HEREDITARY COLORECTAL CANCER:
ASSESSMENT OF GENOTYPE-PHENOTYPE CORRELATIONS AND ANALYSIS OF RARE SUSCEPTIBILITY GENES IN FAMILIAL ADENOMATOUS POLYPOSIS (FAP) AND HEREDITARY NONPOLYPOSIS COLORECTAL CANCER (HNPCC)

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TABLE OF CONTENTS

ABBREVIATIONS

AC	Amsterdam criteria
ACF	Aberrant crypt foci
AFAP	Attenuated familial adenomatous polyposis
APC	Adenomatous polyposis coli
ATP	Adenine tri-phosphate
BER	Base excision repair
BG	Bethesda guidelines
CHRPE	Congenital hyperpigmentation of the retinal pigment epithel
CRC	Colorectal cancer
dHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribo nucleic acid
FAP	Familial adenomatous polyposis
hMLH	Human MutL homolog
hMSH	Human MutS homolog
hPMS	Human post meiotic segregation
HNPCC	Hereditary nonpolyposis colorectal cancer
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
MCR	Mutation cluster region
MLPA	Multiplex ligation-dependant probe amplification
MMR	Mismatch repair
MSI	Microsatellite instability
NCI	National cancer institute
PCR	Polymerase chain reaction
PTT	Protein truncation test

ABSTRACT

Each year 3500 people in Switzerland are diagnosed with colorectal cancer. Approximately 20 percent of all affected patients have two or more first or second-degree relatives with colorectal cancer (at-risk family members). About five percent of these are inherited in an autosomal dominant manner. This thesis has focused on genotype-phenotype correlations in two hereditary colorectal cancer syndromes, familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). In addition, rare susceptibility genes were analyzed: *MYH* in FAP and *PMS2* and *MSH3* in HNPCC. The works encompassed investigations of a consecutive series of 101 Swiss polyposis patients and establishment of genotype-phenotype correlations, delineation of somatic *APC* alterations in attenuated familial adenomatous polyposis (AFAP), genetic characterization of the *MYH* gene recently associated with a multiple colorectal adenoma and carcinoma phenotype, and finally, the assessment of the role of rarely mutated mismatch repair genes *PMS2* and *MSH3* in HNPCC.

In the first part of the thesis, phenotypic differences between *APC* germline mutation carriers and *APC/MYH* mutation-negative individuals in a consecutive cohort of 101 FAP patients were characterized. Furthermore, we wanted to assess possible genotype-phenotype correlations in *APC* mutation carriers. In our study population, no genotype-phenotype correlations with regard to polyp number or extracolonic disease manifestations could be established. The data challenge the prevailing view on genotype-phenotype correlations and advise great caution when basing clinical management decisions for an individual patient on the site of the *APC* germline mutation.

In the second part of the thesis 235 tumors from 35 AFAP patients out of 16 families were screened for *APC* mutations to find out the somatic *APC* mutation spectrum, to determine phenotypic differences among AFAP families, and to delineate the pathways of somatic *APC* mutation in AFAP. It has been shown that colonic polyp number varies greatly among AFAP patients, but members of the same family tended to have more similar disease severity. 5'-mutants generally had more polyps than the other

patients. In some polyps bi-allelic changes ("third hits") have been found, which probably initiated tumorigenesis. Taken together, AFAP is phenotypically and genetically heterogeneous and modifier genes may be acting on the AFAP phenotype.

Biallelic changes in the *MYH* gene have been shown to predispose to a multiple adenoma and carcinoma phenotype. In the third part of the thesis, 79 unrelated *APC*-negative Swiss polyposis patients were screened for germline mutations in *MYH* to assess the frequency of *MYH* mutations and to identify phenotypic differences between *MYH* mutation carriers and *APC/MYH* mutation-negative polyposis patients. Colorectal cancer was significantly more frequent in biallelic as compared to monoallelic mutation carriers or those without *MYH* alterations. With regard to other phenotypic properties (age of onset, extracolonic disease manifestations), it is virtually impossible to discriminate biallelic from monoallelic *MYH* mutation carriers and *MYH* mutation-negative polyposis patients.

In HNPCC alterations in *PMS2* have been documented only in extremely rare cases. In the fourth part of the thesis, DNAs of colorectal cancer patients with immunohistochemically proven loss of PMS2 in the tumor (n = 16) were screened for *PMS2* germline mutations. It was possible to identify heterozygous *PMS2* germline mutations in six patients. To detect germline mutations in the remaining 10 patients, additional mutation screening methods (cDNA sequencing and MLPA technique) have been applied. In conclusion it was shown that PMS2 defects account for a small but significant proportion of CRCs.

In the fifth part of the thesis *MSH3*, a MMR gene, which has thus far not been implicated in HNPCC, has been investigated in a 46 years old colorectal cancer patient with immunohistochemical loss of MSH3 only. A *MSH3* missense mutation (c.2383C>T, p.Arg795Trp) was identified and the possible pathogenicity of the alteration was assessed. It was found that the mutation is present in a hemizygous state in the tumor. Furthermore, 100 healthy probands did not carry the alteration and sequence and amino acid alignment with vertebrates showed that it is located in a conserved region of the gene. Taken together, our findings indicate that the alteration in *MSH3* may indeed be pathogenic.

INTRODUCTION

This thesis aimed to investigate genotype-phenotype correlations and to assess the role of rare susceptibility genes in the two most common hereditary colorectal cancer predispositions, familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC).

In the first study of this thesis genotype-phenotype correlations were investigated in a consecutive cohort of 101 Swiss FAP patients. Differences between adenomatous polyposis coli (*APC)* germline mutation carriers and *APC/MYH* mutation-negative individuals were characterized and possible genotype-phenotype correlations in *APC* mutation carriers were assessed. The manuscript will be submitted for publication.

The second study describes the analysis of somatic *APC* mutations in 235 tumors from 35 AFAP patients (out of 16 families). The main goals of this study were to characterize the somatic APC mutation spectrum in AFAP patients with 3'-mutations, to compare this with other AFAP-associated regions of *APC,* and to delineate the pathways of somatic *APC* mutations in AFAP. It was found that disease severity and genetic pathways vary greatly but depend on the site of the germline mutation. This study was published in Gut, volume 55, no. 10, pages 1440 – 1448, 2006.

Recently, another FAP susceptibility gene besides *APC* was identified. It was shown 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.

This third part of this thesis aimed to assess the frequency of *MYH* mutation carriers within a Swiss cohort of 79 unrelated polyposis patients. In addition, comparisons have been made between *MYH* mutation carriers and *APC* mutation-negative individuals to establish genotype-phenotype correlations in this cohort of patients. The results of the study were published

in the International Journal of Cancer, Volume 118, No. 8, p.1937 – 1940, 2006.

In HNPCC the main susceptibility genes are *MLH1* and *MSH2*. But it is known that also mutations in other MMR genes like *MSH6* and *PMS2* contribute to the disease. The role of MSH3, the heterodimeric partner of MSH2 in the MutSβ complex, in HNPCC is not known so far.

In the fourth study of this thesis, a collaborative study with G. Marra, Institute of Molecular Cancer Research Zurich, 1048 tumors from 5 Swiss hospitals were collected and immunohistochemically tested for loss of MLH1, MSH2, MSH6 and PMS2. Our part of the collaboration included the molecular genetic screening of patients with loss of MMR proteins, in particular the technically demanding screening for *PMS2* germline mutations. The identified high frequency of patients affected by cancers with a primary defect of PMS2 strengthens the role of PMS2 in CRC. This study was published in Gastroenterology, Volume 128, No. 5 p.1160 – 1171, 2005.

The addendum to this study describes further screening efforts for mutations using additional methods in patients with loss of PMS2 in their tumors but without identified *PMS2* germline mutation. cDNA analysis was applied to identify mutations that were possibly masked by the presence of pseudogenes and multiplex ligation dependant probe amplification (MLPA) was used to detect large genomic insertions or deletions.

The fifth study aimed to find out more about the role of the MMR repair gene *MSH3* in HNPCC. It was possible to detect a germline missense mutation in *MSH3* in a CRC patient with immunohistochemical exclusively loss of MSH3 in the tumor. The missense mutation (p.Arg795Trp) was present in a hemizygous state in the tumor of the patient and LOH analysis indicates loss of the wildtype allele. In addition, the missense mutation was not identified in 100 healthy control patients. Thus, there is good evidence that the identified missense mutation may actually have a pathogenic meaning.

1. GENERAL INTRODUCTION

Colorectal cancer incidence

Worldwide more than 10 million people develop cancer each year with approximately 1 million of them being diagnosed of colorectal cancer (www.krebshilfe.de). In industrialized countries colorectal cancer is the third most frequently observed cancer in men (after lung and prostate cancers) and the second most in women (after breast cancer). The lifetime risk in the general population for developing colorectal cancer is 5% but by the age of 70 years almost half of the Western population will have developed an adenoma. In Switzerland, about 3500 people are diagnosed of colorectal cancer each year (Swiss Cancer Registries' Association Database, 2003).

Colorectal carcinogenesis

Colorectal carcinogenesis is one of the best studied cancer models how normal cells become tumor cells due to two main reasons: 1) Most colorectal tumors develop within more than 10 years in histologically well-defined steps. 2) The colon is well observable by colonoscopy and different cancer stages can be easily identified[1].

Different stages of colorectal tumorigenesis:

1. Aberrant crypt foci (ACF)

   are clusters of abnormal tube-like glands in the colon and rectum and can be dysplastic or nondysplastic. Methylene blue staining or microscopic examination of the colonic mucosa can detect these lesions. ACFs are one of the earliest changes visible in the colon that may lead to cancer.

2. Adenomas

   Adenomas are believed to develop from dysplastic aberrant crypt foci. They are often referred to as adenomatous polyps. There are different types of adenomas: tubular, tubular-villous, and villous adenomas. Most of carcinomas develop from villous adenomas.

3. Carcinomas

Carcinomas are thought to develop from adenomas and are therefore called adenocarcinomas. These lesions are highly dysplastic and invade the surrounding tissue. The different stages of adenocarcinomas (Dukes stages, see appendix) are very important for the prognosis. Main factors for the classifications are the grade of infiltration into the tissue and the presence or absence of metastasis.

During the development from a normal colonocyt to a cancer cell additional mutations in oncogenes and tumor suppressor genes give rise to clonal expansion (Figure 1). It is thought that at least 4 sequential genetic changes are necessary for colorectal cancer evolution. Primary targets for these genetic changes are *KRAS*, *APC*, *SMAD4* and *TP53*[2,3].
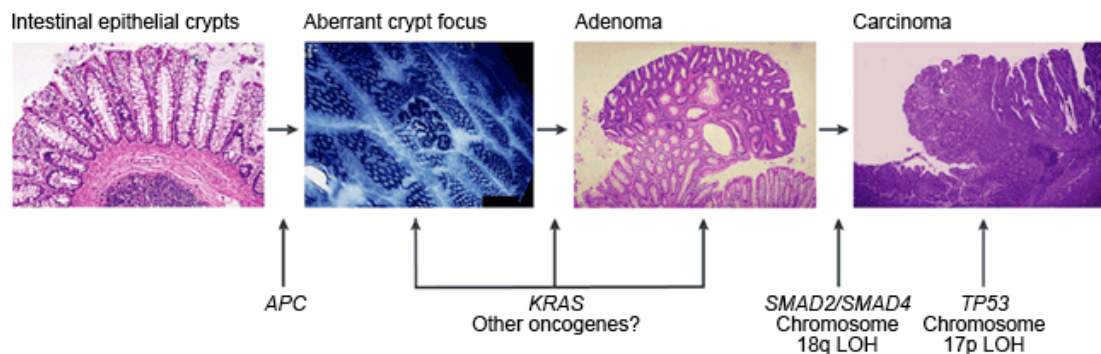
Figure 1: **Histopathology of colorectal cancer and accumulation of different mutations during cancerogenesis (from Fodde et al.[4])**

Genetic basics of colorectal cancer

In most of the colorectal cancer patients (approximately 80%) the disease appears to have occurred sporadically due to environmental or dietary factors. In the remaining 20% CRC seems to be attributed to a definable genetic component[5]. Evidence for a genetic factor in colorectal cancer is given in persons with a familial aggregation of colorectal cancer consistent with autosomal dominant inheritance. In addition, it has been demonstrated recently that biallelic germline mutations in the human homologue of the base excision repair gene *MutY* (*MYH*) cause colorectal carcinomas, thus describing an autosomal recessively inherited CRC predisposition[6,7].

There are two well-defined autosomal dominant inherited colorectal cancer predisposition syndromes: familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). FAP is estimated to account for less than 1%[8] and HNPCC for 2 – 5%[9] of all colorectal cancers in Western countries. Other colorectal cancer predispositions include e.g. the juvenile polyposis syndrome and the Peutz-Jeghers-syndrome. But there are still many familial aggregations of colon cancer remaining etiologically undefined.

In this thesis the main focus is on the assessment of genotype-phenotype correlations in FAP and HNPCC and on the molecular analysis of rarely mutated genes in these colorectal cancer predispositions.

Familial adenomatous polyposis (FAP) and attenuated familial adenomatous polyposis (AFAP)

FAP is an autosomal dominantly inherited colorectal cancer predisposition, which accounts for ca. 1% of all colorectal cancers. The syndrome is caused by germline mutations in the *adenomatous polyposis coli (APC)* gene. *APC* mutation carriers typically develop hundreds to thousands of polyps throughout the rectum and the colon and, if left untreated, colorectal cancer in the third or fourth decade of life[10]. Patients frequently develop benign extracolonic lesions including polyps of the gastric fundus and duodenum, osteomas, dental anomalies, congenital hypertrophy of the retinal pigment epithelium (CHRPE), soft tissue tumors, and desmoid tumors. In addition, several extracolonic cancers occur with a higher incidence in FAP than in the general population[11]. These cancers include tumors of the upper gastrointestinal tract, liver, thyroid and adrenal gland, pancreas, and brain[12-14].

Attenuated familial adenomatous polyposis (AFAP) is characterized by a significant risk of colorectal cancer but patients usually develop fewer than 100 and more proximally located colonic polyps often detected at later age compared to classical FAP (reviewed in[15]).

As mentioned above, most of FAP cases are attributed to germline mutations in the *APC* gene. It is located on chromosome 5q21 consisting of 15 exons which is part of the Wnt signaling pathway (see below). The tumor suppressor

gene or "gatekeeper" encodes a protein essential in cell adhesion, signal transduction and transcriptional activation, with c-myc and β-catenin having established as downstream targets[16]. The majority of *APC* germline mutations occur in the first half of the APC coding region[17] and to date almost 700 different alterations have been described according to the human genome mutation database (http://www.hgmd.cf.ac.uk/ac/index.php). About 95% of all detected alterations are insertions, deletions and nonsense mutations leading to a frameshift or a premature stop codon and result in the truncation of the APC protein[18].

Much effort has been done into making genotype-phenotype correlations that link the site of the germline mutation with the severity of the disease. Profuse polyposis has been associated with *APC* germline mutations between codons 1240 and 1464[19]. In contrast, patients present more often with attenuated FAP if they carry the mutation at the extreme 5' end (codons 1 – 177)[20-23], the alternatively spliced exon 9 (codons 312 – 412)[24-26], and the 3' end (codons >1580)[23,27,28]. In addition, certain extracolonic manifestations as desmoid tumors or CHRPE have been correlated with alterations between codons 1403 and 1578 or 463 and 1387, respectively[29-31]. However, even in patients with identical germline alterations there is a high inter- and intrafamilial phenotypic variability in the individual patients[32,33].

