the RNeasy spin column.

RNA was absorbed by the column membrane and contaminants were washed away with Qiagen buffers. Subsequently, total RNA was eluted from the column with 50μl RNase-free water.

MSI Analysis:

For MSI analysis, matched normal (ie. leukocyte-extracted) and tumor DNA were investigated using a panel of 14 microsatellite markers in two stages. Initial screening consisted of microsatellite markers BAT 25, BAT 26, D10S197, D18S58, D2S123, D5S346 and MFD15. In cases where none or only one of the markers was unstable an additional set of markers were employed to detect low degree instability: BAT 40, D18S69, D19S210, D22S257, D3S1265, D4S243, and MYCL1.

PCR amplifications were performed with approximately 100ng of genomic DNA and 200ng of tumor DNA, in a total volume of 50μl, using a Hybaid Omn-E Thermocycler (Catalys AG, Wallisellen, CH); 94°C-3mins for 1 cycle, 94°C-20 secs, 56°C-30 secs, and 72°C-45secs for 35 cycles, and 72°C-5mins for 1 cycle. Subsequently, PCR products were loaded onto an ABI PRISM 310 Genetic Analyser using the POP4 polymer (PE Applied Biosystems, USA), a HEX, TET, FAM and TAMRA matrix, and the GENESCAN software for analysis. Although experiments were repeated several times, PCR amplification was not possible in 9 tumor specimens. These patients were therefore omitted from further study. In addition, another 2 patients, with an MS-Stable status, were eliminated from the study since their tumor content was below 70% and thus too low for reliable assessment of MSI status. MSI was allocated with respect to the number of microsatellite markers displaying allelic expansions or contractions. Assessment was based on the recommendations of the NCI workshop on microsatellite instability (*17*): >30% of the investigated loci unstable were classified as being MSI-High (MSI-H), >0% and <30% unstable loci MSI-Low (MSI-L) and no unstable microsatellite loci defined MSI-Stable (MSS). Loss of heterozygosity

(LOH) was defined as a ≥50% reduction in relative intensity of one allele compared to the other.

Protein Truncation Test (PTT):

Three micrograms of total RNA were reverse transcribed into complementary DNA with 2μl of random primer, 1.5μl of Rnasin 10000U/μl, 10μl of 5x Buffer (1x buffer: 10mM/L Tris, 50mM/L KCl, and 0.2mg/ml BAS, pH 8.5), 5μl of 10mM dNTPs (Promega Corporation, WI, USA) and 3μl Reverse Transcriptase 600U/μl (Gibco, Maryland, USA). The procedure was completed by heating the samples for 2 hours at 37°C. Polymerase chain reaction (PCR) amplifications were performed in 50μl total volumes, containing the following: 100ng cDNA, 0.2U Taq (Gibco/PWO, Gibco USA/Boehringer Mannheim, USA), 2.5μM each dNTP, 5mM $MgCl_2$, 10x reaction buffer (1x buffer: 10mM/L Tris, 50mM/L KCl, and 0.2mg/ml BAS, pH 8.5) and 0.5μM each primer. PTT primer sequences for hMLH1 and hMSH2 were taken from Luce et al. (*18*) and used to amplify each gene into two overlapping segments of 1.2 and 1.3 kb, and 1.7 kb and 1.3 kb, respectively. The cycling conditions were as follows: 94°C-4 min. for 1 cycle, 94°C-45 secs, 55/56°C-1 min. (for hMSH2 and hMLH1, respectively), and 72°C-150 secs for 45 cycles, and 72°C-10 mins for 1 cycle on a Hybaid Omn-E Thermocycler (Catalys AG, Wallisellen, CH). The banding patterns of the PCR products were primarily assessed on a 1.4% agarose gel. Subsequently, the PTT was run by adding 4μl PCR Product to 6μl PTT Mix (200μl TNT T7 coupled Reticulocyte Lysate System, 8μl RNasin, 16μl TNT reaction buffer, 16μl ^{35}S-Methionine) and heating for 60 mins at 30°C. The reaction was stopped with 10μl of 1x sodium dodecyl sulfate (SDS) sample buffer. Subsequently, the products were loaded onto a 12% SDS-polyacrylamide gel and run for 110 mins at 35mA. The gels were then fixed (10% glacial acetic acid, 30% methanol) for one hour and dried for 45 minutes at 80°C before exposure on a Biomax film (Kodak, Rochester, NY).

IHC

Four micrometer serial sections from paraffin blocks were mounted on silanized slides, deparaffinized and rehydrated. Antigen retrieval was obtained by heating the sections in a pressure cooker at 120°C for 2 min in 10mM citrated-buffered solution (pH 6.0). DAKO peroxidase blocking reagent and goat serum were sequencially used to suppress nonspecific staining due to endogenous peroxidase activity and nonspecific binding of antibodies, respectively. Incubations with primary monoclonal antibodies were performed as follows: anti-hMSH2: 24 hours at 4C with Ab NA26 (Oncogene Research), 1µg/ml; anti-hMSH6: 2 hours at RT with Ab G70220 (Transduction Laboratories), 4µg/ml; anti-hMLH1: 1 hour at RT with Ab 13271A (PharMingen), 1.2 µg/ml; anti-hPMS2: 24 hours at 4C with Ab 65861A (PharMingen), 3 µg/ml. After washing, anti-mouse secondary antibodies conjugated to peroxidase labelled polymer (DAKO EnVision+kit) were applied for 30 min at RT, and the peroxidase activity was developed by incubation with 3,3,diaminobenzidine (DAB) chromogen solution (DAKO). Sections were then counterstained slightly with hematoxylin.

Sequence Analysis

Exon specific primer pairs (sequences as reported by Kolodner (*19,20*) were used to amplify the 16 exons of hMSH2 and the 19 exons of hMLH1, including the respective exon-intron boundaries, from genomic DNA. Fifty microlitres of PCR reaction mixture contained 100ng of genomic DNA, 0.5µM each primer, 2.5µM each dNTP, 5mM $MgCl_2$, 10x reaction buffer, and 0.2U Taq polymerase (Qiagen, Switzerland). The reaction parameters were; 94°C-3 mins for 1 cycle, 94°C-30 secs, 53°C-30 secs and 72°C-45 secs for 35 cycles, and 72°C-3 mins for 1 cycle, for a Hybaid OmnE Thermocycler (Catalys AG, Wallisellen, CH). The sequencing reaction was completed using the Thermosequenase Sequencing Kit (Amersham Pharmacia, Switzerland). PCR amplicons were diluted 1:3 and enzymatically purified with shrimp alkaline phosphatase and exonuclease I (Amersham Pharmacia, Switzerland). The purified PCR products were run

through a cycle sequencing reaction with primers labeled with an infrared dye; T7-IRD800 and SP6-IRD-800 for forward and reverse sequencing, respectively. Cycle sequencing parameters were 95°C-3min. for 1 cycle, 95°C-30 sec. 55°C-30 sec. and 72°C-1min. for 30 cycles. The resulting products were loaded onto a 6% denaturing polyacrylamide gel and analysed on a LiCor 4000L automated DNA Sequencer (LiCor, Lincoln, NE).

Statistical Analysis

Statistical comparison of patients' features, encompassing referral criteria, phenotypic characteristics (sex, age at diagnosis of CRC, tumor location, extracolonic cancers and degree of differentiation), MSI and mutational status, was performed using the Chi-square and Fisher exact test for categorical variables, or Student's t-test for continuous variables, with all of the probabilities reported as two-tailed Ps, considering a P of <0.05 to be statistically significant. To assess the value of the referral criteria and the screening methods employed calculations for sensitivity, specificity, false positive and negative rates as well as diagnostic accuracy were performed according to Jaeschke, Guyati and Sackett (*21*).

Results

Of the consecutive series of 222 patients, 82 (37%) fulfilled the Amsterdam Criteria I/II (ACI and ACII; 76 ACI and 6 ACII) and 76 (34%) complied with the restricted Bethesda Guidelines (rBG) (Table 1). Sixty-four (29%) of the patients studied fulfilled neither the AC nor the rBG due to exceeding the age limit (>50 and >45 years of age at diagnosis, respectively) and were hence assigned to the Neither Criteria group (NC).

Phenotypic Features

As depicted in Table 1, the male to female distribution of the 222 cases was relatively evenly balanced with 44% men and 56% women. If subdivided according to referral criteria however, a statistically significant difference was observed, with women being more prevalent in the rBG (42%) group compared to the AC (35%) and the NC (23%; $p<0.05$ and $p<0.01$, respectively). Consequently, all statistical comparisons were performed for each sex separately to account for possible gender bias; unless stated otherwise, the statistically significant findings were sex-independent.

		REFERRAL CRITERIA			
		Total	**Amsterdam Criteria**	**rBethesda Guidelines**	**Neither Criteria**
SEX:	Female	124	43	52	29
	Male	98	39	24	35
TUMOUR LOCATION:	Proximal	121	40	48	33
	Distal	81	36	26	19
	Not Colon Cancer	20	6	2	12
ADDITIONAL CANCERS:	Syn-/ meta-chronous CRC	19	9	7	3
	Extracolonic Cancer	27	13	9	5
	none	176	60	60	56
TOTAL:		222	82	76	64

Table 1. Phenotypical features of a consecutive series of 222 patients suspected of having HNPCC, and subdivided according to referral criteria.

The average age at diagnosis differed significantly ($p<0.01$) between all 3 referral criteria groups, being 47 years (±11.6 SD; range 25-79, median 45) for AC, 38 years (±9.2 SD; range 19-68, median 38) for rBG and 59 years (±12.4 SD; range 31-90, median 57) for NC patients (Figure 1).

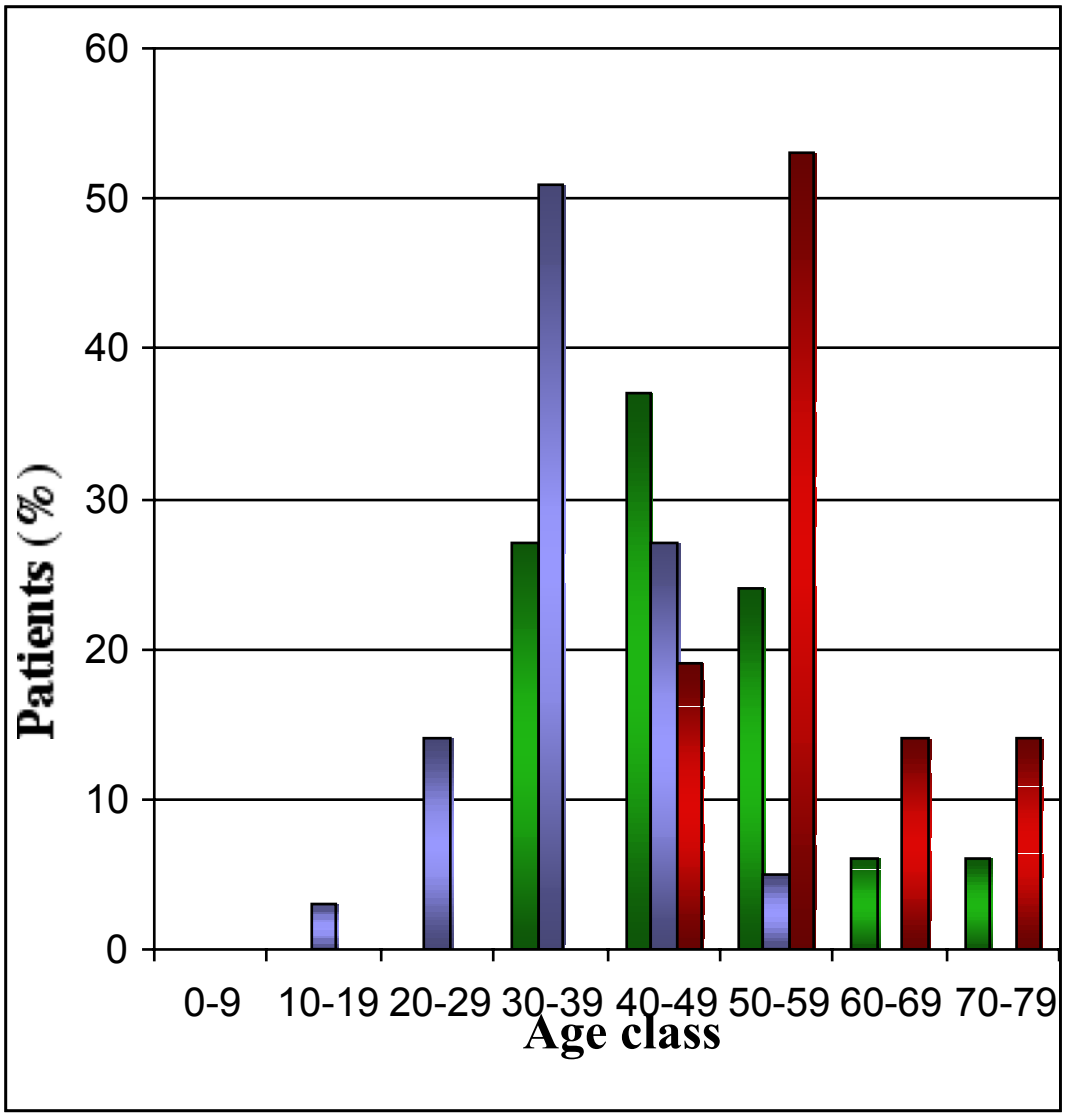

Figure 1: The age distribution of 222 HNPCC suspected patients according to referral criteria (Green bars: Amsterdam Criteria I/II, Blue bars: restricted Bethesda Guidelines and Red bars: Neither Criteria).