The canonical Wnt-pathway

The Wnt signaling pathway is highly conserved and controls numerous decisions during animal development. In unstimulated cells, free β-catenin is destabilized after binding to axin, conductin, glycogen synthase kinase 3β (GSK3β) and APC[34-37] (Figure 2). The main tumor suppressor function of APC resides in its capacity to properly regulate intracellular β-catenin levels[38-40]. As mentioned above, most *APC* mutations result in truncated proteins that lack all axin/conduction-binding motifs and a variable number of the 20-amino-acid repeats that are associated with the downregulation of intracellular β-catenin levels[41,42]. Therefore β-catenin is accumulated due to *APC* mutations, diffuse into the nucleus, where it acts as a co-activator for TCF-responsive genes.
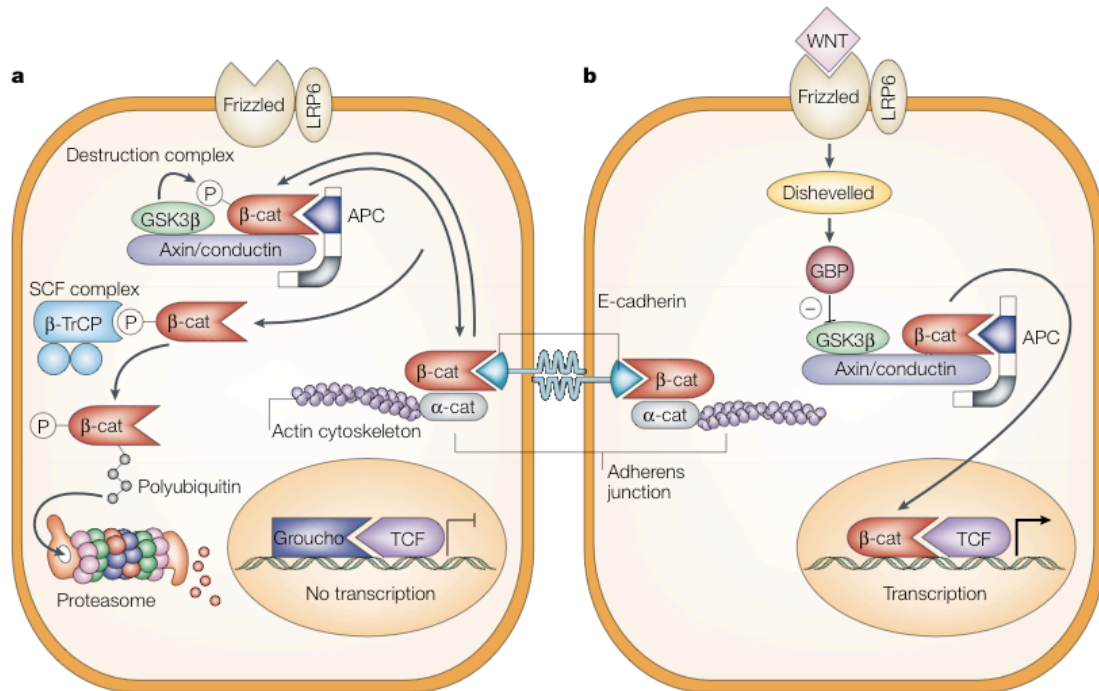
Figure 2:   Model of the wnt-signaling pathway (from Fodde et al.[4]). a) in the absence of a Wnt signal β-catenin is degraded b) in the presence of a wnt signal β-catenin is accumulated.

MYH associated polyposis (MAP)

The phenotype of MAP is similar to FAP and AFAP, but the disorder is inherited in an autosomal recessive manner. MAP is caused by biallelic mutations in the human homolog of the *E. coli* base excision repair gene *mutY* (*MYH*)[6] and has been shown to account for a substantial portion (10 – 30%) of *APC* mutation-negative polyposis patients[24]. The DNA glycosylase MYH is part of the base excision repair (BER) pathway and 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[43].

Base Excision Repair (BER)

BER is a pathway that corrects DNA modifications that arise either spontaneously or from attack by reactive chemicals, e.g. reactive oxygen species (superoxide, hydroxyl peroxide). 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).

In the prevention of 8-oxoG induced mutagenesis, proteins from three 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 oxidized 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[44].
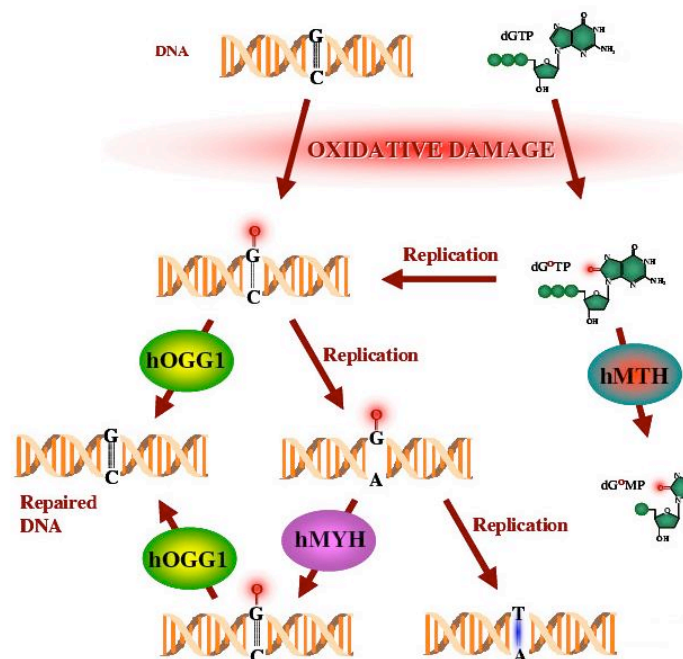
Figure 3: **Base excision repair pathways for oxidative DNA damage. *hOgg1*, *hMYH*, and *hMTH* and their respective repair function[45].**

Hereditary nonpolyposis colorectal cancer (HNPCC)

Hereditary nonpolyposis colorectal cancer (HNPCC) is characterized by an increased risk of colon cancer and other cancers that include cancers of the endometrium, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin. Individuals with HNPCC have an approximately 80% lifetime risk for colon cancer. Two-thirds of these cancers occur in the proximal colon. The average age of colorectal cancer diagnosis is 44 years.

In 1990, the international collaborative group on hereditary nonpolyposis colorectal cancer established the Amsterdam criteria to identify HNPCC

families and to detect susceptibility genes for the disease. These criteria were thought to be too restrictive for clinical purposes and were later modified (Amsterdam criteria II) to include the other HNPCC-related cancers. To suit the Amsterdam criteria I or II (see appendix), all the criteria must be fulfilled.

To identify those individuals whose tumors are candidates for MSI testing, the Bethesda guidelines (see appendix) were developed in 1997[46] and later updated to increase their sensitivity[47]. To fulfill the Bethesda guidelines, just one of the criteria need to be met.

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[48], *hMLH1* (human mutL homolog 1) on chromosome 3p21[49], *hMSH6* (human mutS homolog 6) on chromosome 2p16[50], and *hPMS2* (human postmeiotic segregation 2) on chromosome 7q11[51]. The majority of HNPCC-causing mutations are estimated to affect *hMLH1* in 50% of the families, *hMSH2* in 39%, *hMSH6* in 7% and *hPMS2* in occasional families[52].

Genetic testing for HNPCC

1) Microsatellite instability

Genes in the MMR pathway are responsible for identifying and repairing single nucleotide mismatches and insertion and/or deletion loops that occur due to DNA polymerase errors introduced to genomic DNA during replication. A defect in the MMR genes leads to multiple errors in repetitive DNA sequences (microsatellites) throughout the genome of tumors[53]. Microsatellites are stretches of DNA with a repetitive sequence of nucleotides (e.g. AAAAA or CGCGCGCG), and these regions of DNA are particularly susceptible to acquiring errors when mismatch repair gene function is impaired. This form of genomic instability is called microsatellite instability (MSI) and is the hallmark of HNPCC. In general, a panel of six microsatellite loci (BAT25, BAT26, BAT40, D5S346, D17S250 and D2S123) recommended by the national cancer institute (NCI) workshop is used to assess microsatellite instability[47]. Matched tumor and normal DNA extracted from formalin fixed tissue and blood, respectively, are analyzed for differences in

the length in the microsatellite motifs. A tumor is classified as MSI-high if at least 30% of the markers show MSI, as MSI-low if only one Marker shows MSI and as MS-stable (MSS) if no MSI can be detected[54]. Approximately 90% of colon cancer families matching Amsterdam criteria carry MSI-high tumors[55,56]. Therefore identifying MSI in a tumor has been found to be a good predictor of an underlying germline mismatch repair mutation[57] and any case with any tumor showing MSI is referred for further testing.

2) Immunohistochemistry

Immunohistochemistry (IHC) is a simple and effective method to screen the loss of one or more protein of the mismatch repair system in the tumor. This loss occurs due to two events. First the germline mutation of a MMR gene on one allele followed by a second somatic event on the remaining wildtype allele (second mutation or loss of heterozygosity). Normal mucosa and tumor tissue from patients suspicious for HNPCC are investigated for loss of hMLH1, hMSH2, hMSH6 and hPMS2. The loss of expression of one of these proteins suggests which MMR gene should be investigated for a germline mutation[58-60].

3) Mutation screening

After IHC analysis the respective gene will be screened for a germline mutation using direct sequencing of exons and exon/intron boundaries to detect point mutations and small insertions or deletions. Large genomic deletions or insertions can be detected by using the recently introduced multiplex ligation-dependant probe amplification (MLPA) assay.

Mismatch repair

DNA mismatch repair (MMR) is responsible for the recognition and repair of mispaired nucleotides. Mispaired bases in DNA can occur as a result of chemical or physical DNA damage, polymerase errors during DNA replication, or recombination between non-homologous parental DNA sequences. The single steps of MMR have been conserved throughout evolution of eukaryotes and have been most extensively studied in *E. coli*.

The first step is the recognition of the mismatch mediated primarily of the MutSα complex consisting of the heterodimer hMSH2 and hMSH6 (Figure 4A). This protein complex efficiently recognizes and binds to base/base mispairs, but its affinity for loops of more than one extrahelical nucleotide is relatively low[50]. A third MutS homologue, hMSH3, is also able to form a heterodimer with hMSH2, the MutSβ complex. This complex efficiently recognizes small insertion and deletion loops[61]. Recognition of mismatched nucleotides provokes ADP for ATP exchange by MutS that defines it as a Molecular Switch. ATP binding by MutS results in the formation of a hydrolysis-independent sliding clamp that is capable of diffusion for at least 1kb along the DNA adjacent to the mismatch. Following the dissociation/diffusion of one ATP-bound MutS sliding clamp the mismatch site is exposed to iterative lading of multiple MutS sliding clamps (Figure 4B). The mismatch bound MutSα complex then recruits another protein heterodimer, the MutLα complex consisting of hMLH1 and hPMS2. Fishel et al. found that the MutL protein only interacts with ATP-bound MutS sliding clamps (Figure 4C). The resulting sliding clamp complex of 4 proteins (hMSH2, hMSH6, hMLH1 and hPMS2) diffuses along the DNA backbone until it encounters a "downstream effector" that drives ATP-binding by the MutL protein. In eukaryotes, the first downstream effector is likely to be the PCNA-polymerase complex on the leading strand (Figure 4D). The goal of these interactions is to identify and/or to introduce a strand scission on the newly replicated DNA strand. The next downstream effector in MMR is likely to be a helicase that recognizes and begins to displace the incised DNA strand (Figure 4E). This would require a protein displacement event on the leading strand. Concerted displacement of the newly replicated strand provides a ssDNA substrate for an exonuclase (hEXO1) responsible for degradation of the error-containing strand until the mismatch has been removed (Figure 4F). The combined actions of MSH-MLH-(Helicase)-Exonuclease results in excision of the newly-replicated strand (Figure 4G). A new error-free DNA strand can then made by DNA polymerase with the help of other proteins (Figure 4H)[62].

A — Mismatch recognition by the MutSα complex (MSH)

B — Clamp formation and signal transfer for MutLα (MLH) recruitment

C — Building of a sliding clamp complex consisting of MutSα and MutLα

D — PCNA polymerase introduce a strand scission

E — A helicase displaces the incised DNA strand

F — Error-containing strand is degraded by exonuclease ExoI

G — Excision of the newly-replicated strand

H — DNA polymerase makes a new error-free DNA strand

Figure 4: **Molecular Switch Model for MMR by Fishel and Schmutte (from http://mmr.med.ohio-state.edu/rfishel/RF2.html and Fishel, 1998[62])**

2. ABSENCE OF GENOTYPE-PHENOTYPE CORRELATIONS IN A CONSECUTIVE SERIES OF PATIENTS WITH FAMILIAL ADENOMATOUS POLYPOSIS

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Prepared for scientific publication

ABSTRACT

In about 20-50% of familial adenomatous polyposis (FAP) patients worldwide no germline mutation in the *adenomatous polyposis coli* (*APC)* gene can be identified. In patients carrying a pathogenic *APC* mutation the position of the alteration has been associated with polyp burden and/or extracolonic disease. By screening a consecutive series of 101 unrelated polyposis patients for *APC/MUTYH* mutations the study aimed i) to compare the phenotypic properties of *APC* mutation carriers with those of *APC*/*MUTYH* mutation-negative polyposis patients and ii) to assess possible genotype-phenotype correlations in *APC* mutation carriers. Patients were screened for mutations in *APC*, applying the protein truncation test, DNA sequencing and gene copy number analysis. *APC* alterations were identified in 56 (63%) polyposis patients (76% with classical and 54% with attenuated polyposis), with 30% of them representing novel mutations. Compared to *APC*/*MUTYH* mutation-negative polyposis patients (51.0 years), *APC* mutation carriers displayed a significantly younger median age at diagnosis (40.0 years; p=0.0002). Twenty-two (48%) patients with an *APC* mutation within the "classical polyposis region" actually displayed an attenuated phenotype (AFAP), independent of age and family history. Similarly, four (40%) out of 10 patients with an "AFAP region" mutation presented with profuse polyposis. In summary, *APC* mutation carriers significantly differ from *APC/MUTYH* mutation-negative polyposis patients with regard to age at diagnosis and polyp number. The fact that no evidence for genotype-phenotype correlations could be observed in this cohort of *APC* mutation carriers advises caution when basing clinical management in an individual patient on the site of the *APC* germline mutation.

INTRODUCTION

Familial adenomatous polyposis (FAP, MIM #175100) is an autosomal dominantly inherited colorectal cancer predisposition caused by germline mutations in the *adenomatous polyposis coli* (*APC*) gene. In classical FAP, *APC* mutation carriers develop hundreds to thousands of colorectal polyps which if left untreated will progress to colorectal cancer in the third or forth decade of life[10]. Attenuated FAP (AFAP), also referred to "multiple" polyps, is characterized by the presence of 5 to 99 colorectal adenomas and later age at diagnosis compared to classical FAP (reviewed in[15,63]).

The *APC* gene is located on chromosome 5q21, consists of 8,535 base pairs organised in 15 exons and encodes a protein of 2,843 amino acids (GenBank accession number NM_000038.3). APC is an essential component of the Wnt signaling pathway involved in ß-catenin down-regulation. Furthermore, it has been implicated in cell adhesion, migration as well as in chromosomal stability, and cell cycle progression[64]. Exon 15 encodes the largest part of the protein (>75%) including the region responsible for binding ß-catenin. The majority of *APC* germline mutations occur in the first half of the *APC* coding region[17,64] whereas most somatic mutations are found between codons 1286 and 1513 – the so-called mutation cluster region[65]. About 95% of all *APC* germline mutations result in a truncated protein product[16,18].