Overall, colorectal cancers (CRC) were most commonly located in the sigmoid colon (31%), followed by the rectum (19%), the transverse (15%) and ascending colon (14%), the coecum (8%) and descending colon (4%). The CRC site did not differ significantly among the 3 referral criteria groups, although adenocarcinomas in the rBG group tended to occur more frequently in the distal part of the colorectum compared to those of AC and NC groups (63% vs 48% and 52% respectively; p=0.08). The histopathological properties of the tumors were consistently similar among the 3 groups with the majority of AC, rBG and NC tumors being moderately well differentiated (66%, 66% and 56%, respectively).

Microsatellite Instability

Tumors from 212 (95%) of the 222 referred patients were available to determine MSI status. The group displayed nearly identical phenotypical properties when compared to the initial study population (data not shown). Overall, 41% (n=87) of the patients were found to be microsatellite unstable (MSI) and 59% (n=125) MS-Stable (MSS). Fifty-seven percent of the 87 MSI tumours stemmed from the AC, 25% from the rBG and 18% from the NC group (p<0.01). Of the MSS tumours, 24%, 42% and 34% were from AC, rBG and NC patients, respectively.

The phenotypic features of the 212 patients with CRCs investigated to date for MSI, are depicted in Table 2. CRCs coming from the AC group were significantly more frequently found to be unstable (54%) when compared to those from the rBG (26%) or the NC (20%) group (p<0.01). MSI CRCs were more often proximally located compared to MSS cancers (60%, vs 26%, respectively; p<0.01). Although not statistically significant, patients with MSI CRCs tended to have a higher prevalence of syn- and metachronous colorectal and extracolonic cancers (19% and 13%, respectively) compared to those with stable tumours (12% and 2%, respectively; Table 2).

		MICROSATELLITE INSTABILITY STATUS		
		Total	**MSI**	**MS-Stable**
REFERRAL CRITERIA:	Amsterdam Criteria	80	50	30
	rBethesda Guidelines	73	21	52
	Neither Criteria	59	16	43
SEX:	Male	95	45	50
	Female	117	42	75
TUMOUR LOCATION :	Proximal	80	49	31
	Distal	116	33	83
	Not Colon Cancer	16	5	11
ADDITIONAL CANCERS:	Syn-/ meta-chronous CRC	19	16	3
	Extracolonic Cancer	26	13	13
	none	167	58	109
TOTAL:		212	87	125

Table 2. HNPCC suspected patients according to the MSI-status of their colorectal cancer, and the phenotypic features related.

No association between MSI status and the degree of CRC differentiation was observed, although this may reflect an ascertainment bias with the majority (74%) of all CRCs investigated being moderately well differentiated. Fourteen (6%) CRCs (7 ACs, 5 rBGs and 2 NCs) were mucinous in appearance, with 72% of these being MSI 28% and 28% MSS. These patients did not however, phenotypically significantly differ from the ones with non-mucinous CRCs.

A panel of 14 markers was used in order to ascertain the MSI status of 56 (26%) of the tumours investigated. The mononucleotide repeat markers BAT26, BAT25 and BAT40 proved to be the most sensitive markers for detecting MSI, predicting high-level microsatellite instability in 100%, 96% and 96% (31/31, 30/31 and 30/31), With similar sensitivity, MFD15 and D2S123 detected MSI-H in 87% (20/23) and 86% (24/28), respectively. Six percent of the analysed tumours proved to have an MSI-L status. These were most efficiently detected with BAT26, which displayed novel alleles in 80% (4/5) of the MSI-L CRCs. The remaining markers presented sensitivity values between 20 and 40% in the detection of low degree MSI. Due to the sensitivity and specificity of the BAT26 microsatellite marker, subsequent screening of HNPCC families was done by employing BAT26 only. Hence, BAT26 analysis alone was employed for the assessment of MSI in the remainder of the tumours studied (n=156, 74%). respectively.

hMLH1/hMSH2 gene alteration screening

All 87 patients with MSI tumours, as well as 10 patients with MSS tumours but a positive family history of nonpolyposis CRC (6 AC, 2 rBG and 2 NC), were enrolled in mutational analysis encompassing at least 2 out of 3 different screening methods, direct DNA sequencing (n=54), RT-PCR and the protein truncation test (PTT) (n=25), as well as immunohistochemical detection of mismatch repair proteins *hMLH1, hMSH2, hMSH6* and *hPMS2* (n=92).

Mismatch repair gene alterations were observed in 82%, 74% and 67% of patients fulfilling the AC, rBG and NC, respectively. Overall, 77% (75/97) were found to have either an *hMLH1* or an *hMSH2* gene alteration, of which 96% were microsatellite unstable. Nine frameshift mutations (single base pair ins/del) were identified, as well as 21 base substitutions and 14 larger deletions (2bp-3 exons) (Table 3).

GENE/ EXON	DNA CHANGE	AMINO ACID CHANGE	REFERRAL CRITERIA	FAMILY NUMBER
hMLH1				
2	1995 G>A	G67R	AC	1652
2	184 C>T	Q61X	rBG	1900
3	292 G>C	G98R	AC	2047
4	341 C>G	T117R	AC	434
4	1050 C>T	T350M	AC	1936
5	IVS4-2 A>G	splice acceptor site	AC	1500
7-9	Exons 7-9 del	frameshift	AC	1806
10	811-815delTCCTT	frameshift	AC	1805
10	IVS9-4/791-5delTTAGATCGT	frameshift	AC	1834
13	1490 insC	frameshift	AC	1754
13	1490 insC	frameshift	rBG	1902
13	1490 insC	frameshift	NC	1906
13	1410-1413 Del AAAG	frameshift	BG	1917
15	1731 G>A	splice donor site	AC	1801

15	1690-1693delCTCA	frameshift	rBG	1808
16	1896 Del G	frameshift	AC	1033
16	1946-1848 Del AAG	K616 Del	AC	2151
16	1946-1848 Del AAG	K616 Del	AC	1848
16	1846-1848delAAG	K616del	rBG	1760
16	1852 A>T	K618X	BG	1956
16	1896+1 G>T	splice donor site	AC	2048
17	1976 G>C	A658P	AC	1921
18	IVS18+1 G>T	splice donor site	AC	1813
19	IVS18-2A>T	splice acceptor site	AC	1121
19	IVS18+1 G>T	splice donor site	AC	1831
hMSH2				
2	261-262delTT	frameshift	rBG	1820
3	388-389 Del TC	frameshift	AC	1097
5	942+3 A>T	splice donor site	BG	2170
5	942+3 A>T	splice donor site	BG	1893
7	1148 C>T	R383X	AC	1587

7	1165 C>T	STOP	AC	2025
7-8	Exons 7-8 del	frameshift	AC	1817
7-8	Exons 7-8 del	frameshift	AC	1835
10	1576 Del A	frameshift	AC	1846
10	1576 delA	frameshift	NC	BItalia1
11	1740 G>T	E580X	AC	1642
11	IVS11+2 T>C	splice donor site	AC	1807
12	1787-1789 AAT Del	frameshift	AC	1383
12	1853delC	frameshift	rBG	1886
12	1760-1 G>A	splice acceptor site	AC	1989
14	2261delC	frameshift	AC	1827
15	2503 A>G	N835D	AC	1991
16	2740 G>T	E914X	AC	1841
16	2646 del A	frameshift	NC	BItalia9

Table 3: List of *hMLH1* and *hMSH2* germline mutations identified (AC: Amsterdam Criteria, rBG: restricted Bethesda Guidelines and NC: Neither Criteria)

Of the 87 patients with unstable colorectal tumours, mismatch repair gene alterations were observed in 88%, 81% and 69% of patients fulfilling the AC, rBG and NC, respectively (Table 4). Eighty-one percent were found to have either an *hMLH1* (n=43, 60%) or an *hMSH2* (n=29, 40%) gene alteration.

		GENE ALTERATION STATUS			
		Total	**MLH1**	**MSH2**	**negative**
REFERRAL CRITERIA:	Amsterdam Criteria	50	25	19	6
	rBethesda Guidelines	21	10	7	4
	Neither Criteria	16	8	3	5
SEX:	Female	42	24	8	10
	Male	45	19	21	5
TUMOUR LOCATION:	Proximal	49	27	18	4
	Distal	38	16	11	11
ADDITIONAL CANCERS:	Syn-/ meta-chronous CRC	16	13	3	0
	Extracolonic Cancer	13	6	5	2
	none	58	24	21	13
TOTAL:		87	43	29	15

Table 4: *hMLH1/ hMSH2* gene alteration status in 87 patients with unstable colorectal tumours.

The phenotypic properties between gene alteration positive and negative, MSI patients, e.g. age at diagnosis (41.5 years ±14.49SD and 46.6 years ±16.33SD, respectively; p=0.17) were consistently similar. Extracolonic manifestations (ECMs) were observed in both the gene alteration positive and gene alteration

negative patients, although the risk of ECMs appeared to be higher in association with a gene alteration. Gene alteration positive patients displayed ECMs in the endometrium (n=5, 8%), the skin (n=1, 2%), the adrenal glands (n=1, 2%) and in the stomach (n=1, 2%). Gene alteration negative patients had ECMs of the endometrium (n=1, 7%) and the stomach (n=1, 7%). Interestingly, only gene alteration positive patients had a history of synchronous or metachronous CRCs (23%).

In contrast to the patients with unstable tumours, in 70% (7 out of 10) of the individuals with a positive family history but MSS tumours, no gene alteration could be determined ($p<0.01$). The average age at diagnosis was 50.4 years (±10.9SD). No extracolonic carcinomas or synchronous or metachronous cancers were observed. Two of the three mutation positive, MSS, patients were males, aged 48 (AC) and 59 (NC), with colorectal carcinomas of the sigma and coecum, respectively. Interestingly, they shared the same mutation in hMLH1; 1490 inserted C. The third mutation positive, MSS, patients was a 39 year old female, classified by AC. She had a CRC of the sigma and harbored a mutation in hMSH2; exons 7-8 deleted.

Sensitivity and Specificity of referral criteria and diagnostic methods to identify MMR gene alterations

Referral Criteria: The AC and the BG (which encompass AC and rBG) were able to correctly identify 46 and 63 of the 72 patients with MMR gene alterations corresponding to sensitivity values of 0.64 and 0.88, respectively. However, they also classified 10 (45%) and 12 (55%) of the 22 mutation negative patients, leading to false positive rates of 0.45 and 0.75, respectively. The overall diagnostic accuracy for the AC and the BG amounted to 0.60 and 0.72, respectively.

Microsatellite Instability Analysis: Out of 87 patients with an unstable CRC 72 (83%) were subsequently established as gene alteration positive. Among the 10 MSS cases with a positive family history, three (30%) patients, 2 ACI and 1 NC, were found to harbor *hMLH1* and *hMSH2* gene alterations. Overall, microsatellite instability analysis displayed a sensitivity value of 0.96 in identifying individuals with MMR gene alterations, and a false positive rate of 0.65. The overall diagnostic accuracy amounted to 0.83.

Immunohistochemistry (IHC): Tumours from 95 patients could be screened for the presence of the mismatch repair proteins immunohistochemically, and verified by RT-PCR/PTT and/or direct DNA sequencing. Seventy-four (78%) stained negative for either hMLH1 or hMSH2 (44 hMLH1, 30 hMSH2) whilst in 21 (22%) tumours (14 MSI, 7 MSS) all MMR proteins were present. Among the 10 MSS tumours studied, 2 (20%) showed loss of hMLH1 and 1 (10%) a loss of hMSH2. None of the investigated samples subjected to IHC showed loss of hPMS2 or hMSH6. IHC resulted in sensitivity and specificity values equal to 1 for both hMLH1 and hMSH2 gene alterations.

Direct DNA sequencing: Through direct DNA sequencing, 54 patients could be analysed. A total of 39 (72%) patients (37 MSI and 2 MSS) were established as having a germline mutation in either *hMLH1* or *hMSH2*, 38 (97%) of which were confirmed by a second analysis technique, IHC and/or RT-PCR/PTT. In the remaining 15 patients (all MSI) no mutation could be identified, all of which were cross-validated by IHC and/or RT-PCR/PTT. Eleven (73%) of these patients (all MSI) appeared to be falsely established as mutation negative by direct DNA sequencing; in all 11 patients (5 *hMSH2* and 6 *hMLH1*) the combined results of IHC and RT-PCR/PTT point to the presence of large, exon-spanning deletions or insertions which are currently under investigation. In the remaining 4 (27%) patients (2 AC, 1 rBG, 1 NC) the negative sequencing results correlated successfully with results cleaved from both RT/PTT and IHC. Both MSS cases (1 AC, and 1 NC) subjected to sequencing proved to be mutation positive for

hMLH1; both harbouring a 1490 inserted C. The direct DNA sequencing method thus had an overall sensitivity of 0.78 (0.79 and 0.76 for *hMLH1* and *hMSH2*, respectively), with no false positive results and a diagnostic accuracy of 0.80.

RT-PCR/protein truncation test (PTT): Through RT-PCR/PTT, 25 patients could be screened for aberrant splice transcripts, large genomic deletions/insertions and truncated proteins. Twenty patients (80%) displayed altered amplification or translation products in hMLH1 or hMSH2, all of which were agreed with results cleaved in parallel investigations by IHC and sequencing. Among the 5 gene alteration negative patients, RT-PCR/PTT yielded a false positive results for 1 (20%) patients. Overall, the RT-PCR/PTT method had a sensitivity of 0.95, with no false positive results and an overall diagnostic accuracy of 0.96.

Discussion

This study investigated a consecutive series of 222 Swiss patients with clinically suspected HNPCC with the aim to (i) assess the phenotypic and molecular differences between patients belonging to different referral criteria groups (Amsterdam Criteria I/II (AC), Bethesda Guidelines (BG) and Neither Criteria (NC)), and (ii) to determine the diagnostic accuracy of the individual referral criteria and screening procedures employed, in identifying individuals with mismatch repair (MMR) gene alterations.

The referral criteria groups, being evenly represented in the overall study population, differed significantly ($p<0.01$) in average age of colorectal carcinoma (CRC) onset, due to age at diagnosis being a key criterion for the AC and BG. Still, with an average age at diagnosis of 59 years (being 9 and 14 years later than AC and rBG patients, respectively), NC patients developed CRC 13 years earlier than the general population (Swiss Cancer Registries' Association database, 1996; $p<0.01$), and 33.3% of these MSS NC patients had a family history of colon carcinoma, hence, indicative of a genetic predisposition rather

than a sporadic event. Except for the 16 NC patients with unstable tumors, 2 of which were found to have mismatch repair (MMR) gene alterations, NC tumors were predominantly microsatellite stable (73%; $p<0.01$) and for these the underlying genetic defect(s) is likely to be unrelated to MMR deficiency.

Microsatellite instability (MSI) was observed in 63% of AC tumors out of which 88% were found to have MMR gene alterations. Encompassing both the AC and rBG, the BG increased the MSI tumour detection by an additional 29% (21 MSI tumours) and the MMR gene alteration detection by 26% (12 *hMLH1* and 7 *hMSH2*). In agreement with previous reports (*22*) proximally located colon tumors displayed significantly ($p<0.01$) more often an MSI status. In addition, patients with MSI CRCs had the highest frequency of synchronous and metachronous cancers, with endometrial carcinomas representing the most frequent extracolonic tumor manifestation (*23*). Mononucleotide repeat markers were the most sensitive in detecting MSI, which is consistent with data from other groups (*9*). However, despite its high sensitivity and specificity in this study, and recent reports that BAT26 has been studied as the sole microsatellite marker, caution needs to be applied as MSI-H tumors have been described that do not display instability at the BAT26 locus (*24*). While our results for BAT25 and BAT40 are in agreement with previous reports stating that MSI-L tumors often lack instability at mononucleotide repeats, BAT26 was successful in detecting instability in 80% of the MSI-L CRC (*9,25*). Among the other microsatellite markers used in this survey, MFD15 and D2S123, located intragenic of *BRCA1* and *hMSH2*, respectively, and with the ability to detect complex repetitive sequences as well as dinucleotide runs, were more sensitive than non complex markers with (CA)n repeats only.

Mutational screening, encompassing direct DNA sequencing, RT-PCR/Protein Truncation Test (PTT) and immunohistochemical analysis, identified 83% of patients with unstable tumors to have an *hMLH1* or *hMSH2* gene alteration, compared to 8% of patients with microsatellite stable CRC ($p<0.01$), which is

consistent with previous observations that 70-100% of unstable tumors from HNPCC patients carry germline mutations in *hMLH1* and *hMSH2*(*26*). The mutation spectrum observed in this study corresponds with results published by the International Collaborative Group on HNPCC, showing 80% (71%) of *hMSH2* and 63% (65%) of *hMLH1* germline mutations in HNPCC patients to be either nonsense or frameshift mutations (*27*). In 4% of patients somatic inactivation of *hMLH1* due to promoter hypermethylation is likely to be responsible for the observed MSI. In 8% of patients no MMR gene alteration could be identified by any of the 3 screening methods, including immunohistochemical analysis of *hMSH6* and *hPMS2*.

Comparison of MMR gene alteration-positive and –negative patients did not show any statistically significant phenotypic differences, with the exception that, and in accordance with research by Lindblom, tumors with an *hMLH1/hMSH2* alteration were more often proximally located than gene alteration negative tumors ($p<0.02$) (*28*).

Assessment of the diagnostic value of the referral criteria clearly demonstrated that the Amsterdam Criteria alone identified considerably less patients with MMR gene alterations than combined with the additional inclusion criteria (in particular no. 4, inclusion of individuals with CRC or endometrial cancer at age <45) from the Bethesda Guidelines. Albeit in the same time increasing the number of false positive results, the overall diagnostic accuracy was improved by 12%.

Microsatellite instability as a pre-screening method proved to be highly predictive (83%) in identifying individuals with MMR gene alterations, displaying equally accurate results for unstable tumors in general (MSI-H and MSI-L) and MSI-H alone, the importance of which has been previously demonstrated by Aaltonen et al. on unselected CRC patients (*2*). Notably, 4 out of 22 (18%) AC patients and 2 out of 14 (14%) NC patients with microsatellite stable tumors was found to harbor

an *hMLH1* alteration, highlighting the need for caution when applying MSI analysis as the sole pre-screening method.

Comparison of the 3 screening procedures applied in this study compiled strong evidence that only a combined approach, incorporating immunohistochemical analysis as well as a DNA and a mRNA-based screening method, is able to reliably identify individuals with MMR gene alterations. This is on one hand due to the shortcomings of each technique (eg. failure of IHC and PTT to detect missense mutations and of direct DNA sequencing to identify large, exon-spanning deletions / insertions as well as hypermethylation of the hMLH1 promoter) and on the other hand a consequence of the diverse mutational spectra and the lack of "hot spot" regions in *hMLH1* and *hMSH2*. It appears that more focused investigations (assessment of promoter hypermethylation, linkage analysis) are warranted only subsequently to the combined approach.

In conclusion, this study of a consecutive series of 222 patients, clinically suspected of HNPCC, established the Bethesda Guidelines as more sensitive and of higher diagnostic accuracy than the Amsterdam Criteria I/II alone in identifying patients with mismatch repair gene alterations. Notably, a third set of individuals, was observed, which did not fulfill either referral criteria; these patients displayed a markedly later age at diagnosis of colorectal cancer (mean 59 years), though still occurring significantly earlier than in the general population, and the tumors were predominantly microsatellite stable, pointing to a genetic predisposition unrelated to mismatch repair deficiency. Based on the evaluation of the different screening techniques employed in this study, we propose the following diagnostic approach to optimally identify MMR gene alterations in individuals clinically suspected of having HNPCC: (1) Testing for microsatellite instability *and* immunohistochemical loss of mismatch repair proteins should be used as initial screening methods and (2) subsequent mutational analysis of the positively scored individuals should encompass both, a DNA and a mRNA-based technique.

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Chapter V

Evidence for genetic anticipation in hereditary non-polyposis colorectal cancer

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Abstract

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited colorectal cancer predisposition syndrome caused by germline mutations in DNA mismatch repair (MMR) genes, predominantly *hMLH1* and *hMSH2*. Thus far, only limited data exist on the occurrence of genetic anticipation in HNPCC, i.e. the earlier age at diagnosis of colorectal cancer (CRC) in successive generations. Performing nonparametric, distribution-free statistical analyses, we investigated 55 parent-child pairs (PCPs) diagnosed of colorectal cancer and coming from 21 Swiss HNPCC families with characterised MMR germline mutation (15 in *hMLH1* and 6 in *hMSH2*). The overall median age at diagnosis was 43 years (interquartile range (IQR)=14), with incidence ages ranging from 18 to 62 years. Descendants of HNPCC patients (median age at diagnosis 39 years, IQR=12) were found to be diagnosed of CRC significantly earlier than their parents (47 years, IQR=10), with the median of the paired age difference amounting to 8 years (IQR=15; $p<0.0001$). Birth cohort effects could be excluded since the same, statistically significant age difference was also observed in the oldest offspring birth cohort (birth year <1916; $p=0.01$). Genetic anticipation appeared to be more pronounced when the disease allele was transmitted through the father than through the mother (median age difference 11 versus 4 years, respectively; both $p<0.01$).

Genetic anticipation appears to occur in HNPCC kindreds with identified MMR gene mutation. If confirmed in larger, ideally prospective studies, these results may have important implications for genetic counselling and clinical management of HNPCC families.

Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited colorectal cancer (CRC) predisposition syndrome, exhibiting a high penetrance (80-85%) and accounting for 2 to 10% of the total CRC burden [1]. HNPCC patients typically present with, mostly right-sided, CRC at early age (mean age 45 years) and often develop syn- or metachronous CRC. Besides CRC the tumour spectrum also includes cancers of the endometrium, stomach, small bowel, ovary, ureter/renal pelvis, brain, hepatobiliary tract, and skin. HNPCC is caused by mutations in DNA mismatch repair (MMR) genes (*hMSH2, hMLH1, hMSH6, hPMS2, hPMS1, hMLH3*), with *hMLH1* and *hMSH2* accounting for more than 90% of all germline mutations identified. Following inactivation of the wild-type allele in the tumour, MMR deficiency ensues leading to genetic instability as exemplified by the occurrence of microsatellite instability due to replication errors at short repetitive DNA sequences.