Genotype-phenotype correlations, linking the site of the germline mutation with the severity of the disease, have been reported by several research groups: Severe polyposis with thousands of colorectal polyps has been associated with *APC* germline mutations between codons 1240 and 1464[19]. In contrast, patients carrying mutations at the extreme 5' end (codons 1 – 177)[20-23], the alternatively spliced exon 9 (codons 312 – 412)[24-26] and the 3' end (codons >1580)[23,27,28] were found to present more often with attenuated polyposis at diagnosis. In addition, certain extracolonic disease manifestations have been correlated with the site of the germline mutation, e.g. alterations between codons 1403 and 1578 with desmoid tumours[29,30].

Despite extensive genetic testing, in about 20% of FAP and almost 70% of AFAP patients worldwide no germline *APC* mutation can be identified[18,66]. The etiology of the AFAP phenotype is largely unknown and likely to be heterogeneous on the molecular genetic level. Recently, biallelic mutations in

the human homologue of the *E. coli* base excision repair gene *mutY* (*MYH*) have been shown to predispose to the autosomal recessively inherited *MYH* associated polyposis (MAP, MIM #608456)[6]. MAP has been shown to account for a substantial portion (10 – 30%)[24] of *APC* mutation-negative polyposis patients[67].

Screening a consecutive series of 101 unrelated polyposis patients, this study aimed (i) to compare the phenotypic properties of *APC* mutation carriers with those of *APC/MYH* mutation-negative polyposis patients and (ii) to assess potential genotype-phenotype correlations in *APC* mutation carriers.

PATIENTS AND METHODS

For this study a consecutive series of 101 unrelated polyposis index patients referred because of either classical ($\geq$100 polyps) or attenuated (5 - 99 polyps) polyposis coli were investigated. Written informed consent was obtained from all patients. Detailed clinical information was gathered from interviews and reports from physicians, pathologists and/or patients.

The family history (FH) was considered positive if a first-degree relative has been reported to have developed either gastrointestinal polyposis or colorectal cancer. Extracolonic disease manifestations included: polyps of the stomach and/or the small bowel, osteomas, desmoid tumours, benign cutaneous lesions (e.g. epidermoid cysts, fibromas), adrenal masses (mostly adrenocortical adenomas without endocrinopathy or hypertension) as well as a defined spectrum of extracolonic cancers (stomach, duodenum, pancreas, CNS, bile duct, adrenal gland, thyroid gland and liver)[11-14].

Mutation analysis

Mutation analysis of the *APC* gene was performed using a combination of screening methods, i.e. the protein truncation test (PTT) and direct DNA sequencing of PTT fragments displaying band shifts. These methods were applied according to published protocols[63,68]. DNA sequencing of exon 15g was carried out in all patients to screen for the common missense mutations p.Ile1307Lys and p.Glu1317Gln. The entire coding sequence as well as the promoter and the 3' untranslated region had been previously sequenced in 9 patients (Patient IDs: 1749, 1762, 1767, 1775, 1803, 1821, 1828, 1842,

1859)[18]. The recently introduced multiplex ligation-dependent probe amplification (MLPA) technique was used to screen *APC* for the presence of large genomic rearrangements (MRC Holland, Amsterdam, the Netherlands). In addition, RT-PCR analysis was carried out to characterize the two donor splice site mutations in intron 4 using primers located at the *APC* exon boundary 2/3 and 7/8 (primers and conditions available from authors upon request).

Patients in whom no pathogenic *APC* germline alteration could be identified were subsequently investigated for germline mutations in *MYH* (GenBank accession number NM_012222.1) by denaturing high performance liquid chromatography (dHPLC) and direct sequencing of exons 7 and 13 for the most frequent pathogenic mutations (p.Tyr176Cys and p.Gly393Asp)[67]. Bi- and monoallelic *MYH* mutation carriers were excluded from further phenotypic analysis.

Statistical analysis

Statistical comparison of patients' features, encompassing phenotypic characteristics (gender, age at diagnosis, polyp number, colorectal cancer, extracolonic disease, family history) and mutational status (*APC* mutation-positive vs. mutation-negative) was performed using Chi square or Fisher's exact test for categorical variables or Student's t-test for continuous variables, considering a p-value <0.05 to be statistically significant.

RESULTS

In this study a consecutive series of 101 unrelated polyposis patients were investigated for the germline mutations in the *APC* gene and, if no pathogenic mutation was detected, the *MYH* gene (Figure 1). Patients with mono- or biallelic germline mutations in *MYH* (n=12) were excluded from subsequent phenotypic analysis.

n=101

Polyp number   <100 n=62 61%   >100 n=39 39%

APC mutation   present n=28 45%   absent n=34 55%   present n=28 72%   absent n=11 28%

MYH mutation   present n=10 29%   absent n=24 71%   present n=2 18%   absent n=9 82%
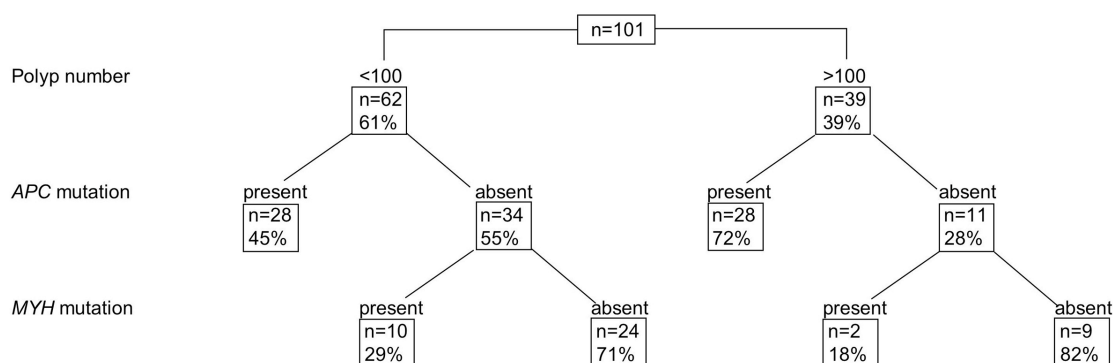
Figure 1:   **Screening results for *APC* and *MYH* mutations in a consecutive series of 101 polyposis patients split according to polyp number at diagnosis**

APC germline alterations

Overall, 56 out of 89 (63%) index patients were found to carry pathogenic mutations in *APC*. The mutation detection rate was 76% (28 out of 37) in patients with classical polyposis ($\geq$100 polyps) and 54% (28 out of 52) in patients with attenuated polyposis (5 to 99 polyps). Subdivision of polyposis patients according to age at diagnosis into those $\leq$40 and those >40 years resulted in similar, statistically significantly different *APC* mutation detection rates of 81% (30 out of 37) and 50% (26 out of 52; p= 0.007), respectively. Importantly, the percentage of *APC* mutation carriers with classical polyposis was very similar (53% (n=16) and 46% (n=12), respectively) in both groups (p=0.59).

Forty-six (82%) *APC* germline mutations were located within the "classical FAP region" (encompassing codons 178 – 1580, except exon 9) with the remainder occurring within the "AFAP regions" (codons 1 – 177, exon 9 and beyond codon 1580).

Overall, 33 frameshifts, 17 nonsense mutations, 3 splice site alterations (c.531+2-531+8delTAAGTAAinsACTTACATTTT, c.531+3insT, c.1547A>G), 2 genomic deletions as well as the missense mutation p.Glu1317Gln, associated with multiple colorectal adenomas, were identified[69].

Seventeen (30%) *APC* mutations represent novel alterations, which, according to the human gene mutation database [Cardiff, http://www.hgmd.cf.ac.uk/] have not yet been reported (Table 1).

Table 1:   Novel pathogenic *APC* germline alterations identified in this study

Mutation	Consequence	Patient ID
c.512_3insG	p.Ser171fs175X	1869
c.531+2-531+8delTAAGTAA insACTTACATTTT	p.Ser142_Asn177del	2366
c.1262G>A	p.Trp421X	1907
c.1374delT	p.Phe458fs465X	1761
c.1547A>G	p.Lys516Arg	2353
c.1726delG	p.Ala576fs577X	1769
c.1960C>T	p.Gln654X	1819
c.2335_6delTT	p.Leu779fs786X	1766
c.2383_4delCT	p.Leu795fs797X	2175
c.2787_8delTA	p.His929fs938X	2097
c.2925_6delAA	p.Lys975fs983X	2074
c.3285delG	p.Gln1095fs1125X	2156
c.3565delT	p.Ser1189fs1264X	1923
c.3767insC	p.Gln1256fs1275X	2132
c.4773delA	p.Ala1591fs1649X	2193
c.36_7delTC	p.Ser246fs249X	1818
c.1312-?_10285del	p.654Gln_283Xdel	1749

Among these we were able to characterize by RT-PCR and cDNA sequencing the nature of two donor splice site alterations in intron 4, c.531+3insT (patient ID 2197) and c.531+2-531+8delTAAGTAA insACTTACATTTT (patient ID 2366). Both were found to result in skipping of exon 4, corresponding to loss of 109 bp, and, consequently, leading to a shift in the reading frame and a first stop codon at amino acid position 148 (p.Leu148X; Figure 2). In index patient 1749 Sieber et al.[70] had previously identified the presence of a large deletion by means of a real-time quantitative multiplex PCR assay coupled with microsatellite marker analysis but without further delineating the extent of the deletion. Applying the MLPA technique the deletion could now be shown to actually encompass exons 10 to 15.

Based on the detailed family history available from 53 out of 56 *APC* mutation carriers, 42% (22 out of 53) of the index patients did not have an affected first-degree relative (parent and/or siblings) at the time of diagnosis which would indicate an unusual high proportion of *de novo APC* alterations in our cohort. Based on the median age at diagnosis of the index patients (39.5 years (IQR 20.0) parents can be assumed to be at least 20 years older (e.g. about 60 years), an age at which about 93% of cases are expected to display

typical symptoms[16]. Importantly, there was no difference with regard to age at diagnosis of index patients with or without a positive family history (40.0 years (IQR 14.5) vs. 39.5 years (IQR 20.0), respectively) and the proportion of *de novo* mutations was similar in both subgroups, those with classical and those with attenuated polyposis (41% vs. 42% respectively).
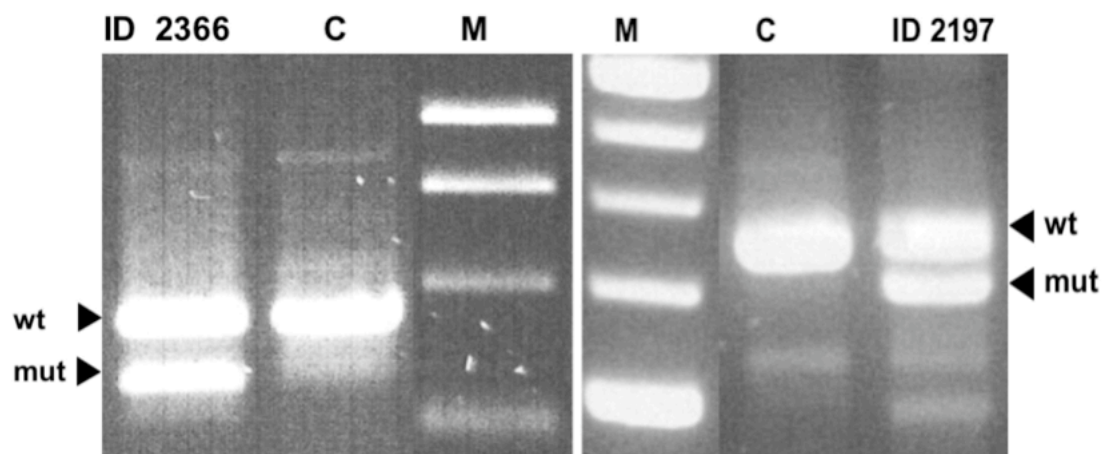
Figure 2: RT-PCR analysis of APC intron 4 donor splice site mutations c.531+3insT (patient ID 2197) and c.531+2-531+8delTAAGTAA insACTTACATTTT (patient ID 2366). The mutant allele corresponds to a loss of approximately 100 bp resulting in complete skipping of exon 4 as shown by cDNA sequencing of the mutant band. (M=DNA molecular weight markers; C=Control-cDNA from a healthy person; wt=wildtype allele; mut=mutated allele)

Phenotypic properties *of APC* mutation-positive and *APC/MUTYH–* negative polyposis patients

The phenotypic properties of *APC* mutation-positive and mutation-negative polyposis patients are depicted in Table 2.

APC mutation-positive patients displayed a significantly younger median age at diagnosis (40.0 years (IQR 18.0) vs. 51.0 years (IQR 18.3); p=0.0002). This could, in theory, be explained by the fact that *APC* mutation carriers presented significantly more often with classical polyposis (50% vs. 27%; p=0.03). The difference in age at diagnosis, however, remained statistically significant when patients were further subdivided according to polyp number count: 40.0 years (IQR 18.0) vs. 50.0 years (IQR 19.5; p=0.0002) in patients with classical polyposis (n=39), and 40.5 years (IQR 17.0) vs. 51.0 years (IQR 17.0; p=0.005) in those with attenuated polyposis (n=52). This age difference could not be explained by either a positive family

history (55% vs. 39%, p=0.38) or by colorectal cancer being present at the time of diagnosis (30% vs. 36%, p=0.56).

Extracolonic disease manifestations were reported in 25 (28%) polyposis patients and encompassed polyps of the upper gastrointestinal tract (n=15), desmoids (n=4), fundic gland polyps (n=2), osteomas (n=1), cancer of the stomach, the thyroid and adrenal gland (one each). The mean age of index patients displaying extracolonic manifestations was 38.5 years (IQR 21.0). Overall, *APC/MYH* mutation-negative patients tended to present with less extracolonic disease manifestations (15%) compared to *APC* mutation carriers (30%), but this difference was not statistically significant (p=0.11) (Table 2).

Table 2:   **Phenotypic properties of *APC* mutation-positive (n=56) and *APC/MYH*– negative (n=33) polyposis patients**

	APC mutation carriers			*APC/MYH* mutation-negative			
	overall n=56	<100 polyps n=28	>100 polyps n=28	overall n=33	<100 polyps n=24	>100 polyps n=9	p-value
Sex							
male	32 (57%)	16 (57%)	16 (57%)	21 (64%)	14 (58%)	7 (78%)	
female	24 (43%)	12 (43%)	12 (43%)	12 (36%)	10 (42%)	2 (23%)	p=0.55
Family history							
positive	31 (58%)	15 (58%)	16 (59%)	13 (48%)	9 (38%)	4 (57%)	
negative	22 (42%)	11 (42%)	11 (41%)	14 (52%)	11 (62%)	3 (43%)	p=0.38
no information	3	2	1	6	4	2	
Age (years)							
median (IQR)	40 (18)	40.5 (17)	40 (18)	51 (18.3)	51 (17)	50 (18)	p=0.0002
Polyp number							
<100	28 (50%)			34 (76%)			
>100	28 (50%)			11 (24%)			p=0.008
Colorectal cancer							
present	17 (30%)	6 (21%)	11 (39%)	12 (36%)	10 (42%)	2 (22%)	
absent	39 (70%)	22 (79%)	17 (61%)	21 (64%)	14 (58%)	7 (78%)	p=0.56
Extracolonic disease							
present	17 (30%)	11 (39%)	6 (21%)	5 (15%)	3 (13%)	2 (22%)	
absent	39 (70%)	17 (61%)	22 (79%)	28 (85%)	21 (87%)	7 (78%)	p=0.11

Genotype-phenotype correlations

To assess possible genotype-phenotype correlations the *APC* mutation-positive index patients were grouped according to the site of the *APC* mutation: group 1 (n=46) encompassing patients carrying a germline mutation within the "classical FAP region" (codons 178 – 1579, except exon 9) and

group 2 (n=10) containing those with a mutation within one of the "AFAP regions" (codons 1 – 177, exon 9 and codons >1580).