The disease shows considerable inter- and intrafamilial phenotypic heterogeneity among *hMLH1/hMSH2* mutation carriers. Despite harbouring identical MMR gene mutations, disease severity and age at diagnosis often vary significantly between family members. In this context genetic anticipation, a "phenomenon in which the age of onset of a disorder is reduced and/or the severity of the phenotype is increased in successive generations" [2] has been put forward to occur in HNPCC. Thus far, only limited and controversial data are available on this issue, ranging from single case reports to few systematic investigations in HNPCC families [3-6]. In this study, we examined 21 Swiss HNPCC kindreds with identified *hMLH1* or *hMSH2* germline mutation for the occurrence of genetic

anticipation with regard to the age at diagnosis of CRC in parents and their descendants (first-degree kinship).

Subjects and Methods

Out of 46 Swiss HNPCC families with an identified MMR gene mutation in *hMLH1* or *hMSH2*, registered between 1990 and 2001 either at the Research Group Human Genetics, Division of Medical Genetics, Department of Clinical and Biological Sciences, Basel, or at the Unit of Genetics, Institut Central des Hôpitaux Valaisans, Sion, Switzerland, 26 (57%) unrelated families were suitable for the study with complete phenotypic information being available from medical and histopathological records on gender, year of birth, age at diagnosis, tumour type and the occurrence of additional (extracolonic) tumours.

The 26 families harboured either a pathogenic *hMLH1* (n=18) or an *hMSH2* (n=8) germline mutation. Out of the 126 individual patients 91 parent-child pairs (PCPs) were created, each consisting of an affected parent and an affected child. Twenty-two parents had more than one child (range: 2 to 5 children) and 14 descendants had themselves affected children included in this study (see Figure 1 with pedigree examples). In 145 (79.7%) *hMLH1*/*hMSH2* mutation carriers, the first tumor diagnosed was a colorectal one compared to 37 (20.3%) patients with an extracolonic tumour. For the study only PCPs with children and parents affected with colorectal cancer (21 families; n=55) were analysed. The PCPs were treated as related data for the statistical analysis. Written informed consent was obtained from all individuals alive.

Statistical analysis

Nonparametric, distribution-free tools were applied for statistical analysis. Related data were compared applying the Wilcoxon matched-pairs signed-ranks test (referred to as paired Wilcoxon test). Independent data were compared with the Mann-Whitney U test. The disproportions of gender frequencies were

analyzed with the Binomial test. The median and the IQR were used instead of the mean and the standard deviation, respectively. The significance level was set to $\leq$0.05 (5%). All statistical tests were applied in their two-sided form. The software-packages used for the statistical analysis were “RS/1” (Research System 1), version 6.1 (Brooks Automation) and “StatXact”, version 4 (Cytel Software Corporation).

Results

To assess the occurrence of genetic anticipation in HNPCC, 55 PCPs, both affected with CRC, coming from 21 Swiss HNPCC kindreds with identified *hMLH1* (n=15) or *hMSH2* (n=6) germline mutations were examined. The PCPs encompassed 52 (62.7%) male and 31 (37.3%) female patients. Twenty-four (28.9%) patients had developed metachronous cancers (range 1 to 4 tumours) encompassing those of the colorectum (n=15; 62.5%), small intestine (n=2; 8.3%), stomach (n=1; 4.2%) and other sites (n=6; 25%). Median age at diagnosis did not significantly differ between genders, neither in parents (p=0.42) nor in descendants (p=0.23).

The overall median age at diagnosis was 43 years (IQR=14), with incidence ages ranging from 18 to 62 years (Figure 2a). Taking the median of the paired age differences, descendants (39 years, IQR=12) were diagnosed 8 years earlier than their parents (47 years, IQR=10.0), the difference being statistically significant (paired Wilcoxon test, $p < 0.0001$), with 76%, 9% and 15% of descendants being diagnosed at an earlier, same and later age, respectively (Table 1). Moreover, the paired age difference was more marked if the parents had developed CRC at later age (Figure 2b).

Comparison of the median age at diagnosis according to the gene mutated found descendants with an *hMLH1* germ-line mutation to be diagnosed statistically significantly earlier than those with an *hMSH2* mutation (38 versus 43.5 years,

p<0.05) in contrast to parents (47 versus 48 years, p=0.91), respectively. Similarly, PCPs with an *hMLH1* mutation displayed a significant median of the paired age difference (p<0.01). The median of the paired age difference in *hMSH2* PCPs with colorectal cancer did not reach statistical significance, possibly owing to the small sample size (n=14; p=0.12; Table 1).

When subdividing PCPs according to parental transmission, sons and daughters who inherited the disease allele from their father showed a significantly increased median age difference of 11 years (p<0.001) compared with 4 years in children who had inherited the germline mutation from the mother (p<0.01; Table 2). Importantly, median age at diagnosis did not significantly differ between mothers (45 years, IQR 14) and fathers (47 years, IQR 10; p=0.5).

In order to test if the observation of apparent anticipation could reflect a birth cohort bias of ascertainment we grouped the PCPs according to the birth year of the children and created 3 birth cohorts: 1) those born before 1916 (n=9), 2) between 1916-1936 (n=20) and 3) those born after 1936 (n=24). As depicted in Table 3, the median of the paired age difference remained statistically significant in the first (15 years; p=0.01) and third cohort (9 years; p=0.001). In the second cohort the median age difference amounted to 5 years but hardly missed statistical significance (two-sided p=0.06).

Discussion

In this study we provide significant evidence for genetic anticipation in 55 parent-child pairs (PCPs) coming from 21 Swiss HNPCC kindreds with characterised *hMLH1* or *hMSH2* germline mutation. Compared to the parents, descendants developed colorectal cancer (CRC) eight years earlier, in particular if the parents had developed cancer at later age.

Besides the first report on HNPCC by Warthin in 1925, only four studies deal in detail with the occurrence of genetic anticipation in this syndrome, i.e. the

progressive decrease in age of onset of CRC in successive generations. Although three investigations did observe anticipation in HNPCC kindreds fulfilling the Amsterdam Criteria I (ACI), they cannot be directly compared with our study since i) no statistical tests were applied, ii) investigations were done either before the actual discovery of the genes responsible [3, 6] or afterwards, but without molecular genetic characterization [4]. The fourth study by Tsai et al. analysed a total of 67 ACI-positive PCPs (38 families) with *hMSH2* and *hMLH1* germline mutations characterised in 14 (7 families) of them [5]. However, they could not detect any difference in mean age at diagnosis between generations in neither of the subgroups analysed. This apparent contradiction to our findings could be due to the small sample size (14 compared to 55 PCPs in our study) leading to a type II error (failure to reject the null hypothesis). In addition, the comparison might be hampered by differences in the way data were collected and the time window analysed.

In contrast to the findings by Tsai et al. [5], a possible birth cohort bias of ascertainment could not be confirmed in our study group (Table 3): the median of the paired age difference remained statistically significant in the youngest as well as the oldest birth cohort (9 years, $p<0.001$, and 15 years, $p<0.01$, respectively) and just failed significance in the middle birth cohort (5 years, $p=0.06$). On the other hand, PCPs with cancers other than CRC (n=36), excluded from the original study, revealed a clear birth cohort effect (data not shown).

Intriguingly, genetic anticipation in our study population was more pronounced if the disease allele was transmitted through the father than through the mother (9.5 vs. 4 years paired median age difference, respectively; Table 2 and Figure 1). This finding does not appear to be due to differences in median age at diagnosis between fathers and mothers which were similar (47 vs. 45 years; $p=0.5$). It remains to be seen, however, whether this apparent parental transmission effect is merely due to ascertainment bias or may actually reflect a true biological phenomenon. If so, it is tempting to speculate that this effect is

related to differences in male and female gametogenesis, in particular given the greater number of cell divisions during spermatogenesis and continuous replication throughout adulthood. Given the data implying hMLH1 in the meiotic recombination process [7, 8] and in analogy to genetic anticipation observed in trinucleotide repeat disorders like Huntington's disease [9], it could be hypothesized that mismatch repair (haplo)insufficiency in the germline could lead to anticipation via low level repeat instability.

Despite the evidence for genetic anticipation presented here, there are several caveats to this retrospective study. First of all, we cannot exclude an ascertainment bias since our study population represents a highly selected group of patients. In particular, a selection bias might have resulted from under-representation of "younger parent-older child" pairs in which the parent had died before producing a "complete" family. Furthermore, the significant difference in age at diagnosis of CRC observed between the generations could i) reflect changes in environmental factors such as dietary and life style habits, ii) be due, at least in part, to earlier and better diagnosis progressively over time and/or iii) greater awareness/anxiety in descendants. It is therefore mandatory that these findings are confirmed in larger, ideally prospective studies on HNPCC kindreds. This could also allow to assess the occurrence of genetic anticipation for other HNPCC related tumours, particularly endometrial cancer.

In summary, we have presented significant evidence for genetic anticipation to occur in HNPCC families harbouring MMR gene mutations, with descendants being diagnosed of CRC 8 years earlier than their parents. Intriguingly, this effect seemed to be more pronounced if the disease allele was transmitted through the father. If our findings on genetic anticipation in general and on paternal transmission in particular are confirmed, they may have important implications for genetic counselling and clinical management of MMR gene mutation carriers and their offspring.

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General Discussion:

This thesis has focused on genotype-phenotype correlations in two hereditary colorectal cancer syndromes, hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) in an attempt to optimise the selection criteria for affected individuals, to establish the sensitivity and specificity of different screening methods, to investigate a relatively new gene associated with a multiple colorectal adenoma and carcinoma phenotype and to assess the role of a modifier gene locus on chromosome 1p33-36. All investigations aimed ultimately to aid clinicians in their selection of patients for different genetic screening programs and give them guidelines for optimal genetic testing, hence helping them maintain an overview on the best surveillance and prevention strategies and genetic counselling schemes.

The preliminary step towards the diagnosis of a hereditary cancer syndrome is the compilation of a detailed account of the family history of cancer (Polednak, 1998). The pedigree should concentrate on all types of cancer and their location; the family member's age at cancer onset; any pattern of multiple primary cancers; any association with phenotypic features that may be related to cancer, eg. colonic adenomas; and whenever possible records of pathological findings. Such a combination of information frequently identifies a hereditary colorectal cancer syndrome in the family, should it exist. Molecular genetic investigations can then produce clarification of the diagnosis, and determine the presence of a germline mutation in the family (Vogelstein et al., 1998; Eng et al., 2001).

On establishing the diagnosis of a hereditary colorectal cancer syndrome, the proband's high risk relatives should be informed. Genetic counselling and DNA testing should be offered and in attempts to reduce morbidity and mortality, surveillance measures may be instigated depending on the nature of the disorder (Lynch et al., 1999).

Genetic counselling is of high importance to a patient with a clear familial form of colorectal cancer. The family concerned should be updated on the details regarding the genetic risks of cancer at specific sites; the available options for surveillance and management; and the necessity for genetic testing (Lynch et al., 2001; Aktan-Collan et al., 2000). It is advised that counselling is conducted face-to-face, although sessions may include multiple family members (Lynch et al., 2001). However, the outcome of mutational analysis should be revealed to a patient on a one-to-one basis (Lynch et al., 1999).

Hereditary Nonpolyposis Colorectal Cancer (HNPCC):

The consistently accurate identification of HNPCC families continues to be a problem despite knowledge concerning the genetic basis of the disease (Scott et al., 2001). At a meeting of the International Collaborative Group (ICG) on HNPCC in 1990, the Amsterdam Criteria (AC) were introduced for the uniform identification of hereditary colorectal cancer patients for genetic screening and surveillance programs (Vasen et al., 1990). Originally drawn up to identify the genes responsible for HNPCC, and exclusively focusing on a family history of CRC, the AC were recently extended to also include endometrial, small bowel and upper renal tract cancers (Amsterdam criteria II, ACII) (Vasen et al., 1991; Vasen et al., 1999). In parallel, the Bethesda guidelines (BG) were set up to define which colorectal tumors should be tested for MSI in order to identify HNPCC patients in the general population (Rodriguez-Bigas et al., 1998). Considering the value of these criteria it is surprising that only limited data are available which assess their sensitivity and specificity, especially in conjunction with MSI testing and various other mutation screening methods. Through the investigation of 222 unrelated patients, referred to the Medical Genetics department because of clinically suspected HNPCC, this thesis reports on the phenotypic and molecular differences between patients belonging to different referral criteria groups, and the diagnostic accuracy of the criteria and screening procedures employed in identifying individuals with mismatch repair (MMR) gene alterations.

Of the referred patients, 37% fulfilled the AC, 34% the rBG and 29% the NC group. Fifty-seven percent of the MSI tumours stemmed from the AC, 24% from the rBG and 18% from the NC group ($p<0.01$). Eighty-three percent of the unstable tumors had gene alterations whilst in comparison 95% of the stable tumors were gene alteration negative ($p<0.01$). Gene alteration positive tumors were more frequently unstable than gene alteration negative tumors ($p<0.001$), and were more often proximally located ($p<0.02$). This study proved the BG to be of superior sensitivity and diagnostic accuracy compared to AC I/II alone, in identifying patients with MMR gene alterations. This observation is in accordance with previous reports stating that the Bethesda Guidelines (excluding the AC) can identify an additional 20% of the HNPCC families which have a condition related to MMR gene mutations (Buerstedde et al., 1995; Wijnen et al., 1998; Heinimann et al., 1999). Furthermore, as a result of assessing the different screening procedures the following diagnostic approach should allow optimal identification of individuals with MMR gene alterations: (1) Testing for MSI combined with immunohistochemical loss of MMR proteins as initial screening methods and (2) subsequent mutational analysis of the positively scored individuals encompassing both a DNA and a mRNA-based technique.

A combination of methods is necessary due to the inadequacies of each technique eg. failure of IHC and PTT to detect missense mutations and of direct DNA sequencing to identify large, exon-spanning deletions / insertions as well as hypermethylation of the *hMLH1* promoter, (Aaltonen et al., 1998) and on the other hand a consequence of the diverse mutational spectra and the lack of “hot spot” regions in *hMLH1* and *hMSH2* (Moslein et al., 1996; Liu et al., 1996; Salovaara et al., 2000).

Whist familial adenomatous polyposis has had numerous genotype-phenotype correlations made and described (Lynch et al., 1996, 1998, 2003; Brensinger et al., 1998) no such relationships appear to have been made for HNPCC. However, subtle associations have been reported describing possible histological

variances between *hMSH2*-mutated tumors and *hMLH1*-mutated tumors in HNPCC (Shashidharan et al., 1999) and some clinical differences that may exist (Vasen et al., 1999), but to complicate matters further, previous reports also suggest that the phenotype in HNPCC varies from individual to individual (Watson et al., 2001).

This thesis aimed to further characterize the phenotype of HNPCC patients by comparing MMR gene mutation carriers to gene alteration negative. Mutation positive patients were found to be on average 3 years younger than mutation negative individuals at the time of their CRC diagnosis. Mutation positive patients had CRCs located more frequently in the proximal region of the colon compared to the mutation negative individuals ($p<0.0001$). In addition, a higher prevalence of extra colonic manifestations was observed and more syn-/metachronous CRCs were found, in mutation positive compared to mutation negative patients ($p<0.03$ and $p<0.05$, respectively).

Using the HNPCC referral criteria as a basis, AC and BG, and subsequently phenotypic differences such as those established in this and other studies, namely age at CRC diagnosis (Watson et al., 2001), CRC location (Lynch et al.,1999), the occurrence of syn-/metachronous cancers (Gryfe et al., 2000), and the presence of extracolonic manifestations (Watson et al., 1993; Lin et al., 1998) families or individual patients can be identified and offered genetic testing for genes associated with HNPCC. Better classification of the mutation negative group could lead to the identification of additional genes associated with this disorder.

Familial Adenomatous Polyposis (FAP):

In a significant subset, 20-50%, of clinically diagnosed FAP patients an *APC* germline mutation cannot be identified, giving rise to the so-called *APC*-negative individuals (Armstrong et al., 1997; Giardiello et al., 1997; van der Luijt et al., 1997). Characteristically, a later age at diagnosis is observed and 50% of *APC*-

negatives have fewer than 100 colorectal polyps. In addition, extracolonic manifestations (eg. polyps of the upper gastrointestinal tract, desmoids, osteomas) are less frequently detected. Several reasons could stand for the failure in world-wide efforts to identify germline mutations within the APC gene of FAP patients. No individual method for mutation detection is sensitive enough for all types of gene alteration. Only a combination of several different screening techniques results in a good detection rate. Furthermore, since current mutation analysis has little focus on the regulatory regions of the APC gene or quantitative tests of

APC gene expression there is a lack of understanding in these areas. And finally, few cases of FAP genetic linkage to the APC locus have been described suggesting that other genes may be responsible for the development of FAP or may lead to a similar clinical phenotype (Stella et al., 1993; Tops et al., 1993).

In light of results from recent studies, implicating biallelic germline mutations in the base-excision-repair (BER) gene *MYH* with a multiple colorectal adenoma and carcinoma phenotype, this thesis reports on the investigation conducted to further correlate *MYH* germline mutations with *APC*-negative individuals and establish any genotype-phenotype correlations. Thirteen from 65 individuals were identified as *MYH* mutation carriers, 7 of which had biallelic mutations. Aside from previously reported mutations, 3 apparently novel gene alterations were established. No specific somatic *APC* mutations were observed although loss of heterozygosity of *APC* was observed in 3 patients with biallelic *MYH* mutations. In addition, 2 biallelic mutation carriers also harboured *KRAS* oncogene mutations in exon 1. The phenotypical characteristics of all patients investigated were similar, although biallelic MYH mutation carriers had a higher prevalence of colorectal cancers diagnosed, compared to the monallelic mutation carriers and the mutation negative individuals. Further distinctions in phenotype have been made by other research groups and hence recommendations can be made for genetic testing and surveillance based on numbers of colorectal adenomas

(Jones et al., 2002; Sieber et al., 2003) and with the observed increased frequency of G to A somatic APC transversions (Al-Tassan et al., 2002). Number and histology are indeed the indicators in the detection of many colorectal cancer predisposition syndromes (Enholm et al., 2003) although it is still clearly apparent that more experience should be derived to entitle a more profound understanding of the natural history of *MYH*-associated colorectal neoplasia.

FAP displays considerable inter- and intr-familial phenotypic heterogeneity, which represents a major problem in genetic counselling of *APC* mutation carriers. Such phenotypic heterogeneity in FAP patients cannot be solely related to the variety of different *APC* gene mutations. Other genetic factors can modify disease expression as illustrated by numerous mouse models of FAP (Dietrich et al., 1993; MacPhee et al., 1995).

The *Min* mouse model indicated a putative disease modifier locus on chromosome 4, which is syntenic to human chromosome 1p35-36 (Dietrich et al., 1993). The recent identification of germline *MYH* mutations, mapped to the 1p33-34 region, in multiple adenoma and carcinoma patients, points to a possible role as a disease modifier in FAP. This thesis documents a study where fine-mapping of the critical region, as well as mutation analysis of the *MYH* gene, were performed on a large Swiss FAP kindred (no.1460). These investigations allowed the significant exclusion of the 1p33-36 region as a modifier locus and *MYH* as a modifier gene for extracolonic disease in this FAP family. The results indicate that linkage analysis of further putative candidate regions is necessary to identify a disease modifier locus in FAP which will prove critical in establishing genetic risk and thus improved accuracy in genetic counseling of FAP patients.

In conclusion it can be said that morbidity and mortality from FAP and HNPCC can be reduced once the patients' familial or hereditary risk is determined and a complex program of cancer surveillance and management is undertaken (Burke et al., 1997; Jarvinen et al., 2000; Ramsey et al., 2001). Prevention will be

improved by the identification of the responsible germline mutation in a family, hence confirming the risk. Advances in technology in cancer screening and the identification of biological markers of cancer susceptibility, eg. microsatellite instability, and also specific germline testing, will also aid physicians in achieving cancer prevention targets. In addition, molecular genetic research on hereditary forms of colorectal cancer must continue and strive to search for new mutations, novel genes, and even modifier genes, in these heterogeneous disorders.

Appendix I

Optimization of the denaturing high performance liquid chromatography (dHPLC) protocol for use in the screening of patients with suspected hereditary non polyposis colorectal cancer (HNPCC)

This procedure was optimised for use in the Human Genetics Lab
by Anna M Russell

Introduction

Hereditary non polyposis colorectal cancer (HNPCC) is an autosomal dominantly inherited cancer predisposition with at least 5 genes associated to the risk of patients developing cancers of the colon, rectum, endometrium, small bowel and urinary tract[1]. These include *hMLH1*[2], *hMSH2*[3], *hMSH6*[4], *hPMS1* and *hPMS2*[5]. In more than 90% of HNPCC families with identified germline mutations, *hMLH1* and *hMSH2* are accountable for the disease (http://www.nfdht.nl). The most sensitive mutation detection technique is considered to be direct DNA sequencing. However, sequencing of the 35 exons of *hMLH1* and *hMSH2* proves to be technically demanding, time consuming and expensive[6].

In the detection of sequence variations in disease genes, high sensitivity is fundamental. The most widely employed mutation scanning techniques in laboratories today range from relatively simple methods eg. single-strand conformation analysis (SSCP) and heteroduplex analysis, to the more complex procedures such as direct sequencing, protein truncation test (PTT) and denaturing gradient gel electrophoresis (DGGE)[7]. SSCP and heteroduplex analyses tend to lack sensitivity, whilst the more sensitive methods are often labour intensive, expensive and time consuming. For the optimal detection of

mutations in large numbers of DNA fragments, the scanning methods should be sensitive, non-hazardous, relatively inexpensive and fully or at least semi-automated to minimise time and labour costs. To satisfy clinicians and their patients, such techniques should also have a rapid turnover time. The above criteria seem to be fulfilled by the recently established denaturing high performance liquid chromatography (dHPLC) method, developed primarily as a pre-screening method in the identification of sequence variations in a number of disease genes.

dHPLC is based on the detection of heteroduplexes in short segments of DNA by ion-reverse phase high performance liquid chromatography[8]. Partial heat denaturation within an acetonitrile gradient leads to the separation of the DNA strands, resulting in the formation of hybrid wild type/mutant heteroduplexes. These heteroduplexes have a reduced column retention time and hence an altered mobility compared to their homoduplex counterpart. The most obvious advantages of the dHPLC method include low cost, the use of automated instrumentation and the speed of the analysis (5 minutes per sample).

This technique has been successfully employed in the detection of mutations and polymorphisms in the Y chromosome[9], exons from the factor IX and neurofibromatosis type 1 genes[10], rearranged transforming (RET), cystic fibrosis transmembrane conductance regulator (CFTR) and phosphatase and tensin homologue on chromosome 10 (*PTEN*) genes[11], *BRCA1* and *BRCA2*[12,13,14] and *hMLH1* and *hMSH2*[15,16]. The sensitivity of dHPLC for *hMLH1* and *hMSH2* mutation detection has been previously described by Holinski-Feder et al[15] to be approximately 97%.

Due to the previously stated advantages of the dHPLC method, the sensitivity reported for the detection of mutations in genes related to the HNPCC syndrome, and the relatively large number of colorectal cancer patients whom this rapid and inexpensive technology may benefit, including those in our research cohort

suspected of HNPCC but proving to be mutation negative after screening was complete, we decided to optimise the protocol for dHPLC for use in our lab.

I was able to develop the dHPLC protocol for the following genes and DNA segments; *hMLH1, hMSH2, hMSH6, and hMYH* genes, the *APC* mutation cluster region and the promoter regions of *hMLH1* and *hMSH2*.

Methods

i) The WAVE System Hardware:

Powering Up the WAVE System:

- Each hardware module should be powered up in the following order:
    - Interface
    - Pump
    - Chiller
    - Autosampler
    - Oven
    - UV detector
    - Degasser

- With the L7200 Autosampler, the chiller module MUST be powered up BEFORE the Autosampler

- Each module performs a self-initialisation and one of the following messages should appear:

    - D-Line System is not ready
    - E5

- **NB 1:** If these messages do not appear, the module is not in D-Line (communication mode). Switch off all hardware modules, in reverse order

ie. Degasser to Interface, and switch on again. Also check all connecting cables at the back of each hardware module for loose wires/plugs.