Nearly half (n=22, 48%) of group 1 patients presented with an AFAP phenotype (<100 polyps) at the time of diagnosis (Figure 3). Attenuated and classical polyposis patients from this group, however, did not significantly differ with regard to median age at diagnosis (41.5 years (IQR 17.0) vs. 38.5 years (IQR 16.5), p=0.79) or any other phenotypic property. Moreover, two out of five index patients (30 and 42 years old, respectively) carrying the c.3927_3931delAAAGA mutation at codon 1309, commonly associated with severe polyposis[71], actually displayed a multiple adenoma phenotype (Figure 3).

In group 2, six (60%) out of 10 index patients displayed an attenuated phenotype. Four (57%) out of seven patients carrying *APC* mutations at the 5' end or in exon 9 presented with a severe phenotype at diagnosis with hundreds to thousands of colorectal adenomas (Figure 3). All group 2 patients harbouring a mutation at the 3' end of the gene (n=3) displayed an attenuated polyposis phenotype. Extracolonic disease was equally frequent in both groups (30% each).
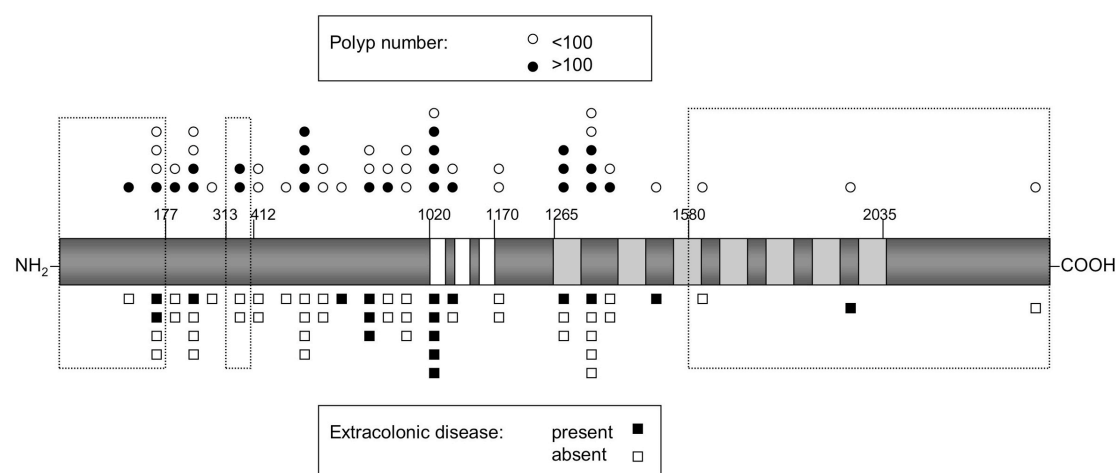
Figure 3: Schematic representation of the APC protein indicating polyp number and extracolonic disease in 56 APC mutation carriers according to the site of the respective germline mutation. White lines delineate 15-amino acid repeats for β-catenin binding. Light grey lines indicate 20-amino acid repeats for β-catenin binding and degradation and for GSK 3β phosphorylation. Dotted squares indicate the "AFAP regions.

DISCUSSION

This study on a consecutive series of 101 unrelated polyposis patients aimed to i) compare the phenotypic properties of *APC* mutation carriers with those of *APC/MUTYH* mutation-negative polyposis patients and ii) assess potential genotype-phenotype correlations in *APC* mutation carriers.

Overall, two thirds of the patients were found to harbour pathogenic germline mutations in *APC*, which is similar to observations by others (48 – 62%[66,72-75]). In accordance with results by Friedl et al.[66], the mutation detection rate in patients with classical polyposis was considerably higher (76%) compared to those with an attenuated phenotype (54%) with most of the *APC* germline mutations being located within the 5' half of the gene (codons 1 – 1309). Interestingly, the mutation detection rate in polyposis patients ≤40 years at diagnosis was significantly higher than in patients >40 years (81% vs. 50%, p= 0.007) and, importantly, was independent from polyp number.

One third of *APC* germline alterations represented novel mutations, consistent with previous reports (35% in average;[66,76-79]). This finding highlights the importance of screening the entire coding sequence of the *APC* gene for mutations.

Ten to 25% of *APC* germline mutations are estimated to occur *de novo*[72,80,81]. The high frequency observed in our study (42%), although independent from either age at diagnosis or polyp number, may actually reflect incomplete family history assessment and/or variable disease penetrance within families and consequently result in an overestimation. To clarify this issue, molecular genetic analysis of the parents would be needed.

APC mutation carriers differed statistically significantly from *APC/MYH* mutation–negative polyposis patients with regard to age at diagnosis (median age of 40 vs. 51 years, respectively) which is similar to previous observations[77,82,83]. This finding could not be explained by differences in polyp number, family history or colorectal cancer occurrence between the groups. Furthermore, although not statistically significant, extracolonic disease manifestations were twice as frequent in *APC* mutation carriers.

Several research groups have reported on genotype-phenotype correlations in *APC* mutation carriers correlating the site of the mutation with

polyp number and/or extracolonic disease[19,20,84]. Severe or classical polyposis (>100 adenomas) has been mainly observed in patients carrying *APC* mutations within the "classical FAP region" (codons 177 to 1580, except exon 9). In our study however, nearly half of our *APC* mutation carriers with alterations in the classical region actually displayed an attenuated polyposis phenotype.

Patients carrying an *APC* germline mutation in the "AFAP regions" (codon 1-177, exon 9 and codons >1580) have been reported to present with attenuated polyposis at diagnosis[15,23,26]. In contrast to these findings, four (57%) out of seven 5' *APC* mutation carriers in our consecutive series actually presented with severe polyposis coli displaying hundreds to thousands of colorectal polyps, similar to a report by Sieber et al.[85]. Despite the small number of patients with "AFAP region" mutations, precluding any meaningful statistical analysis, these observations clearly illustrate the considerable phenotypic variability in this group of mutation carriers.

Occurrence of desmoid tumours, upper gastrointestinal polyps and osteomas in polyposis patients has previously been correlated with *APC* alterations at codons 976 – 1067 and beyond codon 1309[86] as well as between codons 1403 and 1578[29]. In our study, extracolonic disease manifestations were evenly distributed among patients with mutations in the "classical FAP" or the "AFAP region". With regard to the above mentioned, specific extracolonic manifestations, 59% (10 out of 17) of patients actually carried mutations outside these regions.

A possible limitation of the current study may concern polyp count assessment in the index patients. Despite the fact that all referring medical centres and practices performed colonoscopy according to well-accepted international guidelines, use of imprecise terms like "multiple" may result in incorrect group assignment (i.e. < vs. $\geq$100 polyps).

In conclusion, our study on a consecutive series of 101 polyposis patients showed that i) *APC* mutation carriers significantly differed from *APC/MYH* mutation-negative polyposis patients with regard to age at diagnosis (40 vs. 51 years) and polyp number and that ii) no evidence for possible genotype-phenotype correlations was observed in our set of *APC* mutation carriers. Our finding that the individual phenotype could not be

predicted with certainty from the genotype has also been reported recently by A.L. Knudsen et al. (1st conference of InSiGHT, Newcastle upon Tyne, June 2005). Taken together, these data challenge the prevailing view on genotype-phenotype correlations and advise great caution when basing clinical management decisions for an individual patient on the site of the *APC* germline mutation.

ACKNOWLEDGMENTS

3. DISEASE SEVERITY AND GENETIC PAHTWAYS IN ATTENUATED FAMILIAL POLYPOSIS VARY GREATLY, BUT DEPEND ON THE SITE OF THE GERMLINE MUTATION

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ABSTRACT

Background: Attenuated familial adenomatous polyposis (AFAP) is associated with germline mutations in the 5', 3' and exon 9 of APC. These mutations probably encode a limited amount of functional APC protein. Methods and Results: We found that colonic polyp number varies greatly among AFAP patients, but members of the same family tended to have more similar disease severity. 5'-mutants generally had more polyps than the other patients. We analysed somatic APC mutations/LOH in 235 tumours from 35 patients (16 families) with a variety of AFAP-associated germline mutations. Like two previous studies of individual kindreds, we found bi-allelic changes ('third hits') in some polyps. We found that the 'third hit' probably initiated tumorigenesis. Somatic mutation spectra were similar in 5'- and 3'-mutant patients, often resembling classical FAP. In exon 9-mutants, by contrast, 'third hits' were more common. Most 'third hits' left three 20-amino acid repeats (20AARs) on the germline mutant APC allele, with LOH (or proximal somatic mutation) of the wild-type allele; but some polyps had loss of the germline mutant, with mutation leaving one 20AAR on the wild-type allele. Conclusions: We propose that mutations, such as nt4661insA, that leave three 20AARs are preferentially selected in cis with some AFAP mutations, because the residual protein function is near-optimal for tumorigenesis. Not all AFAP polyps appear to need 'three hits', however. AFAP is phenotypically and genetically heterogeneous. In addition to effects of different germline mutations, modifier genes may be acting on the AFAP phenotype, perhaps influencing the quantity of functional protein produced by the germline mutant allele.

INTRODUCTION

Classical familial adenomatous polyposis (FAP) is caused by germline mutations in the *adenomatous polyposis coli (APC)* gene between codons 178 and 1580. FAP patients typically develop hundreds to thousands of adenomatous polyps in the colon and rectum by the third decade of life. If left untreated, one or more adenomas progress to carcinoma by 45 years of age. Extracolonic features, such as polyps of the upper gastrointestinal tract, desmoid tumours and osteomas, are also common. Attenuated FAP (AFAP or AAPC) patients generally present with a lower number (<100) of colorectal adenomas by their fourth decade and have a later age of onset of colorectal cancer (mean age 55 years)[15,87,88]. In some AFAP patients, extracolonic features have been reported to be infrequent[24], although other AFAP patients – such as those with hereditary desmoid disease – have severe extra-colonic disease[89,90]. AFAP is associated with germline mutations in specific regions of the *APC* gene (Figure 1): the 5'-end (codons 1-177, exons 1-4); the 3'-end (distal to codon 1580); and the alternatively spliced region of exon 9 (codons 311-408)[15,23,26]. The molecular mechanism(s) underlying these genotype-phenotype associations for *APC* remains largely unknown.
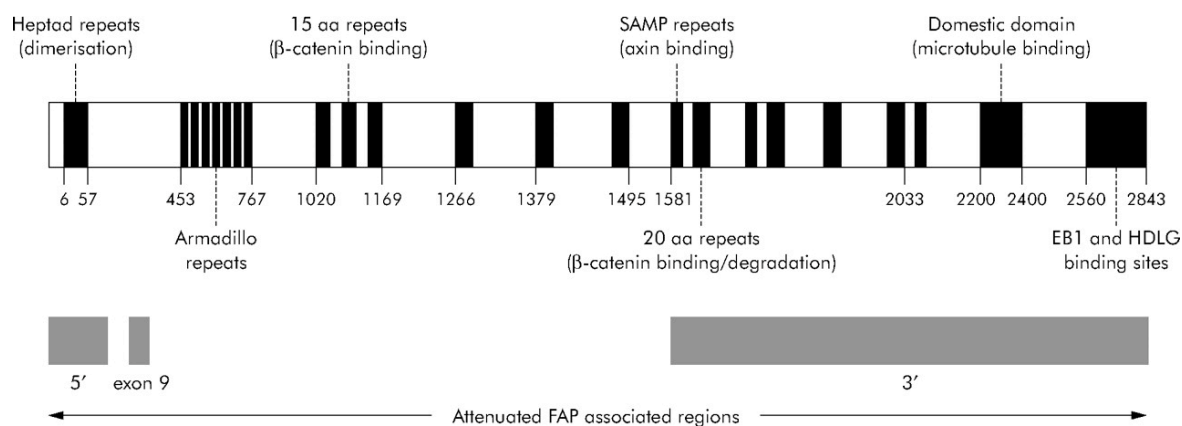
Figure 1   **Representation of the adenomatous polyposis coli protein comprising important functional domains and showing regions of the protein germline mutation, which are associated with attenuated familial adenomatous polyposis (FAP).**

APC is a tumour suppressor gene and almost all mutations truncate the protein or take the form of allelic loss (loss of heterozygosity, LOH). Several genetic studies of colorectal adenomas from FAP patients have shown that

somatic *APC* mutations are dependent on the position of the germline *APC* mutation (Figure 1)[91-93]. The APC protein contains seven 20-amino acid repeats (20AARs), which are involved in degrading the transcriptional cofactor beta-catenin and hence negatively regulate Wnt signalling. In colorectal polyps, germline mutations between codons 1285 and 1378 leave only one 20AAR intact and are strongly associated with somatic loss of the wild-type *APC* allele. LOH usually occurs through mitotic recombination, thus leaving two identical alleles and a total of two 20AARs in the tumour cell[94]. FAP patients who carry germline mutations before codon 1285 (no 20AARs) tend to have somatic mutations which leave one or, more commonly, two 20AARs in the protein. Finally, patients with germline mutations after codon 1398 (two or three 20AARs) tend to have somatic mutations before codon 1285. The same associations are also found in sporadic colorectal tumours[95]. This interdependence of 'first' and 'second hits' shows that selective constraints on *APC* mutations are active and that an optimum level of beta-catenin mediated signalling must be achieved for the tumour cell to grow[92]. There is no reason to expect that AFAP polyps are not subject to the same selection for optimal Wnt signalling as other colorectal adenomas.

The 'first hit-second hit' associations can explain why FAP patients with germline *APC* mutations between codons 1285 and 1378 have particularly severe colorectal disease, because the associated allelic loss occurs at a higher spontaneous frequency than the somatic truncating mutations selected in other FAP patients[91]. Conversely, the milder disease in AFAP patients may be explained if the mutations required to give the polyp cell a strong selective advantage are difficult to acquire. Spirio et al.[87] studied colorectal tumours from a single AFAP family with a germline *APC* mutation in the 5'-end of the gene (codon 142FS). About 12% of their polyps showed loss of the germline mutant allele, implying that this was a 'third hit' subsequent to a mutation on the germline wild-type allele. Furthermore, a large proportion (36%) of the truncating somatic mutations detected were 1bp insertions at an A6-tract between nucleotides 4661-4666 (codons 1554-1556). Spirio et al.[87] concluded that germline mutations in the 5' region of *APC* encode proteins that retain residual activity, owing to alternative splicing or initiation of translation. Somatic mutations would be required not only to inactivate the wild-type

allele, but also to reduce the residual activity of the mutant germline allele. Su et al.[96] studied 9 adenomas from an AFAP family with a germline mutation (R332X) in exon 9. They found 'third hits', including loss of the germline mutant allele and 4661insA, and showed the latter to occur on the germline mutant chromosome. The APC isoprotein lacking exon 9 retained at least partial ability to downregulate beta-catenin-mediated transcription, providing a reason for the 'three hits' and thus attenuation of the phenotype. Su et al.[96] suggested that exon 9-mutant AFAP patients develop more tumours than the general population because the germline mutant *APC* allele could be inactivated by a broad spectrum of somatic mutations, including some, such as nt4661insA, that would not normally affect a wild-type *APC* allele.

The existing studies only analysed single families, but established the important principle that 'third hits' can occur in AFAP. These 'third hits' could be LOH or mutation at codon 4661. In this study, we analysed a larger number of AFAP families with the following aims

• to search for phenotypic differences among AFAP families, both between and within kindreds with mutations in each of the three AFAP-associated regions of *APC*

• to determine whether the two families reported were typical of AFAP

• to find out the somatic *APC* mutation spectrum in AFAP patients with 3'-mutations and to compare this with the other AFAP-associated regions of *APC*

• to find out why 4661insA is such a common 'third hit'

• to delineate the pathways of somatic *APC* mutation in AFAP, with emphasis on whether polyps end up with the optimal genotype as predicted by studies of classical FAP

• to determine whether 'three hits' are always needed in AFAP.

MATERIALS AND METHODS

Study population and samples

We contacted Polyposis Registries in the United Kingdom, Switzerland, Germany and Denmark with a request to study colorectal tumours from AFAP patients with characterised germline *APC* mutations in the 5'- or 3'-regions of the gene (codons 1-177 and 1580-2843) or in the alternatively spliced region

of exon 9 (codons 311-408). In total, 235 fresh-frozen or formalin-fixed, paraffin-embedded colorectal tumours were obtained from 35 individuals in 16 families. All patients gave written informed consent. 231 of the tumours were colorectal adenomas, almost all of tubular morphology and with a median diameter of 3mm (range=117mm); four tumours were colorectal carcinomas (median diameter=5mm, range=2-20mm). 30 tumours were from 6 AFAP patients from 5 families with germline *APC* mutations in the 5'-region of the gene (G126X, 141FS, Q163X, 170FS, 173FS). 79 tumours were from 10 AFAP patients from 5 families, each of which carried the relatively common R332X nonsense mutation in the alternatively spliced region of APC exon 9. 126 tumours were from 19 AFAP patients from 6 families with germline *APC* mutations in the 3'-region of the gene (1597FS, 1738FS, 1919FS, 1943FS, 1982FS, 2078FS). Clinical details (*APC* germline mutation, gender, age at presentation, polyp count) were obtained and are being analysed as part of a larger study of phenotype in AFAP (A.L.Knudsen, in preparation); numbers of polyps analysed per patient are summarised in Table 1. Paired normal tissue was available for all patients. H&E-stained sections were prepared from each tumour to confirm the presence of at least 60% neoplastic tissue. DNA was extracted from tumour and normal tissue using standard methods.

Table 1   **Characteristics of the 35 patients with germline adenomatous polyposis coli (*APC*) mutations in the three attenuated familial adenomatous polyposis (AFAP) associated regions (5', exon 9, and 3'; codons 1–177, 311–408, and >1580)**

Patient ID	Germline *APC* mutation	Sex	Age at presentation (y)	Polyp count	Polyps analysed
AFXMK	G126X	M	36	834	6
DFAP48	141FS	F	56	2	1
AVC.III.2	Q163X	M	51	2100	4
55.iv.2/1112	170FS	F	32	1357	11
554.iii.2	170FS	M	50	1077	5
1464/1	173FS	M	39	"multiple"	3
673.iii.3/1132	R332X	F	49	200-300	6
1571.ii.2	R332X	F	68	5	5
578.AA	R332X	F	n/a	n/a	4
578.FPL	R332X	F	27	20	6
578.iii.9	R332X	M	41	n/a	17
578.iv.1	R332X	F	43	130	14
578,uv,4	R332X	F	27	n/a	9
578.iv.7	R332X	M	n/a	n/a	15
DFAP16	R332X	M	52	"multiple"	1
DFAP81	R332X	M	32	<100	2
344-40	1597FS	M	47	99	4
344-44	1597FS	M	43	50	3
01/266	1738FS	F	53	29	19
2233/3	1919FS	n/a	n/a	n/a	15
MD2976	1919FS	F	43	>100	21
77-11	1943FS	M	66	500	24
77-12	1943FS	M	56	500	2
77-40	1943FS	M	39	500	2
1460/28	1982FS	F	65	>100	5
1460/42	1982FS	F	33	8	1
1460/88	1982FS	M	48	<100	1
1489/10	1982FS	F	29	n/a	1
1624/04	1982FS	F	40	<100	3
S73119	2078FS	F	52	<100	1
DW20284	2078FS	F	n/a	n/a	1
J42424	2078FS	F	n/a	n/a	3
L12562	2078FS	F	60	"numerous"	3
110.2.vi	2078FS	F	n/a	33	14

FS, frameshift; n/a, not available.

Mutation screening

All samples were screened for somatic *APC* mutations using fluorescence single strand conformational polymorphism (SSCP) analysis on the ABI3100 sequencer (details available from authors upon request). Fresh-frozen samples were screened between codons 1 and 1779. Owing to the limiting quantity of DNA, formalin-fixed, paraffin-embedded samples were screened

between codons 1220 and 1603, an area encompassing the somatic mutation cluster region[16] and extending beyond the first SAMP repeat involved in axin binding. Samples with bandshifts on SSCP analysis were sequenced in both forward and reverse orientations from a new PCR product.

Cloning

We wished to determine the phase of somatic *APC* mutations with respect to the germline wildtype or mutant allele, but the quality of DNA available from archival tumours was insufficient to allow long-range PCR amplification. We therefore identified a SNP (nt4479A>G) within *APC* which was close enough to most somatic mutations of interest to be PCR-amplified, and which was informative and linked to the disease-causing mutation. After amplification of a region encompassing the somatic *APC* mutation and the SNP, the PCR product was cloned and multiple clones were sequenced using the pGEM-T Easy Vector System II (Promega).

Loss of heterozygosity analysis

In the case of germline nonsense mutations in *APC*, loss of heterozygosity (LOH, allelic loss) analysis was performed using three microsatellite markers, D5S346, D5S421 and D5S656, which map close to *APC*. Where linkage information was available for the microsatellites studied, the allele targeted by the allelic loss was assigned as germline mutant or wild type. Where no linkage information was available, the allele targeted was determined by inspection of the sequencing electropherogram in constitutional and tumour DNA for the region containing the mutation. In the case of germline (and somatic) frameshift mutations, LOH analysis was performed using oligonucleotide primers, which encompassed the germline insertion/deletion, which was then used as a pseudo-polymorphism for assessing loss. Standard methods of fluorescence-based genotyping on the ABI3100 sequencer were used. Allelic loss was scored at any informative marker if the area under one allelic peak in the tumour was reduced by more than 50% relative to the other allele, after correction for the relative peak areas of the alleles found in constitutional DNA of the same patient.

Multiplex ligation-dependent probe amplification (MLPA) analysis and real-time quantitative multiplex (RQM-)PCR

MLPA analysis to determine the copy number of the *APC* promoter and individual exons was performed on polyps with allelic loss at *APC* using the Salsa MLPA kit P043 APC (MRCHolland) according to manufacturer's instructions. RQM-PCR to determine the copy number of APC exon 14 (normalised against human serum albumin (Alb) exon 12) was performed as previously described[70]. The assay has previously been shown to be sensitive for tumour samples containing less than 30% contaminating normal tissue[93].

RESULTS

Overall phenotypic assessment

We have previously shown that disease severity (number of colorectal adenomas) in classical FAP patients varies considerably independent of the germline mutation, but that family members tend to have similar severities of disease[97]. In order to test for the same tendency in AFAP, we searched the published literature (details available from authors) for all patients who had germline mutations in the AFAP-associated regions of *APC* and with precisely reported colorectal polyp counts at presentation. We then combined these data with our own. Patient age had no significant effect on polyp number. We then tested for familial aggregation of disease severity and found good evidence for this, both when all families were considered together (p<0.00001, Kruskal-Wallis test) and when families with germline mutations in the three AFAP-associated regions of *APC* were analysed separately (p=0.0002, p=0.045, p=0.0005 respectively for 5'-, exon 9- and 3'mutants, Kruskal-Wallis test). Whilst some effects of local clinical practice are possible, such strong associations are unlikely to result from systematic errors in polyp counting. We then calculated the median polyp count for each family irrespective of size, and tested whether this varied among the three groups with mutations in different regions of the APC gene. The 33 families with 5' mutations had significantly more severe disease (median of medians=69 polyps, IQR=45475; p=0.047, Kruskal-Wallis test) than the 16 exon 9-mutant and 26 3'mutant families, who had similar disease severities (median of

medians=16, IQR=8-130 for exon 9; median of medians=15, IQR=4-150 for 3').

Somatic mutations in tumours of patients with AFAP-associated germline *APC* mutations

Given that our data showed aggregation of disease severity within families, it became more likely that the individual kindreds analysed by previous studies[87,96] had provided only a partial description of the genetic pathways of tumorigenesis in AFAP. We first screened colorectal tumours from 5'-mutant patients for somatic *APC* changes (Supplementary Table 1). We found truncating somatic mutations in 9 of 30 (30%) adenomas. Similar to adenomas from classical FAP patients with germline mutations before codon 1285[91-93], all of the truncating mutations left either one or two 20AARs in the protein. Just two of the adenomas (7%) harboured a detected 'third hit', each in the form of loss of the germline mutant allele (Supplementary Table 1). Our results were consistent with those reported by Albuquerque et al.[92] on the polyps of a single 5' mutant-patient, but differed from those of Spirio et al.[87] in that we found no mutations at nucleotides 4661-6 or at any other site after the third 20AAR. It was notable that while most of the patients of Spirio et al.[87] had presented with attenuated polyposis, the patient of Albuquerque et al. had been reported to have about 100 adenomas and most of our 5'-mutant patients had presented with a classical FAP phenotype (Table 1). The family of Spirio et al.[87] cannot therefore be considered representative of all patients with mutations in the AFAP-associated 5' region of *APC*.

For patients with exon 9 germline mutations, we found truncating somatic mutations in 47/79 (59%) adenomas (Table 2, Supplementary Table 2). Of the total of 50 truncating mutations, 33 (66%) were nt4661insA at codon 1554, and this change was always present on the germline mutant allele where assignment was possible. (An uncharacterised defect in DNA mismatch repair as a cause for this observation was excluded by analysing the microsatellite marker BAT26.) Three other mutations leaving three 20AARs (at codons 1518, 1530 and 1537) were found. LOH was found in 13/79 (16%) adenomas; this affected the wildtype allele in 9 cases and the mutant allele in 4 cases. Thirty-one (39%) adenomas had evidence of 'thirds hits', either two detected

somatic changes or a single identified somatic change on the germline mutant allele. The data allowed three main genetic pathways to be identified in the exon 9-mutant patients' polyps with evidence of 'third hits' (Table 2):

(i)   mutation leaving three 20AARs on germline mutant allele, plus loss of the wildtype allele;

(ii)   mutation leaving three 20AARs on germline mutant allele, with undetectable mutation of the wildtype allele (most likely towards the 5' end of the gene, which could not be screened in all polyps, and leaving zero 20AARs);

(iii)   mutation leaving one 20AAR on the wildtype allele plus loss of the germline mutant allele

Table 2   Numbers of tumours with evidence of "third hits" (somatic mutation of germline mutant allele) at the adenomatous polyposis coli (*APC*) gene in exon 9 and 3' mutant patients' polyps

Somatic mutation on germline wild-type allele ("second hit")	Somatic mutation on germline mutant allele ("third hit")	No of 5' mutant patients	No of exon 9 mutant patients	No of 3' mutant patients
LOH	20AAR3	0	6	0
LOH	20AAR2	0	0	2
20AAR0	20AAR3	0	2	1
20AAR1	20AAR3	0	2	0
20AAR0	LOH	0	0	1
20AAR1	LOH	2	4	3
20AAR2	LOH	0	0	2
Not found	20AAR3	0	20	0
Not found	20AAR2	0	2	2
Not found	LOH	0	0	9

LOH, loss of heterozygosity

20AAR1, truncating mutation before first 20 amino acid beta-catenin binding and degradation repeats (20AAR), etc. Note that these are minimum estimates of the true frequency, not only because we could not screen the entire gene for mutations in small archival polyps but also because it was not possible to assign all mutations to the germline mutant or wild-type allele.

For patients with 3' germline mutations, we found truncating somatic mutations in 35/126 (28%) adenomas (Table 2, Supplementary Table 3). Of the total of 36 truncating mutations, only 2 (6%) were nt4661insA. Three other

mutations leaving three 20AARs (at codons 1537, 1576 and 1570) were found. LOH was found in 30/126 (23%) adenomas, equally affecting the wildtype and mutant alleles. Twenty (16%) adenomas had either two detected somatic changes or an identified somatic change of the germline mutant allele. There was no clear tendency for different families to acquire different somatic mutations (Supplementary Table 3). The data only allowed one consistent genetic pathway to be identified in the 3'-mutant patients' polyps with evidence of 'third hits', namely a mutation leaving one (or two) 20AARs on the germline wildtype allele, plus loss of the mutant allele (Table 2).

Comparison between somatic mutations in the three groups of patients

The somatic mutation spectra of the 5'- and 3'-mutant patients' tumours did not differ significantly from each other as regards: (i) proportion of mutations leaving one, two or three 20AARs (p=0.074, χ^2 test); (ii) overall LOH frequency (2/9 versus 30/126, p=0.64); and (iii) proportion of tumours with detected 'third hits' or an identified somatic change on the germline mutant allele (2/9 versus 20/126, p=0.45). However, whilst exon 9-mutant patients had a similar frequency of LOH (13/79, 22%, p=0.14) to the other patients, germline exon 9 mutants had a higher frequency of mutations that left three 20AARs (36/50 versus 5/45, p<0.001, χ^2 test) and a higher frequency of tumours with detected 'third hits' (31/79 versus 22/156, p<0.0012, χ^2 test). In large part, these associations reflected the fact that nt4661insA was particularly common in the exon 9-mutant patients' tumours and exclusively targeted the mutant germline allele. Overall, our data were consistent with a large proportion of polyps in the 5'- and 3'-mutant patients developing along the 'classical' FAP pathway, their polyps showing similar somatic mutations to individuals with germline mutations which leave zero 20AARs[91]. Exon 9-mutant patients were, however, significantly different from the other two groups of patients. Although not all nt4661insA mutations could be assigned to a germline allele, if we made the reasonable assumption that all of these mutations were on the germline mutant allele, over half of all tumours from exon 9-mutant patients had 'third hits' (Supplementary Table 2). These differences could not readily be explained by features such as the size or

dysplasia of the tumours analysed, which did not differ significantly among the three patient groups (details not shown).

Mechanism of LOH

We tested the possibility that different LOH events (for example, those involving the germline wild type and mutant alleles) were caused by different mechanisms, such as mitotic recombination and deletion, which resulted in different gene dosages and functional consequences. However, none of 17 tumours with allelic loss (10 with mutant LOH and 7 with wild-type LOH) showed copy number changes in the APC promoter region or exons using MLPA analysis. We selected for RQM-PCR analysis 10 further tumours (2 with mutant LOH and 8 with wild-type LOH) with mean LOH ratios below 0.3 (indicating that contamination with normal tissue was low enough not to confound the detection of deletion[93]), but, all adenomas showed copy number values between 0.79 and 0.97, consistent with diploid *APC* copy number and LOH by mitotic recombination.

Early pathways of tumorigenesis in AFAP polyps with 'three hits'

Our data, combined with previous findings[87,96], showed that a substantial proportion of AFAP adenomas have acquired two somatic APC changes, one targeting the germline wildtype and one the germline mutant allele. Consideration of the order in which these somatic changes occur and their respective effects on tumour growth has important implications for determining the molecular genetic mechanism underlying AFAP. In AFAP adenomas, initiation of tumour growth might require all 'three hits' to be present in the tumour cell of origin ('kick-start' model). In this case, the two somatic mutations could occur in either order without functional consequence. The 'kick-start' model implies that a mechanism exists which results in an increase of the intrinsic or effective mutation rate in order to explain the relatively high frequency of such tumours as compared to the general population.