Powering up the WAVE System computer and WAVEMAKER Software:

- Press the power button on the computer to start
- Press **CTRL + ALT + DEL** simultaneously for the LOGIN window to appear
- In the *User Name* field type **ADMINISTRATOR**. Leave the *Password* field blank
- On the desktop Transgenomic folder, double click the **D-7000 HSM** icon
- Click on the hardware status button on the left side of the screen: ⓘ
- Click the **Initialise** button on the toolbar
- WAVEMAKER Software initialises each of the systems' modules. The following values should appear in the hardware boxes, after 2-3 minutes, if all components have been properly connected and turned on:
    - Interface Module: D-7000
    - Autosampler : L-7250
    - Pump A : L-7100
    - Pump B : /
    - Pump C : /
    - Oven : L-7300
    - Detectors : L-7400
- If these values do not appear, do as previously ie. NB 1, and repeat the Initialisation step
- Once all components are confirmed and values appear correctly on the hardware status dialog box, click **OK** and close the HSM software

Preheating the Oven:

- Press the **SET MONIT** button on the keypad of the oven
- Press the **UP/DOWN** arrows to set the temperature to 50°C

WARNING: Failure to preheat the oven before commencing buffer flow may result in damage to the DNASep Cartridge

Purging the Pump:

- The pump should be purged daily
- Open the drain valve on the front right of the pump, anticlockwise
- Manually set the pump to purge the lines by pressing **MANUAL SET** on the L7100 keypad. Enter the following percentages: **B 33%, C 0%, D 33%**. Line **A** will automatically be set at **34%**
- Enter **0.9 ml/min** for pump flow rate and an upper pressure limit of **3600 psi**.
- Press **ENTER**
- Press **PUMP ON/OFF** to start the pump
- Press **PURGE**
- Continue purging until all air bubbles have been eliminated from the eluant lines; 1-5 minutes
- Switch off pump and close drain valve again

Equilibrating the System/DNASep Cartridge:

- Ensure the oven temperature is greater than 40°C before starting the buffer flow through
- Press the **MANUAL SET** button on the autosampler keypad to set the following conditions: **Buffer A 50%, Buffer B 50%, flow rate 1.5 ml/min** and **3600 psi**
- Press the **PUMP ON/OFF** button on the keypad to start the pump
- Allow the system to equilibrate for 10 minutes
- Longer equilibrium times, eg. 40-60 mins are necessary for DNASep Cartridges that have been stored, new columns, after Active Clean™ flush or if the buffer bottles have run dry.

NB. If the buffer bottles have been left to run dry, equilibrate until the Sample and Reference numbers displayed on the UV Detector are similar ie. within 10-20 units of eachother

Priming the Autosampler:

- The Autosampler can be primed during the purging step to save time
- Press the **WASH** button on the Autosampler **3-15 times**, until all air is removed from the syringe inlet line

NB. The Autosampler should be primed before the beginning of every project. If the Autosampler is not primed, inconsistent injections, loss of intensity or air spikes may be detected in the chromatograms.

ii) Setting up Methods:

Mutation Detection Analysis using the Rapid DNA Option:

1. Open the **WAVEMAKER Software** main window
2. Click the **App. Type** button
3. Select the **Mutation Detection** button
4. Select the **Rapid DNA** check box
5. Click the **Apply** button
6. Click the **Sequence** button. The DNA sequence page appears
7. In the *sequence box*, enter the DNA sequence of the DNA sample to be investigated. This can be copied and pasted from a word file. NB. ensure the sequence includes the primer and T7/SP6 sequences. See Tables 1- 6 for sequences used in our analyses for
   the *MLH1, MSH2, MSH6,* and *MYH* genes, the APC mutation cluster region and the *MLH1* and *MSH2* promoter regions
8. Click the **apply** button. The *Oven Temp* field on the Navigator Bar is automatically updated
9. Click the **Gradient** button. The gradient page appears

10. Verify that the entries in the *Gradient Parameters* area are appropriate. If any changes need to be made check the *Edit Table* box and enter the desired values. Ensure the *Stop Equilibrate Time* is set to 3 minutes and the F*low Rate* is 1.5 ml/min
11. If any changes are made, click the **Apply** button
12. Save method. You must make and save a separate method for each exon of each gene, under **File, Save Method**
13. Give the method name as: **Gene, exon** and **temp.**
14. At least 2 temperatures for each exon should be run in order to incorporate all melting domains. A second temp. can be set as follows:

- Carry out steps 2-6 as previously stated
- Click the **Melting** button. The *Melting Domains* page appears
- The first temp. you set should be automatically shown as you click the **Calculate** buttons
- In the first temperature box, change the value to be ca.3°C more. The melting domain graph should change accordingly
- In the **Oven Temp**. field on the *Navigator Bar* change the temp. to the value you have chosen as your second analysis temperature. Click on any other field in the Navigator bar to have this change accepted
- Continue with steps 9-13 as previously stated
- See Tables 7–12 for the melting temperatures we set for our use in analysing the exons of *MLH1, MSH2, MSH6,* and *MYH* genes, the APC mutation cluster region and the *MLH1* and *MSH2* promoter regions

Creating a Project:

- Open the **WAVEMAKER Software** main window
- Click the **App. Type** button
- Select the **Mutation Detection** button
- Select the **Rapid DNA** check box
- Click the **Apply** button
- Select the *Sample Table* tab. The *Sample Table* appears

- Review the *Vial, Volume, Injection, Sample Name* and *Method* fields on the Sample Table. All other fields can be left blank and are filled automatically
- Set up at least 2 blank samples to give the column and the oven enough time to reach equilibrium. Two rows in the sample table with the same, following values:
    - Vial = 1
    - Volume = 0
    - Injection = 1
    - Sample Name = Blank
    - Method = Mutation

- The *Vial* field should correspond to the position on the 96 well plate ie.
  Sample 1 = Vial 1 on the Sample Table = Well A1 on the 96 plate
  Sample 2 = Vial 2 on the Sample Table = Well B1 on the 96 plate
  Sample 3 = Vial 3 on the Sample Table = Well C1 on the 96 plate etc
- The *Volume* field should be set to 5 to allow an uptake of 5μl from each sample
- The *Injection* field should be set to 1 to permit only one injection per sample
- The *Sample Name* field should contain all details concerning the sample being run. The details stated here will be the only ones related to the chromatogram in the results section. Hence , it is important here to state the *sample name, gene, exon and run temp.*
- The *Method* field should correspond to the sample exon being analysed. Go to **File** on the menu bar and **open method**. A copy of the method is made in the project's folder and can be selected from *Meth. Name* column drop-down list. The *Sequence field* will be automatically filled and will relate to the method file selected
- When the Sample Table is complete, select File from the menu bar. Select **Save Project As** and type the name of the project in the *File Name* field
- Click the **Save** button. Samples cannot be run until the project has been saved

- If the Samples are already loaded into the autosampler, click **Run Samples** to begin the analysis

III) *Viewing Results:*

To View Chromatograms:

- On the *results table*, click on the sample row for which you want to view a chromatogram
- If you want to view all chromatograms in a subset, select the *Show All* check box above the assigned subset
- To fine-tune chromatogram displays, click the right mouse button on the displayed chromatogram and select **Chart** from the menu. Chart details, axes and off-set values can be changed

Configuring and Printing Reports:

- Select **File** from the menu bar
- Select **Print Report**. The report window appears and allows you to select which page of information to display or print
- Click the **Build Report** button
- If you want to print the report, click the **Print** button on the top of the *Report Preview* area. Select the appropriate options and click **OK**. The report prints
- If you do not wish to print the report, click **Cancel**

IV) *Shutting Down the WAVE System:*

Shut Down for 2-4 Days:

- When the WAVE system is not in use, it is important not to shut down the system completely but keep the pump running at a very low flow rate
- The column can be maintained for 2-4 days by pumping the column at a flow rate of 0.05 ml/min with 50% Buffer B

Shut Down for More than 4 Days:

- Place all buffer lines in Solution D
- Flush the system, including the column, for 30 minutes
- Remove the column and close the black screws
- Place the black union into the position of the removed column
- Now shut down the system top to bottom ie.Degasser to Interface

V) *Trouble Shooting:*

1. NO PEAK:

♦ Failure of the PCR reaction: repeat PCR

♦ Air in the syringe: wash syringe at least 15 times

♦ Failure of syringe to take up sample: watch the syringe when either washing or whilst it is taking up sample. If no sample is taken up, check the syringe seal and replace if necessary. Also, check the syringe is tightly screwed into place. If this doesn't solve the problem, replace syringe

♦ Check the syringe is going to the correct Z value within the sample tube:

96 well plate = 35

PCR tube = 32

2. HIGH SPIKES DURING BLANK RUN

♦ Air in the syringe: WASH syringe at least 15 times

♦ Air in the system: run buffers 1 by 1, 100% through the system for 2-5 minutes. Then run A, B and D at 100% for 10 minutes. Continue until the **Reference** and **Sample** values on the UV detector are roughly the same ie. within 20 units of eachother

VI) General Etiquette:

Please remember that we are using the lab space and equipment of another research group. Hence, recycle empty cardboard boxes and empty bottles in the room next to the 7th floor elevators.

All waste solutions should be taken to the waste solutions room on the 7th floor (ask Anna or Jian for directions).

Tables of primers used in PCR amplification of genes and DNA segments:

MLH1 Exon	*Sense Strand Primer 5'-3'*	*Antisense Strand Primer 5'-3'*
1	aggcactgaggtgattggc	ctcacttaagggctacga
2	aatatgtacattagagtagttg	gagtcaggacctttctctg
3	agagatttggaaaaatgagtaac	cctgtgatgacattgt
4	aacctttccctttggtgagg	gcctaggtctcagagtaatc
5	gattttctcttttcccctttggg	gtaaattgttgaagctttgtttg
6	gggttttattttcaagtacttctat	gctcatacattgaacagttgctgagc
7	ctagtgtgtgtttttggc	ggtggagataaggttatg
8	ctcagccatgagacaataaatcc	ccatcacattattttggaac
9	caaaagcttcagaatctc	ccactcacaggaaacacccacag
10	catgactttgtgtgaatgtacacc	cagatgttctatcaggctctcctc
11	ggctttttctcccccctccc	cgtgagagcccagatttt
12a	ctctccactatatatatatatata	gcagcctctgagcaaac
12b	gatggttcgtacagattcccg	ctacctcctttattctgtaataa
13	tgcaacccacaaaatttggc	ggttttggaaatggagaaag
14	tggtgtctctagttctgg	gcagagctactacaacaatg
15	cccattttgtcccaactgg	ctgaaatttcaactgatcg
16	catttggatgctccgttaaagc	caaataaaatttccagccgggtg

17	ggaaagcactggagaaatggg	cggtacatgcatgtgtgctggaggg
18	taagtagtctgtgatctccg	aaacggagatcacagactac
19	gacaccagtgtatgttgg	catcccaacatacactggt

Table 1: Primers sequences used in the dHPLC analysis of *hMLH1*. Sense strand primers and antisense strand primers also had the T7 and SP6 sequences attached, respectively

MSH2 Exon	*Sense Strand Primer 5'-3'*	*Antisense Strand Primer 5'-3'*
1	tcgcgcattttcttcaacc	gcgtgctggggagggac
2	gaagtccagctaatacagtgc	gagtagaaaaataaaaatgtgaag
3	gcttataaaattttaaagtatgttc	ggagattccaggcctaggaaaggc
4	tttcatttttgcttttcttattcc	gaaggatatttctgtcatat
5	ccagtggtatagaaatctt	gggttaaaaatgttgaatgg
6	gttttcactaatgagcttgcc	cccacatgattataccac
7	gacttacgtgcttagttg	ccttcaactcatacaatatatac
8	atttgtattctgtaaaatgagatc	gttatttttaaaaagcaaaggcc
9	gtctttacccattatttatagg	ggaataattcttttgtct
10	ggtagtaggtatttatggaatac	ccctaaatgctctaacatg
11	cacattgcttctagtacac	gttctgaatgtcacctgg
12	attcagtattcctgtgtac	gctttgtgggggtaacg
13	cgcgattaatcatcagtg	gatagaaatgtatgtctctgtcc
14	taccacattttatgtgatgg	gggaaacttactaccc
15	ctcttctcatgctgtccc	gtttaacttagcttctctat
16	taattactaatgggacatt	aaaatcccagtaatggaatgaaggta

Table 2: Primers sequences used in the dHPLC analysis of *hMSH2*. Sense strand primers and antisense strand primers also had the T7 and SP6 sequences attached, respectively

MSH6 Exon	*Sense Strand Primer 5'-3'*	*Antisense Strand Primer 5'-3'*
1	agatgcggtgcttttaggag	agttggcttgaatgagtgca
2	tgccagaagacttggaattg	ctgccatgtgtgtgtgtttg
3	gatggggtttgctatgttgc	gaagaaaggggagggtgta
4a	aatgaaaaacagtggctgca	gcatttcatcagaaaccaag
4b	ttaaaaggaaaagctctagg	ggctttcctgaaattgcattt
4c	attcatgaaaggcaactggg	ggaaatctctcaaaggaaac
4d	caagttttatttgaaaaagg	gcagtgacattaaacaactt
4e	aagcctatcaacgaatggtg	cctgatttgactgtagaatt
4f	ctctctgcagacaaaaaatcc	gcttgttctcaggctttgat
5	ctgataaaacccccaaacga	ggtgatcattttccaaacacag
6	ttgtgaaagttgttttagagtgcc	aagctggagtgcaatggc
7	gcccagccaataattgcata	actcaccattgtggcacaga
8	tgctaagcagactcgtgtag	gctagcacatgtatcgctaa
9	attcggttttttgagaggg	gg aagggatgatgcactatga
10	taaaaggggaagggatgatg	tctgaatttaccacctttgtcaga

Table 3: Primers sequences used in the dHPLC analysis of *hMSH6*. Sense strand primers and antisense strand primers also had the T7 and SP6 sequences attached, respectively

MYH Exon	*Sense Strand Primer 5'-3'*	*Antisense Strand Primer 5'-3'*
1	tgaaggctacctctgggaag	aggagacggaccgcaagt
2	ggctgggtctttttgtttca	gggccacaacctagttcctt
3a	ctgtgtcccaagaccctgat	ttggtcgtaccagcttagca
3b	agctgaagtcacagccttcc	cacccactgtccctgctc
4	cctccaccctaactcctcatc	aaagtggccctgctctcag
5	caggtcagcagtgtcctcat	gtctgacccatgacccttcc
6	gtctctttctgcctgcctgt	tcacccgtcagtccctctat

7	cgggtgatctctttgacctc	gttcctaccctcctgccatc
8	tcttgagtcttgcactccaatc	aaagtgggggtgggctgt
9	gctaactctttggcccctct	cacccttgttaccccaacat
10	ctgcttcacagcagtgttcc	gacttctcactgccccttcc
11	acactcaaccctgtgcctct	ggaatggggcttctgactg
12a	cttggcttgagtagggttcg	ggctgttccagaacacaggt
12b	gagtggtcaacttccccaga	cacgcccagtatccaggta
13	agggaatcggcagctgag	gctattccgctgctcactta
14	aggcctatttgaaccccttg	caacaaagacaacaaaggtagtgc
15	ccctcacctccctgtcttct	tgttcacccagacattcgtt
16a	ctacaaggcctccctccttc	gctgcactgttgaggctgt
16b	gccagcaagtcctggataat	acatagcgagacccccatct

Table 4: Primers sequences used in the dHPLC analysis of *hMYH*. Sense strand primers and antisense strand primers also had the T7 and SP6 sequences attached, respectively

APC MCR Exon	*Sense Strand Primer 5'-3'*	*Antisense Strand Primer 5'-3'*
1	tgcaaagtttcttctattaaccaa	atttaggtgacactattctgcttcctgtgtcgtctg
2	ttcattatcatctttgtcatcagc	atttaggtgacactattggaacttcgctcacaggat
3	gcagaaataaaagaaaagattggaa	atttaggtgacactatctttgtgcctggctgattct
4	ctagaaccaaatccag cagact	atttaggtgacactatgaacatagtgttcaggtggactttt
5	agcgaaatctccctccaaaa	atttaggtgacactatctggcaatggaacgactctc
6	cccactcatgtttagcagatg	atttaggtgacactatgtttgtccagggctatctgg
7	tggaatggtaagtggcattat	atttaggtgacactatcagcagtaggtgctttatttttaggg
8	tcctcaaacagctcaaacca	atttaggtgacactatagcatctggaagaacctgg

		a
9	a agcaagctgcagtaaatgct	atttaggtgacactatatggctcatcgaggctca
10	aagtactccagatggattttcttg	atttaggtgacaqctatggctgctctgattctgtttca
11	atgcctccagttcaggaaaa	atttaggtgacactattcaatatcatcatcatctgaat catc
12	aaaaactattgactctgaaaaggac	atttaggtgacactatggtggaggtaattttgaagca

Table 5: Primers sequences used in the dHPLC analysis of the *APC* mutation cluster region (MCR). Sense strand primers and antisense strand primers also had the T7 and SP6 sequences attached, respectively

Promoter Region	*Sense Strand Primer 5^{l}-3^{l}*	*Antisense Strand Primer 5^{l}-3^{l}*
MLH1 promoter region	agtagccgcttcaggga	ctcgtccagccgccgaataa
MSH2 promoter region	gctgagtaaacacagaaa	ctcctggttgaagaaaatgc

Table 6: Primers sequences used in the dHPLC analysis of the *hMLH1* and *hMSH2* promoter regions. Sense strand primers and antisense strand primers also had the T7 and SP6 sequences attached, respectively

Tables of melting temperatures used in dHPLC analysis

MLH1 Exon	*Tm 1 °C*	*Tm 2 °C*
1	62.3	63.1
2	56.1	59.1
3	55.6	57.6
4	55.7	58.7
5	55.8	58.8

6	56.1	58.1
7	56.0	57.0
8	53.8	57.8
9	56.9	57.9
10	58.6	59.0
11	57.7	62.7
12a	55.5	58.5
12b	59.5	60.5
13	57.7	59.7
14	58.1	59.1
15	56.7	57.7
16	57.5	59.5
17	58.0	60.0
18	56.8	59.8
19	55.5	58.5

Table 7: Melting temperatures (Tm°C) for *MLH1* heteroduplex fragment analysis

MSH2 Exon	*Tm 1 °C*	*Tm 2 °C*
1	65.7	66.1
2	55.0	56.0
3	57.4	59.4
4	50.6	53.6
5	53.1	57.1
6	55.5	58.5
7	54.2	57.2
8	54.7	57.7
9	56.4	57.4
10	55.9	57.9
11	55.0	56.0
12	56.0	59.0

13	55.8	57.8
14	55.7	57.7
15	57.7	58.3
16	55.3	57.3

Table 8: Melting temperatures (Tm°C) for *MSH2* heteroduplex fragment analysis

MSH6 Exon	*Tm 1 °C*	*Tm 2 °C*
1	65.3	67.3
2	56.2	60.2
3	58.1	60.1
4a	57.7	58.7
4b	58.2	59.2
4c	57.6	58.6
4d	57.6	58.6
4e	56.8	57.8
4f	57.7	58.1
5	57.3	58.3
6	55.1	56.1
7	53.9	54.9
8	56.1	57.1
9	55.5	57.5
10	54.0	57.0

Table 9: Melting temperatures (Tm°C) for *MSH6* heteroduplex fragment analysis

MYH Exon	*Tm 1 °C*	*Tm 2 °C*
1	62.7	66.7
2	60.9	63.9
3a	61.7	62.7
3b	62.5	64.5

4	61.4	62.4
5	61.7	63.7
6	60.4	63.4
7	60.7	64.7
8	61.1	66.1
9	60.9	64.9
10	61.5	65.5
11	62.2	64.2
12a	62.5	64.5
12b	62.1	65.1
13	63.6	65.6
14	59.6	63.6
15	59.6	63.6
16a	60.8	62.8
16b	56.3	61.3

Table 11: Melting temperatures (Tm°C) for *MYH* heteroduplex fragment analysis

APC Exon	*Tm 1 °C*	*Tm 2 °C*
1	54.3	56.3
2	57.6	58.1
3	58.0	60.5
4	59.4	60.0
5	59.7	60.5
6	59.1	59.6
7	58.5	60.5
8	58.5	60.5
9	59.2	60.2
10	58.0	58.8

11	56.1	57.9
12	55.5	57.8

Table 12: Melting temperatures (Tm°C) for *APC* mutation cluster region heteroduplex fragment analysis

Promoter Region	*Tm 1 °C*	*Tm 2 °C*
MLH1 promoter region	53.3	55.3
MSH2 promoter region	56.7	58.7

Table 13: Melting temperatures (Tm°C) for Promoter Region region heteroduplex fragment analysis

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Appendix II

Optimization of the Laser Capture Microdissection (LCM) protocol for use in the screening of patients with familial colorectal cancer syndromes

This procedure was optimised for use in the Human Genetics Lab
by Anna M Russell

Introduction

A long standing hurdle for researchers in the field of cancer genetics is the difficulty in characterising molecular changes that result during cancer progression, where specific normal epithelial cells become premalignent cells and then further transform into invasive and metastatic cancer (1 Liotta 2000). In attempts to understand the molecular forces driving such an evolution, comparisons are made between samples taken from the healthy appearing epithelium, the premalignant cells, and the invasive carcinoma, all from the same tissue sample, taken from the same patient. This enables fluctuations of expressed genes or alterations in the cellular DNA to be correlated to the transition from one disease stage to the next. However, for this to be accomplished, it is desirable to sample pure cells in different stages of cancer development without the contamination of neighbouring, non specific cells.

The procurement of pure cells from specific microscopic regions of tissue sections is achievable by the method of Microdissection. Tissue samples are heterogenous and complicated structures with many different cell types interlocked in morphologic units with dense adhesive interactions with adjacent cells, connective stroma, blood vessels, glandular and muscle structures,

adipose cells, and inflammatory or immune cells. The diseased cells of interest are surrounded by these heterogenous tissue compositions and epithelial cells, precancerous cells or invading cancer cells may account for less than 5% of the total volume of the tissue biopsy sample. Hence, microdissection is paramount to the study of evolving tissue lesions in healthy tissues.

A new technology, known as Laser Capture Microdissection (LCM) was initiated by NIH (2 Emmert-Buck, 1996) and subsequently commercially developed through a Collaborative Research and Development Agreement (CRADA) partnership with Arcturus Engineering Inc. (650-962-3020).

The theory behind LCM is very simple and has been developed to provide a fast and dependable method of capturing and preserving specific cells from tissue, under direct microscopic visualisation. A laser beam and special transfer film are used to extract a microscopic homogeneous cellular subpopulation from its complex tissue milieu. This subpopulation can then be compared with adjacent interacting, but distinct, subpopulations of cells in the same tissue.

The LCM method affords a number of advantages.

1. Under microscopic direction it is possible to separate multiple identical cells, and catapult only those cells of interest. The rest of the tissue remains intact and ready for further dissection.
2. The elegance of this technique is that no tissue is destroyed in the process. LCM operates by positive rather than negative selection. LCM creates no chemical bonds to the targeted tissue that may alter subsequent molecular analysis.
3. The morphology of the transferred cells is preserved and can be readily visualised under the microscope.
4. Targeting precision of cells is 1μm with the targeted spots as small as 3-5μm.
5. The user can capture from 1000-3000 shots on one transfer cap.

6. Depending on the size of the cells the total number of captured cells can be up to 6000.
7. As each shot takes less than a second to perform, a large number of pure cells can be captured from a heterogeneous tissue sample in a very short period of time.

However, it should be noted that the LCM method employs specialised, expensive technology and requires training and practice for protocol optimisation.