Alternatively, the 'second hit' - necessarily involving the germline wild-type allele - might be sufficient for early adenoma growth, with the 'third hit'

(involving the germline mutant allele) being required for subsequent tumorigenesis prior to clinical presentation. This 'stepwise' model postulates that mutation of the germline wild-type allele induces limited clonal expansion (thereby increasing the effective mutation rate), and is followed by mutation of the germline mutant allele to give an optimal APC genotype.

APC mutation data from individual adenomas can be used to distinguish between these possibilities, because the 'kick-start' and 'stepwise' models are expected to leave distinct footprints as regards the proportion(s) of somatic mutant allele(s), since these proportions depend on the order in which the somatic changes have occurred and some residual adenoma with 'two hits' is expected in the 'stepwise' case (Figure 2).
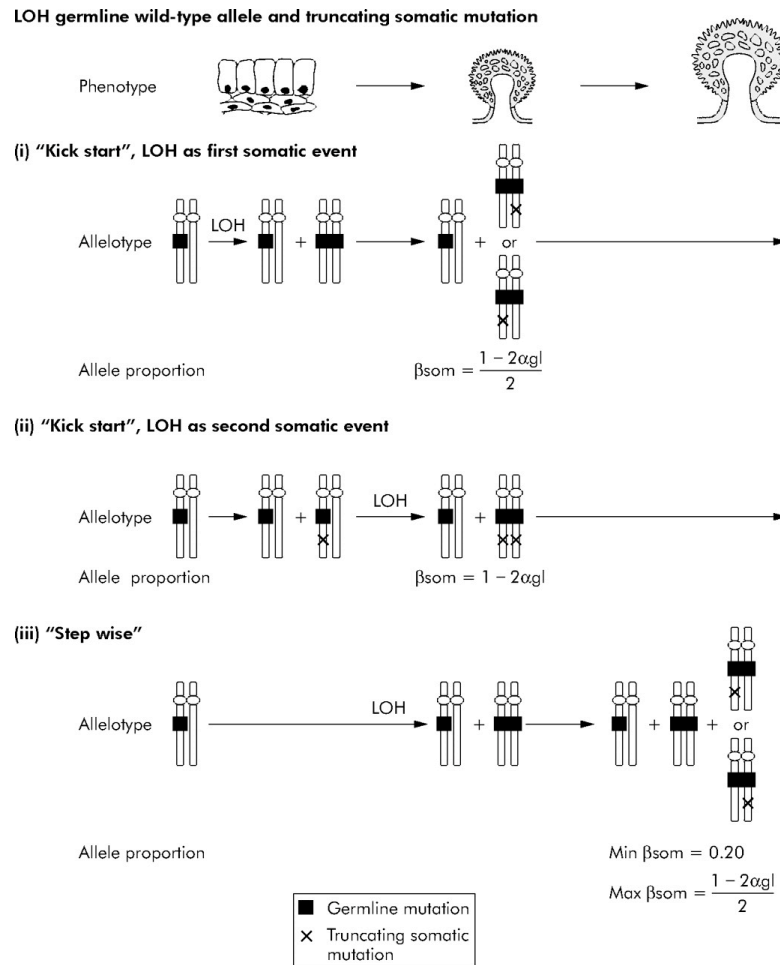
Figure 2   Pathways of tumorigenesis in attenuated familial adenomatous polyposis (AFAP) polyps with "three hits", illustrating the possible sequences in which somatic mutations/allelic loss may occur in AFAP polyps with "three hits" as well as the possible functional effects of these changes. Loss of the germline wild-type allele and truncating somatic mutation are shown. In a "kick-start" model, these changes can occur in either order (i) or (ii) and tumour growth ensues once both somatic changes have occurred; in a "step wise" model (iii), loss of the germline wild-type allele leads to limited clonal expansion and is followed by the truncating somatic mutation which promotes further tumour growth. The expected proportions of ßsom and gl are shown. ßsom = proportion of somatic mutant allele in polyp; gl = proportion of germline wild-type allele in polyp. LOH, loss of heterozygosity by mitotic recombination. For each model, the expected proportion of the somatic mutant allele (ßsom) in the polyp can be determined from the proportions of the germline wild-type (gl) allele as shown. gl can be estimated from the LOH ratio.

LOH germline mutant allele and trucating somatic mutation on wild-type allele

Phenotype

(i) "Kick start"

Allelotype

Allele proportion

$$\beta som = 1 - 2\beta gl$$

(ii) "Step wise"

Allelotype

Allele proportion

■ Germline mutation
✗ Truncating somatic mutation

$$\text{Min } \beta som = 1 - 2\beta gl$$
$$\text{Max } \beta som = 1 - \beta gl$$
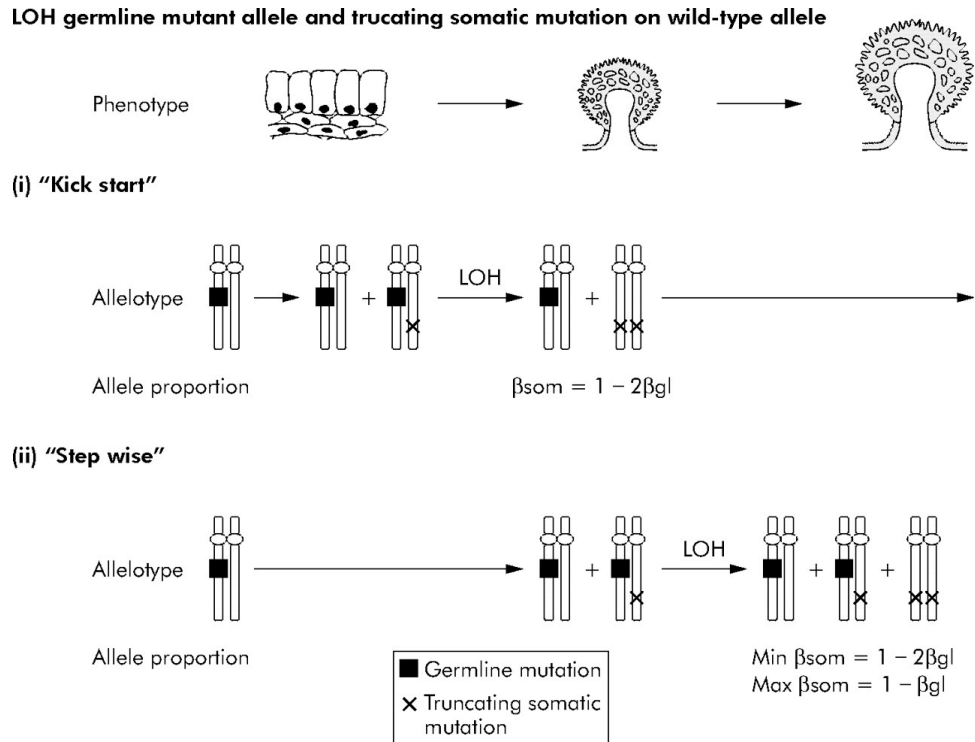
Figure 3   **Pathways of tumorigenesis in attenuated familial adenomatous polyposis (AFAP) polyps with "three hits", illustrating the possible sequences in which somatic mutations/allelic loss may occur in AFAP polyps with "three hits" as well as the possible functional effects of these changes. Loss of the germline mutant allele and truncating somatic mutation are shown. In both the "kick-start" (i) and "step wise" (ii) models, the truncating somatic mutation precedes loss of the germline mutant allele but in the "kick-start" model, both changes are required for tumour growth. The expected proportions of ßgl and ßsom are shown. ßsom = proportion of somatic mutant allele in polyp; ßgl = proportion of germline mutant allele in polyp. LOH, loss of heterozygosity by mitotic recombination. For each model, the expected proportion of the somatic mutant allele (ßsom) in the polyp can be determined from the proportions of the germline mutant (ßgl) allele as shown. ßgl can be estimated from the LOH ratio.**

Figure 4   **Pathways of tumorigenesis in attenuated familial adenomatous polyposis (AFAP) polyps with "three hits", illustrating the possible sequences in which somatic mutations/allelic loss may occur in AFAP polyps with "three hits" as well as the possible functional effects of these changes. Two truncating somatic mutations are shown. In a "kick-start" model (i), these changes can occur in either order and tumour growth ensues once both somatic changes have occurred; in a "step wise" model (ii), somatic mutation of the germline wild-type allele causes limited clonal expansion and is followed by somatic mutation of the germline mutant allele, which promotes further tumour growth. The expected ratio of the two somatic alleles is 1:1 for the "kick-start model" but lies between 1:4 and 1:1 for the "step wise" model with the minimum estimate (*) assuming a mutation detection sensitivity of 20%.**

Consider, for example, polyps with loss of the germline wild-type allele and a somatic insertion/deletion mutation on the germline mutant allele. We can measure two ratios of relative allelic dosage, one for the germline mutation and the other for the truncating somatic mutation, and use these to estimate the proportion of each allelotype in the tumour. Furthermore, we can calculate the expected values of these ratios by predicting the proportion of somatic mutant allele expected in the tumour under different models of tumorigenesis (Figure 2). By comparing the observed proportion of the somatic mutant allele with that expected, we can determined whether the 'stepwise' or 'kickstart' model fits better (see Figure 2 for details). Similarly, observed and expected

allele proportions can be determined for adenomas with one somatic insertion/deletion mutation on the germline wildtype allele and loss of the germline mutant allele (Figure 2). For tumours with two truncating somatic mutations, the expected ratio of the two mutant alleles under each model can be compared to the ratio measured directly by cloning a PCR product encompassing both changes, sequencing multiple clones and counting how many times each allele is represented (Figure 2).

For seven tumours with loss of the germline wild-type allele and a somatic insertion/deletion mutation, the observed and expected proportions of the somatic mutant allele were very similar to those expected under the 'kick-start' model, assuming that the 'second hit' was the insertion/deletion (on the somatic mutant allele) and the 'third hit' was the allelic loss (Table 3). Similar results in favour of a 'kick-start' model were obtained for three adenomas with one somatic insertion/deletion and loss of the germline mutant allele, and for one adenoma with two truncating somatic mutations (Table 3).

Table 3   Observed and expected frequencies of somatic mutant *APC* alleles in attenuated familial adenomatous polyposis (AFAP) polyps with "three hits" for "kick-start" and "step wise" models of tumorigenesis. Polyp with one somatic insertion/deletion and loss of the germline wild-type allele

A. Polyp with one somatic insertion/deletion and loss of the germline wild-type allele.

Model	Allele frequencies	Polyp 1	Polyp 2	Polyp 3	Polyp 4	Polyp 5	Polyp 6	Polyp 7	χ^2
	Observed αgl	0.21	0.27	0.21	0.29	0.3	0.26	0.28	
	Observed βsom	0.64	0.52	0.49	0.52	0.33	0.4	0.27	
"Kick-start", LOH first	Expected βsom = [(1-2αgl)/2]	0.29	0.23	0.29	0.21	0.2	0.24	0.22	1.59
"Kick-start", LOH second	Expected βsom = (1-2αgl)	0.58	0.46	0.58	0.42	0.4	0.48	0.44	0.14
"Step wise"	Expected minimum βsom*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	2.72
	Expected maximum βsom =[(1-2αgl)/2]	0.29	0.23	0.29	0.21	0.2	0.24	0.22	1.59

*Assuming that the somatic insertion/deletion can be detected if it comprises >20% of all alleles in the polyp.

B Polyp with one somatic insertion/deletion and loss of the germline mutant allele.

Model	Allele frequencies	Polyp 8	Polyp 9	Polyp 10	χ^2
	Observed βgl	0.33	0.1	0.24	
	Observed βsom	0.41	0.83	0.61	
"Kick-start" (LOH must be second)	Expected βsom = (1-2βgl)	0.34	0.8	0.52	0.03
"Step wise"	Expected minimum som = (1-2βgl)	0.34	0.8	0.52	0.03
	Expected maximum som = (1-2βgl)	0.67	0.9	0.76	0.14

C Two truncating somatic mutations.

Model	"Third" to "second hit" ratios*	Polyp 11	χ^2
	Observed	27:31	
"Kick-start"	Expected	1:1	0.03
"Step wise"	Expected minimum	1:4	14.4
	Expected maximum	1:1	0.03

* Assuming a detection sensitivity of 20% for the somatic 'third hit'.

αgl = proportion of germline wild-type allele in polyp; ßsom = proportion of somatic mutant allele in polyp. Observed αgl frequency was determined from the loss of heterozygosity (LOH) ratios. Observed ßsom frequencies were similarly determined from LOH ratios generated by polymerase chain reaction amplification of a region encompassing the somatic insertion/deletion and subsequent Genescan analysis (using constitutional DNA from patients with germline mutations identical to the somatic change for normalisation). The observed "third" to "second hit" ratio for polyp 3 was determined by sequencing 58 clones of a polymerase chain reaction product encompassing both somatic changes.

DISCUSSION

Our analysis of a relatively large set of AFAP families has shown complexity in the phenotype and early genetic pathways of tumorigenesis. The two previous analyses of somatic *APC* mutations in AFAP each focussed on single families, one with a germline mutation in the 5' region of the gene[87] and the other with a mutation in exon 9[96]. These two studies unequivocally provided the important and original finding that 'three hits' - that is, two somatic mutations, including loss or mutation of the germline mutant allele - can occur in AFAP tumours. The restricted size of the two studies meant, however, that they were unable to provide further conclusions.

We have found that patients with germline *APC* mutations in the 5' and 3' regions of the gene or the alternatively spliced region of exon 9 have a highly variable large-bowel phenotype, in that the number of colorectal adenomas varies from almost none to the hundreds or thousands of lesions found in classical FAP[15]. Although assessment methods necessarily differ among clinical centres, our analysis shows that patients with 5' *APC* mutations (codons 1-177) are likely to have a more severe phenotype than those with mutations in exon 9 or the 3' end of the gene (>codon 1580). Phenotypic severity also tends to be similar within families, suggesting that restricting analyses to single kindreds may not provide accurate assessment of AFAP patients.

Our study has confirmed that 'three hits' at *APC* often occur in AFAP adenomas. In such polyps, the 'third hit' appears to be required for the initiation of tumorigenesis. Although 'third hits' might occur at loci other than *APC*, we have previously found no mutations at beta-catenin in AFAP polyps (unpubl. data). In polyps with 'three hits' from exon 9-mutant and 3'-mutant patients, we have been able to identify specific combinations of *APC* mutations, which tend to occur. Exon 9 is alternatively spliced in all normal and neoplastic tissues, which we have examined (not shown). The combinations of APC mutations almost certainly produce a near-optimal level of Wnt signalling, comparable with those found in classical FAP[91]. Some of the combinations – such as R332X-nt4661insA/LOH – strongly suggest that the tumour has developed as a result of the functional effects of the germline mutant allele, but other combinations of mutations – such as truncating

mutation leaving one 20AAR on the wildtype with LOH of the germline mutant – might simply be indicative of a 'sporadic' tumour occurring on the background of AFAP.

In our families, 'third hits' were much rarer in 5'- and 3'-mutant patients than in the exon 9 mutants. These former families' somatic mutations usually - but not always - resembled those of classical FAP patients who have germline mutations before the first 20AAR of the APC protein. In many ways, this is the result which would be predicted were the 5' or 3' mutations simply to cause absent or non-functional protein. 5' APC mutations probably produce a small amount of partially functional APC through use of an internal ribosome entry site (IRES) at codon 184[98]. 3'-mutant proteins have been reported as being unstable[27], although the reasons for this are unknown. It is entirely plausible that the levels of functional APC protein vary among individuals with both 5' and 3' mutations, for example as a result of modifier alleles. Thus, for an adenoma to form, some patients would tend to require 'third hits' and others would not. The family of Spirio et al.[87], for example, may have been relatively efficient at use of the IRES. Formal testing of this hypothesis *in vivo* would require an exceptionally large, unselected series of tumours and patients.

Our analysis of exon 9-mutant cases further provides further evidence to show that not all AFAP patients are the same. 'Third hits' were common in these patients' tumours. There was a markedly increased frequency of mutations which left three 20AARs on the germline mutant allele, particularly – but not exclusively - at nt4661, which appears to be a relatively hypermutable site. Our view differs somewhat from that of Su et al.[96], who proposed that insAnt4661 mutations were over-represented in AFAP polyps because both 'strong' and 'weak' mutations were sufficient to severely reduce function of the exon 9-mutant allele. We suggest that mutations leaving three 20AARs on the germline mutant allele are common because the resulting allelotype R332X-4661insA gives a near-optimal genotype, taking into account loss of the germline wildtype allele and alternative splicing of exon 9. Variation in splicing efficiency – again through modifier allele action - could explain phenotypic variability in exon 9-mutant AFAP, but it appears that many of these patients produce sufficient functional protein by splicing out exon 9 that 'third hits' are necessary in most polyps.

The reason why AFAP patients develop fewer polyps than classical FAP patients is evident, in that 'three hits' are often needed to produce the near-optimal genotype. We do not, however, claim that all polyps from patients with AFAP-associated *APC* mutations require 'three hits'. Even allowing for the imperfections of mutation screening and LOH analysis in archival specimens, we were able to analyse the fresh-frozen adenomas comprehensively and found many without 'three hits'. Moreover, several polyps from our patients had somatic mutations which would have been predicted from a 'two hit' model of optimal Wnt signalling. Currently, we cannot explain why in a single patient, some polyps seem to require 'three hits' and others do not, but it is possible that 'third hits' at other loci can substitute for *APC* mutation. Another possibility is that selective constraints on the diminished APC function needed for tumorigenesis are 'just right'[87,92] at some times, but weaker at others, for example during development or when tissue is undergoing repair.

The genetic analysis of colorectal tumours from patients with germline mutations in AFAP-associated regions of *APC*, by this study and others, has revealed a novel mechanism underlying the genotype-phenotype association in this tumour syndrome, namely a requirement for 'three hits' in at least some AFAP adenomas. This finding must be viewed in the framework of the model of optimal combinations of *APC* mutations, rather than simple loss of protein function. More than one different combination of *APC* mutations can provide near-optimal Wnt signalling in AFAP. However, not all AFAP patients are the same. Given that assembling a very large series of AFAP patients is extremely difficult, it is not easy to decide on what is the 'typical' AFAP phenotype or somatic genotype. In the seven families with 5' *APC* mutations studied to date[87,92] and this study), about 15-20% of polyps seem to acquire 'three hits', but only Spirio et al.[87] found a high frequency of nt4661insA. In the six 3'-mutant families studied (all from this study), the frequency of 'third hits' seems similar to that of the 5'-mutants. Six exon 9-mutant families have been studied ([96] and this study) and almost all of these show evidence of a high frequency of 'third hits' – we estimate a minimum of 50% in our study. In addition, there appear to be genetic factors apart from the germline *APC* mutation that influence disease severity, as evidenced by the tendency for polyp numbers to be similar within families. The phenotypic and somatic

molecular heterogeneity in AFAP means that clinical management of patients with AFAPassociated mutations must be empirical. Accurate prediction of phenotype may only be possible when factors, such as modifier genes, that influence genetic pathways and disease severity are identified.

4. PREVALENCE OF *MYH* GERMLINE MUTATIONS IN SWISS *APC* MUTATION-NEGATIVE POLYPOSIS PATIENTS

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* The first 3 authors equally contributed to this article

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. Seventy-nine unrelated *APC* mutation-negative Swiss patients with either classical (n = 18) or attenuated (n = 61) polyposis were screened for germline mutations in *MYH* by dHPLC and direct genomic DNA sequencing. Overall, 7 (8.9%) biallelic and 9 (11.4%) monoallelic *MYH* germline mutation carriers were identified. Among patients with a family history compatible with autosomal recessive inheritance (n = 45), 1 (10.0%) out of 10 classical polyposis and 6 (17.1%) out of 35 attenuated polyposis patients carried biallelic MYH alterations, 2 of which represent novel gene variants (p.R171Q and p.R231H). Colorectal cancer was significantly ($p < 0.007$) more frequent in biallelic mutation carriers (71.4%) compared with that of monoallelic and MYH mutation-negative polyposis patients (0 and 13.8%, respectively). On the basis of our findings and earlier reports, *MYH* mutation screening should be considered if all of the following criteria are fulfilled: (i) presence of

classical or attenuated polyposis coli, (ii) absence of a pathogenic *APC* mutation, and (iii) a family history compatible with an autosomal recessive mode of inheritance.

INTRODUCTION

Familial adenomatous polyposis (FAP; OMIM entry no.175100) is an autosomal dominantly inherited colorectal cancer (CRC) predisposition caused by germline mutations in the *adenomatous polyposis coli (APC)* gene and characterized by the development of hundreds to thousands of adenomatous polyps throughout the intestinal tract[16]. 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[16,87,96]. With routine screening techniques failing to detect pathogenic *APC* germline mutations in 10–30% of classical FAP patients and in up to 90% of AFAP patients[99], 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[43]. The observation of an excess of transversion mutations in tumors eventually led to the discovery of *MYH*-associated polyposis (MAP). A number of studies have already been conducted in attempts establish the extent to which germline mutations in the *MYH* gene may contribute to individuals with an AFAP phenotype[7,100,101]. As a result, biallelic *MYH* germline mutations have been attributed to ~1–3% of all unselected CRC patients. This nation-wide study aimed to (i) assess the frequency of *MYH* mutation carriers in 79 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 mutation carriers, monoallelic mutation carriers and *APC/MYH* mutation-negative patients.

PATIENTS AND METHODS

This nation-wide study investigated 79 ostensibly unrelated Swiss index patients referred between 1994 and 2004 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 ($\geq$100 polyps, n = 18) or multiple adenomas/attenuated (5 – 99 polyps) FAP (AFAP; n = 61). In all patients, no germline *APC* mutation could be identified by means of the protein truncation test, single strand conformation polymorphism or direct DNA sequencing (patients thereafter referred to as *APC* mutation-negative)[18]. Forty-five patients displayed a family history compatible with autosomal recessive inheritance; in the remainder there was either evidence for vertical transmission or no detailed family history available. In addition, 100 control Swiss individuals were enrolled so as 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 to cases, and the results of the various genetic analyses were independently checked by at least 2 assessors.

DNA extraction

Genomic DNA was isolated from EDTA blood, using methods previously described by Miller et al.[102]. Briefly, 10ml blood was mixed with 30ml of EL buffer (155mM NH_4Cl, 10mM $KHCO_3$, 1mM EDTA, pH 7.4) and left on ice for 15 min. The lysate was centrifuged at 2000g for 10 min, washed twice with EL buffer and the intact leukocyte pellet resuspended in NL buffer (10mM Tris.HCl, pH 8.2, 400mM NaCl, 2mM Na2EDTA, 1%SDS and 200μg/ml proteinase K) and incubated overnight at 37°C. The next day, 1ml of 6M NaCl was added and vigorously shaken, followed by centrifugation to remove cellular proteins. The supernatant containing the DNA was placed in a fresh

tube and the DNA precipitated with ethanol. The resulting DNA pellet was washed with 70% ethanol, dried briefly and then suspended in 1ml of TE buffer (10 mM Tris.HCl, pH 7.5, 0.1 M EDTA).

MYH mutation analysis

In 57 (72%) patients (15 FAP and 42 AFAP), the entire _MYH_ coding sequence was analyzed by direct DNA sequencing. An additional 22 patients (3 FAP and 19 AFAP) were exclusively screened for mutations in exons 7 and 13 in which the most common pathogenic mutations in the Caucasian population, p.Y165C and p.G382D, occur. Each time a heterozygous _MYH_ mutation was identified, the entire gene was subsequently analyzed by direct DNA sequencing (exons 2, 5, 8 and 12) and dHPLC (exons 1, 3, 4, 6, 9, 10, 11, 14, 15, 16) to identify/exclude the presence of a second germline mutation.

Exon specific primer pairs were used to amplify the 16 exons of _MYH_ (HUGO ID: MUTYH; Genbank accession no. NM_012222), including the respective exon–intron boundaries (primer sequences and PCR conditions available from the authors upon request). Twenty-five microliters of PCR reaction mixture contained 50ng of genomic DNA, 10pmol of each primer and a PCR mastermix at 1.5mM $MgCl_2$, according to the manufacturer's instructions (Invitrogen, Basel, Switzerland). All PCR reactions were done on a Hybaid OmnE thermocycler (Catalys AG, Wallisellen, Switzerland).

As a prescreening method to detect DNA sequence changes, 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 with the control samples run in parallel, direct DNA sequencing was performed to characterize 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 manufacturers' guidelines. Following purification using the DyeEx 2.0 Spin Kit (Qiagen, Basel,

Switzerland), sequencing products were analyzed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Germline mutations identified in *MYH* were confirmed by sequencing in both, forward and reverse, directions, and from at least 2 independent PCR products. Germline mutations p.Y165C and p.G382D were independently confirmed by restriction enzyme digests, using IIaI and BglII, respectively.

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 χ^2 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

Seventy-nine APC mutation-negative Swiss polyposis patients from the Basel (n = 58) and Sion (n = 21) medical genetic centers were investigated for the presence of *MYH* germline alterations. Twenty-three percent of the individuals were referred because of suspected classical FAP (n =18), whilst the majority exhibited an attenuated or multiple adenoma phenotype (n = 61).

***MYH* mutation analysis**

The complete coding sequence of the *MYH* gene was investigated in 57 index patients. In addition, 22 patients were screened for alterations in exons 7 and 13, which harbor the most common pathogenic mutations, p.Y165C and p.G382D. Overall, 7 (8.9%) biallelic and 9 (11.4%) monoallelic *MYH* germline mutation carriers were identified. According to the clinical classification, 1 (5.6%) out of 18 FAP and 6 (9.8%) out of 61 AFAP patients harbored a biallelic *MYH* mutation. If only individuals with a family history compatible with autosomal recessive inheritance were considered (n = 45), 10.0% (1/10) of patients with classical polyposis and 17.1% (6/35) of AFAP patients harbored biallelic *MYH* germline mutations.

Table 1   Phenotypic Features and germline mutations identified in *MYH* mutation carriers[1]

Patient ID	Sex	Age	Polyp No.	CRC	Extracolonic disease	*MYH* 1st Mutation	*MYH* 2nd Mutation
Biallelic *MYH* mutation carriers							
1775/01	M	38	<100	Yes	Yes	p.G84fs	p.W138_M139insIW
1828/01	F	42	<100	Yes	No	p.Y165C	P.Y165C
1859/01	M	33	<100	No	No	p.Y165C	P.Y165C
2013/01	M	50	<100	Yes	No	p.G382D	p.G382D
2073/01	F	60	50	No	No	p.Y165C	p.R171Q
2184/01	M	48	>100	Yes	No	p.G382D	p.G382D
2185/01	M	48	74	Yes	No	p.Y165C	P.R231H
Monoallelic *MYH* mutation carriers							
1384/01	F	20	Multiple	No	Yes	p.G382D	None detected
1665/01	F	54	>100	No	No	p.I209V	None detected
2145/01	M	40	70	No	No	p.Y165C	None detected
2243/01	M	49	50	No	No	p.Y165C	None detected
2261/01	F	69	>100	No	No	p.Y165C	None detected
DFAP 17	F	34	20	No	Yes	p.G382D	None detected
DFAP 82	M	58	>100	No	No	p.G382D	None detected
DFAP 99	F	63	43	No	No	p.G382D	None detected
SA453	M	41	5	No	No	p.G382D	None detected

CRC Colorectal Cancer

[1] Patient 1775/01 has previously been reported by Sieber et al.[100]

In addition to the mutations p.Y165C and p.G382D, which accounted for 43% and 29% of mutant alleles in the biallelic patients, respectively, 2 novel alterations were detected in AFAP patients compound heterozygote for p.Y165C/p.R171Q and p.Y165C/ p.R231H (Figs. 1a and 1b). One FAP patient, who was found to be a compound heterozygote with a p.G84fs/p.W138_M139insIW mutation, has been previously reported by Sieber et al.[100] The healthy parents of this individual were available for investigation and were found to be heterozygous carriers of the p. W138_M139insIW and the p.G84fs alteration, respectively. Although the pathogenicity of p.R171Q and p.R231H remains to be established by functional studies, such gene alterations were not observed in 200 chromosomes from Swiss control samples. Furthermore, the amino acid positions are evolutionary highly conserved across distantly related species (*E. coli*, *S. pombe*, mouse, rat and human).
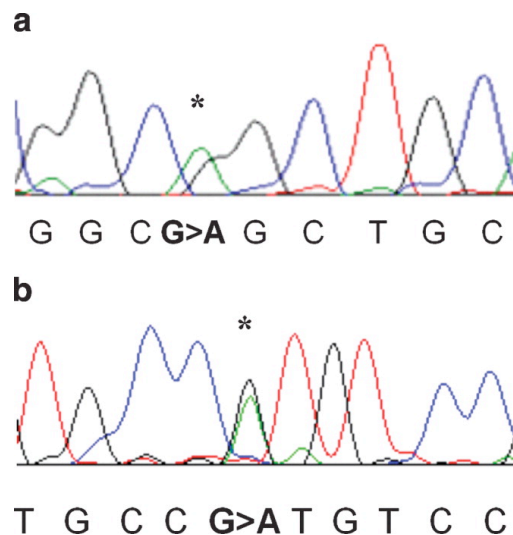
Figure 1   **Sequencing chromatograms displaying the 2 novel MYH germline variants marked by an asterisk (*). (a) c.512G>A, p.R171Q (heterozygote); (b) c.693G>A, p.R231H (heterozygote)**

Nine patients were identified as monoallelic *MYH* mutation carriers, with the p.G382D mutation present in 5 (56%) of them (Table I). In the remaining 63 (80%) patients, no pathogenic *MYH* mutations could be identified. The previously described polymorphisms in exon 2 (c.64G > A; p.V22M) and exon 12 (c.972G > C; p.Q324H) were detected with allele frequencies of 6% and 17%, respectively, similar to that of a Swiss control sample population (200 chromosomes) assessed in parallel (2% p.V22M and 12% p.Q324H).

Genotype–phenotype comparisons

The phenotypic features of the 7 biallelic *MYH* mutation carriers are depicted in Table I, with one of them displaying classical FAP. In 5 (71%) patients, CRC had been diagnosed at a median age of 48 years (IQR 10.5, range 33–60 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 (p.Y165C/p.R171Q) a CRC had been diagnosed at a median age of 51 years (range 49–54). Except for patient 1775, in whom duodenal adenomas had been detected, no apparent extracolonic disease manifestations were observed in the other biallelic mutation carriers.

Among the 9 monoallelic *MYH* mutation carriers (Tables 1 and 2), 4 patients (no. 1384/01, 2243, DFAP17 and DFAP 82) had siblings with either CRC or polyps reported. With respect to extracolonic disease manifestations,

a facial lipoma was observed in patient DFAP17 and a duodenal adenocarcinoma at age 20 in patient 1384/01.