Figure 1: The Laser Capture Microdissection system and methodology

A number of different molecular analyses have been conducted successfully on cells procured by LCM. These include genomic analyses such as loss of heterozygosity analysis, restriction fragment length polymorphism analysis, DNA methylation analysis, fluorescence in situ hybridization, and comparative genomic hybridization.

Gene expression analysis (ie. RNA analysis) has been achieved from LCM samples using reverse transcription PCR, the construction of cDNA libraries and differential hybridization on high density spotted nylon filters, glass microarrays, and recently on high density oligonucleotide arrays after amplification of RNA.
Successful proteomic analysis has been performed by carrying out LCM in conjunction with western immunoblotting, solid-phase sequential chemiluminescent immunometric assay, and one dimentional and two dimentional polyacrylamide gel electrophoresis (PAGE). See references for the above application details.

With the LCM technique established in our laboratory for colorectal tumour tissue samples, we aim to isolate pure microscopic clusters of cells from the colon and rectum, in order to investigate the clonal evolution, both inter-tumoral and also between patients.

Methods

Preparation of Slides for LCM: *in flow cabinet:*

1. Dip slides in 100% EtOH
2. Place the LCM membrane directly ontop of slide NB. to facilitate membrane mounting without wrinkles, the membrane should be smaller than the object slide and the slide should be wet with alcohol
3. With backing paper from the membrane, smooth out membrane creases
4. Leave to dry well

5. Apply glue (Entellan) to 2 opposite membrane edges (top + bottom of slide). Use a pipette with tip
6. Leave to dry well
7. Apply ca. 20ul of poly-L-lysine to the membrane and spread carefully with a pipette tip NB. avoid any leakage underneath the membrane, as this may result in problems with the LCM later.

Tissue Sectioning:

- Fix paraffin tissue blocks securely into the Microtome
- Cut tissue sections 10μm thick
- Allow sections to settle in a heated waterbath (40°C)
- Transfer the sections from the waterbath onto the LCM pre-prepared slides (see above)
- Smooth out the sections to remove wrinkles and aid adhesion to slides
- Allow to dry overnight at room temperature and in flow cabinet
-

    - Some histopahthology labs use an adhesive in the water bath to better adhere the tissue section to the slide, but this may result in reduced LCM transfer of tissue. Also, baking the sample onto the slide may bond it too strongly and prevent LCM transfer.
    - Careful attention should be given during sectioning to prevent 'carryover'. Carryover contamination of one specimen from another or transfer of material from one region of a section to another can lead to spurious results. The microtome used to cut sections should be kept clean and excess paraffin and tissue fragments should be wiped from the area with xylene between each block. Alternatively, a fresh microtome blade should be used for each block.

Tissue Staining:

Deparaffinization:

Xylene	2 x 30s
Ethanol absolute	1.5 min
Ethanol 96%	1.5 min
Ethanol 70%	1.5 min

Use a pipette to carefully wash away alcohol with dH_2O (take care not to wash dissolving glue from the membrane over the tissue section)

Staining:

Toluidine Blue	10s

Rinse with water, again using a pipette

Fixation:

Ethanol 96%	30s
Ethanol 96%	30s
Ethanol absolute	30s
Ethanol absolute	30s

Allow sections to dry for at least 30 minutes (preferable overnight) at 37°C before attempting LCM.

Laser Capture Microdissection:

1. The operator is able to view the tissue and select the desired microscopic clusters of cells for analysis. This is done by drawing around the desired cell(s) with the mouse pointer (Figure 1)
2. For laser microbeam microdissection the objective lens of the microscope converges the laser light to produce extremely high density focal energy. The focused laser beam induces a localised photodecomposition, without

detectable heating, ablating the tissue in the narrow focal point without damage to the surrounding tissue (Figure 2)

3. By increasing the light intensity and delivering a pulse of laser energy just below the focal plane of the dissected specimen, the energy pulse created, drives the specimen up and out of the section into a waiting collection vessel (Figure 3)
4. By re-focusing the microscope lense, the catapulted cell(s) can be visualised in the collection cap above the slide. If the tissue sample has been stained and there is solution in the collection cap, it is possible that the solution has changed colour with the dye, confirming the cell(s) was/were successfully captured.

Table 1: Values for microdissection of 10 μm sections of colorectal tumour tissue stained with Toludine Blue and mounted on LCM membrane covered slides.

LCM Values on Software Program	*Values for cutting colorectal tumour tissue*	*Values for catapulting cell(s)*
Cut Focus	80 micron diameters	70 micron diameters
Cut Energy	70 micron diameters	100 micron diameters

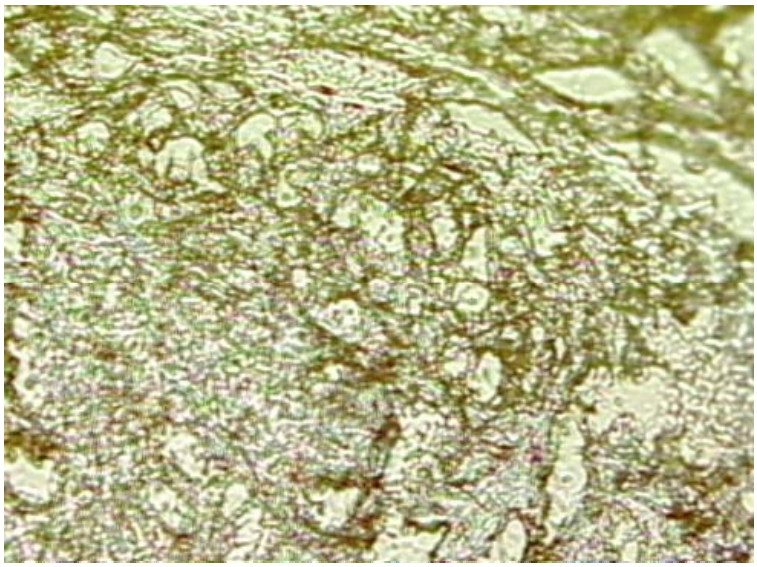

Figure 1:
The operator views the tissue section (colorectal tumour), under the microscope and selects the desired cluster of cells to be catapulted

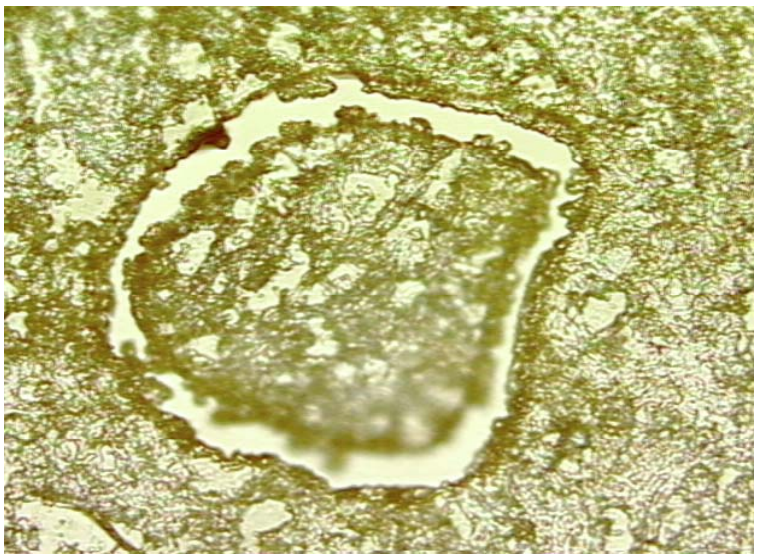

Figure 2:
The focused laser beam induces a localised photodecomposition ablating the tissue in the narrow focal point without damaging the surrounding tissue. The desired cluster of cells are ready to be catapulted.

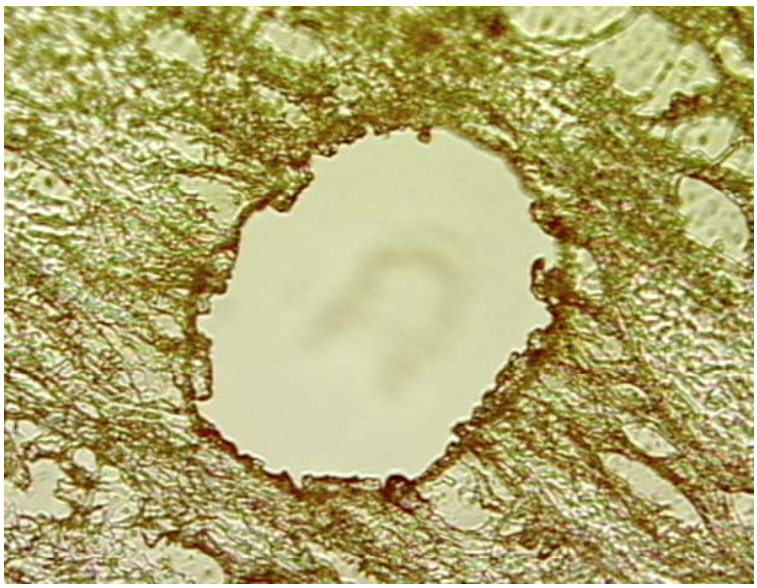

Figure 3:
By increasing the light intensity and delivering a pulse of laser energy just below the focal plane of the dissected specimen, the energy pulse created, drives the specimen up and out of the section into a waiting collection vessal.

*All actual pictures taken during optimization procedure: well differentiated colorectal carcinomas from the sigma.

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PERSONAL INFORMATION

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PROFILE

- Conversant with many molecular biology techniques. Computer literate in a number of software applications
- Able to devise experimental procedures with careful, independent planning, conduct them with precision, monitor their progress and analyse all results thoroughly and critically
- Proven ability to work both independently and within a team, under pressure
- Skilled in communication and presentations, also at international conference level. Have conducted and taken part in numerous different collaborations in both research and diagnostics
- Scientific writing skills acquired through preparation of protocols, clinical reports and scientific research papers (published)
- Leadership and management skills gained by assisting the running of the Hereditary Colon Cancer diagnostics laboratory, Basel, CH
- Friendly, humorous and easygoing nature although hardworking, focused and dedicated. Flexible and self motivated. Ability to adapt quickly to new situations and learn rapidly

EDUCATION

- Rugby High Grammar School for Girls, Rugby, England. 1988-1994
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SUMMARY OF QUALIFICATIONS

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PUBLICATIONS

- **Anna Marie Russell** and Giancarlo Marra. Inherited Colon Cancer. Swiss Cancer Research Bulletin, Dec.2001, vol.4, pg166-169
- Hansjakob Müller, Martina Plasilova, **Anna Marie Russell**, Karl Heinimann. Genetic Predisposition as a Basis for Chemoprevention, Surgical and Other Interventions in Colorectal Cancer. Recent Results in Cancer Research, 2003, vol.163, pg235-247
- Petr Cejka, Lovorka Stojic, **Anna Marie Russell**, Karl Heinimann, Giancarlo Marra and Josef Jiricny. Methylation Induced G2/M Arrest Requires a Full Complement of the Mismatch Repair Protein hMLH1. The EMBO Journal, 2003, vol.22, No.9, pg2245-2254
- **Anna Marie Russell,** Martina Plasilova, Angela Wolf, Zuzanna Dobbie, Hansjakob Müller and Karl Heinimann. Exclusion of a Modifier Gene Locus on Chromosome 1p33-36 in a Large Swiss Familial Adenomatous Polyposis Kindred. European Journal of Human Genetics, 2004, vol 12 pg365-371
- **Anna Marie Russell,** Jian Zhang, Pierre Hutter, Pierre Chapuis, Oliver Sieber, Laura Lipton, Hansjakob Müller and Karl Heinimann. Prevalence of MYH germline Mutations in Swiss APC Mutation Negative Polyposis Patients. Paper prepared for publication, August 2004
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POSTER PRESENTATIONS

- Karl Heinimann, **Anna Marie Russell**, Giancarlo Marra, Josef Jiricny, Hansjakob Müller and Zuzanna Dobbie. Evaluation of Referral Criteria and Diagnostic Testing in a Prospective study on Hereditary Nonpolyposis Colorectal Cancer Kindreds. 12th ICG-HNPCC Meeting, Galilee, Israel, Sept.20-24th 2000.

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