Table 2   **Phenotypic characteristics of biallelic *MYH* mutation carriers, monoallelic 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=9)	MYH mutation negative patients (n=29)
Sex			
Male	5 (71)[1]	5 (56)	18 (62)
Female	2 (29)	4 (44)	11 (38)
Clinical classification			
FAP ($\geq$100 polyps)	1 (14)	3 (33)	6 (21)
AFAP (<100 polyps)	6(86)	6 (67)	23 (79)
Age at diagnosis (y)			
Median	48	49	48
IQR	10.5	20.8	20
Range	33-60	20-69	22-77
Colorectal cancer			
Present	5 (71)	0	4 (14)
Absent	2 (29)	9 (100)	25 (86)
Extracolonic disease			
Present	1 (14)	2 (22)	4 (14)
Absent	6(86)	7 (78)	25 (86)

FAP, Familial Adenomatous Polyposis; AFAP, attenuated FAP.
[1] Values given in parentheses indicate percentages.

Twenty-nine (46%) out of 63 *MYH* mutation-negative patients had a family history of CRC and/or multiple polyps/polyposis compatible with an autosomal recessive mode of inheritance and could, therefore, be included in the genotype–phenotype analysis (Table II). Comparing the phenotypic properties of biallelic *MYH* mutation carriers, monoallelic mutation carriers and *APC/MYH* mutation-negative polyposis patients, colorectal cancer was significantly more frequent in biallelic mutation carriers than in the other subgroups (71% vs. 0% and 14%, respectively; χ^2 14.5, p < 0.001). Median age at diagnosis was similar between the 3 subgroups (48, 49 and 48 years, respectively). No further statistically significant phenotypic differences with respect to polyp number, age at diagnosis or extracolonic disease were observed.

DISCUSSION

In this nation-wide survey on 79 Swiss APC mutation-negative polyposis patients, 9% were found to harbor biallelic (n = 7) and 11% monoallelic (n = 9) germline mutations in the base excision repair gene *MYH*. Considering only patients with a family history compatible with autosomal recessive inheritance, biallelic *MYH* mutation carriers were observed in 10% (1/10) of patients with classical and in 17% (6/35) 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 common pathogenic missense mutations, p.Y165C and p.G382D [6,7,100,103], 2 novel alterations in the *MYH* gene p.R171Q and p.R231H were detected. Two hundred control chromosomes, assessed in parallel, did not harbor these missense changes, which proved to be target amino acids highly conserved across 5 distantly related species. Furthermore, whilst p.R171 constitutes part of a 6 helix barrel domain that contains the Helix–Hairpin–Helix motif, p.R231 lies within the alpha-8 helix making up the cluster domain. Together they form part of a DNA binding complex, where 9 lysines and 5 arginines form an electrostatically positive DNA interaction surface[104]. Clearly, functional studies are needed to ascertain the pathogenicity of these novel mutations. Moreover, since the parents of the individuals harboring 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 p.Y165C and p.G382D amounted to 5.7% (9/158) and 5.1% (8/158), respectively; if only patients with a family history compatible with an autosomal recessive mode of inheritance were considered, the allele frequencies raised to 10% (9/90) and 8.9% (8/90). 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)[6,103]. This further substantiates the view that the frequency of the p.Y165C and p.G382D mutations in the general population is too low to justify large-scale mutation screening[43].

The overall frequencies of biallelic mutation carriers did not significantly differ between patients displaying a classical (5.6%) and those displaying an

attenuated (9.8%) FAP phenotype, which is similar to reports by Sieber et al. who identified biallelic mutations in 7.5% and 5% of patients, respectively[100]. The frequency of monoallelic mutation carriers, however, was significantly higher in our study group (11.4% compared with that of 3.9% as reported by Sieber et al.[100]) which may reflect ethnic and geographic differences between the populations studied. Six (86%) out of 7 biallelic *MYH* mutation carriers were found to have less than 100 polyps at the time of diagnosis and 5 (71%) had developed 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 data from Enholm et al.[103], who investigated a population-based series of Finnish CRC patients.

On the basis of clinicopathological features, it is virtually impossible to discriminate 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 the occurrence of extracolonic disease was similar. Colorectal adenocarcinomas, however, were significantly ($p < 0.001$) more frequent among biallelic as compared to that of monoallelic *MYH* mutation carriers and *MYH* mutation-negative polyposis patients.

In conclusion, biallelic *MYH* germline alterations were identified in 15.5% of Swiss *APC* mutation-negative patients with a family history compatible with autosomal recessive inheritance. Biallelic mutation carriers were more frequently observed in AFAP patients compared to those with classical FAP (17% vs. 10%). 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: (i) presence of classical or attenuated polyposis, (ii) absence of an *APC* germline mutation and (iii) a family history compatible with an 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[105].

5. IMMUNOHISTOCHEMICAL ANALYIS REVEALS HIGH FREQUENCY OF PMS2 DEFECTS IN COLORECTAL CANCER

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K.T., M.M. and J.L. contributed equally to this study.

ABSTRACT

Background & Aims: Germline mutations in the DNA mismatch repair (MMR) genes MSH2, MSH6 or MLH1 predispose to colorectal cancer (CRC) with an autosomal dominant inheritance pattern. The protein encoded by PMS2 is also essential for MMR; however, alterations in this gene have been documented only in extremely rare cases. We addressed this unexpected finding by analyzing a large series of CRCs. Methods: Expression of MSH2, MSH6, MLH1, and PMS2 was studied by immunohistochemistry in 1048 unselected, consecutive CRCs. Where absence of MMR proteins was detected, microsatellite instability and cytosine methylation of the respective gene promoter were analyzed. The DNA of patients presenting with PMS2-deficient cancers was examined for germline and somatic alterations in the PMS2 gene. Results: An aberrant pattern of MMR protein expression was detected in 13.2% of CRCs. Loss of expression of MSH2, MSH6, or MLH1 was found in 1.4%, 0.5%, and 9.8%, respectively. PMS2 deficiency accompanied by microsatellite instability was found in 16 cases (1.5%) with a weak family history of cancer. The *PMS2* promoter was not hypermethylated in these cases. Despite interference of the *PMS2* pseudogenes, we identified several heterozygous germline mutations in the *PMS2* gene. Conclusions: PMS2 defects account for a small but significant proportion of CRCs and for a substantial fraction of tumors with microsatellite instability. However, the penetrance of heterozygous germline mutations in *PMS2* is considerably lower than that of mutations in other MMR genes. The possible underlying causes of this unorthodox inheritance pattern are discussed.

INTRODUCTION

Repair of mismatches, non-Watson-Crick base pairs arising during DNA replication or recombination, requires the mismatch repair (MMR) proteins MSH2, MSH3, MSH6, MLH1, and PMS2 and several factors involved in DNA replication[106]. Most base-base mismatches (G/T, G/G, and so on) are recognized by the MSH2/MSH6 heterodimer, whereas small insertion/deletion loops arising in mononucleotide and dinucleotide repeats (the so-called microsatellites) through strand misalignments can be bound either by the MSH2/MSH6 or the MSH2/MSH3 heterodimer. The partial redundancy of MSH3 and MSH6 in the repair of insertion/ deletion loops has a profound effect on microsatellite instability (MSI), one of the key phenotypic traits of MMR-deficient tumors[107,108]. Thus, whereas MSH2-deficient tumors invariably display MSI because both heterodimers are defective, the degree of MSI in MSH6deficient tumors can vary[109-111] because the MSH2/MSH3 heterodimer partially compensates for the loss of MSH2/ MSH6 in insertion/deletion loop repair[112,113]. The converse is not true, however, because the MSH2/MSH6 heterodimer compensates for the absence of MSH2/MSH3 in insertion/deletion loop repair. Correspondingly, primary alterations in the MSH3 gene have not been found in tumors with MSI. Like their biological roles, the biochemical characteristics of the 3 MSH polypeptides also differ, inasmuch as MSH2 deficiency leads to the proteolytic degradation of both MSH3 and MSH6, whereas MSH2 is largely stable in the absence of one of its cognate partners[106]. This means that cells with destabilized MSH2 will also appear as MSH3 and MSH6 negative in immunohistochemistry, whereas cells with mutated MSH3 or MSH6 will be seen to lack solely the affected polypeptide.

The MLH1/PMS2 heterodimer was suggested to act as a matchmaker between the mismatch recognition complex and the downstream MMR factors[106]. Interestingly, as in the case of the MSH proteins, the stabilities of the MLH1 and PMS2 polypeptides are different. Thus, when MLH1 is not expressed or when it is mutated such that it is either destabilized or it cannot interact with PMS2, the latter protein is degraded. In contrast, MLH1 remains stable in the absence of PMS2[113,114], possibly through interactions with other MutL homologues such as PMS1 or MLH3.

MSI analyses of tumor DNA suggested that 10%– 20% of colorectal cancers (CRC) may be associated with MMR system malfunctions[115]. In sporadic (i.e., nonfamilial) CRCs, the MMR defect is associated with the silencing of MLH1 transcription by cytosine methylation[116]. In families affected by hereditary nonpolyposis colon cancer (HNPCC, also called Lynch syndrome), MMR malfunction has been linked primarily with heterozygous germline mutations in *MSH2* or *MLH1*. Due to the partial functional redundancy of MSH3 and MSH6, germline alterations in *MSH6* are less frequent and are associated with colon and endometrial cancers in families that often do not fulfill Amsterdam criteria for diagnosis of HNPCC[117,118] (sometimes referred to as "HNPCC-like" or "attenuated HNPCC"). Nevertheless, the inheritance pattern in families carrying *MSH6* germline mutations is autosomal dominant, as in families with germline alterations in *MSH2* or *MLH1*. Germline mutations in the *PMS2* gene have been reported in 6 subjects presenting with a severe childhood cancer syndrome characterized by CRC and brain tumors in the first 2 decades of life (3 cases were diagnosed as Turcot's syndrome)[51,119-123]. A careful mutational analysis in these cases showed that the germline alterations were biallelic, suggesting a recessive inheritance pattern reminiscent of similar syndromes observed in children found to be compound heterozygotes or true homozygotes for mutations in *MSH2* or *MLH1*[124-128]. To date, heterozygous germline mutations in *PMS2* have not been reported in HNPCC families as defined by Amsterdam criteria. This finding is contrary to expectations, because PMS2 is essential for MMR. MLH1 can interact with other partners, notably PMS1[114] and MLH3[129] (Cannavó et al., manuscript in preparation); however, neither heterodimer could be shown to play a major role in human MMR. Correspondingly, cell lines not expressing PMS2 display MSI and their extracts are MMR deficient to an extent similar to that observed in cells mutated in *MLH1* or *MSH2*[114,130,131]. It might therefore be anticipated that, similarly to *MSH2*, *MLH1*, and *MSH6*, germline mutations in a single *PMS2* allele would predispose to CRC. We addressed this curious finding by analyzing the expression of PMS2 along with that of its heterodimeric partner MLH1 and with that of MSH2 and MSH6 in a large series of unselected CRCs. This approach, supported by the analysis of the PMS2 gene, allowed us to

identify patients affected by cancers with a primary defect of PMS2 (i.e., not secondary to an MLH1 defect) and to describe their phenotype.

PATIENTS AND METHODS

Patients

Between January 2000 and June 2002, 1048 consecutive CRCs were collected from patients who underwent surgery at 5 Swiss hospitals: Triemli Hospital Zurich (F.B.), University Hospital Zurich (H.-M.R.), Cantonal Hospital of Lucerne (J.-O.G.), Cantonal Hospital of Aarau (H.Y.), and Cantonal Hospital of St Gallen (J.N.). No tumors were excluded from this study. In the first instance, the expression of MSH2, MSH6, MLH1, and PMS2 was investigated by immunohistochemistry. In tumors with aberrant patterns of MMR protein expression, MSI analysis was performed using the mononucleotide marker BAT26. We then analyzed the methylation status of the *MLH1* or *PMS2* gene promoters in tumors not expressing MLH1 or PMS2, respectively. Finally, for patients presenting with PMS2-deficient tumors, blood and tumor DNA were analyzed for the presence of germline and somatic mutations and for the loss of heterozygosity (LOH) at the *PMS2* locus.

Information about the family history of cancer was obtained from a questionnaire sent to the family doctors and to the patients. In our study, cases fulfilling the Amsterdam criteria II (or revised Amsterdam criteria)[117] for diagnosis of HNPCC are designated "AC," whereas cases not complying with AC but fulfilling the revised Bethesda guidelines for MSI testing (Table 2 of Umar et al.[47]; guidelines for the identification of tumors that should be tested for MSI) are designated "BG." Cases fulfilling neither AC nor BG are termed "sporadic."

The study protocol was approved by the institutional review boards.

Immunohistochemistry

Tumors were fixed in buffered formalin and embedded in paraffin. Four-micrometer sections were mounted on glass slides coated with organosilane (DakoCytomation, Glostrup, Denmark), deparaffinized, and rehydrated. Antigen retrieval was accomplished by heating the sections in a pressure

cooker at 120°C for 2 minutes in 10mmol/L citrate-buffered solution (pH 6.0). DakoCytomation peroxidase blocking reagent and goat serum were sequentially used to suppress nonspecific staining due to endogenous peroxidase activity and unspecific binding of antibodies, respectively. Incubations with primary monoclonal antibodies were performed as follows: antihMSH2, 24 hours at 4°C with 1μg/mL antibody NA26 (Oncogene Research, Darmstadt, Germany); anti-hMSH6, 2 hours at room temperature with 4μg/mL antibody G70220 (Transduction Laboratories, Basel, Switzerland); anti-hMLH1, 1 hour at room temperature with 1.2μg/mL antibody 13271A (PharMingen, Basel, Switzerland); anti-hPMS2, 24 hours at 4°C with 3μg/mL antibody 65861A (PharMingen, Basel, Switzerland). After washing, anti-mouse secondary antibodies conjugated to peroxidase-labeled polymer (Dako EnVision+ kit) were applied for 30 minutes at room temperature, and the peroxidase activity was developed by incubation with 3,3'-diaminobenzidine chromogen solution (Dako). The sections were then counterstained with hematoxylin. Lack of protein expression was clearly evident as absence of nuclear staining in tumor cells despite nuclear staining in proliferating cells in normal crypts and stroma.

MSI, LOH, and PMS2 Sequencing

DNA was extracted from histologic sections and blood with QIAamp DNA and DNeasy, whereas RNeasy and Omniscript/Sensiscript RT kits (Qiagen, Basel, Switzerland) were used for RNA isolation and complementary DNA synthesis. MSI analysis was performed by analyzing the mononucleotide repeat BAT26 and, for PMS2-deficient tumors, several dinucleotide repeats on chromosome 7p22. LOH analysis was performed using 8 microsatellite markers flanking *PMS2* on chromosome 7p22. LOH was scored at any informative marker if the area under one allelic peak in tumor DNA was reduced by >50% relative to the other allele after correcting for the relative peak areas in DNA from normal tissue. Search for germline mutations was performed on genomic DNA and complementary DNA by sequencing all 15 *PMS2* exons and their intron-exon boundaries. Primers described by Nicolaides et al.[132] were used except for exons 1, 3, 5, and 10–14, for which new primers were designed to allow a more specific amplification and to avoid coamplification of *PMS2*

pseudogenes. All primers and reaction conditions are reported in supplementary table 1 (see appendix).

Promoter Methylation Status

Genomic DNA was subjected to sodium bisulfite conversion. Briefly, 2 μg of DNA was denatured in 0.3N NaOH at 37°C for 20 minutes and then incubated in 3mol/L sodium bisulfite and 500 μmol/L hydroquinone at 50°C for 16–18 hours. After desalting with Wizard DNA Clean-Up System (Promega, Walliselen, Switzerland), the bisulfite-treated DNA was desulfonated with 0.3N NaOH for 20 minutes at 37°C, neutralized with ammonium acetate, ethanol precipitated, and resuspended in 20 μL of water. The methylation status of 2 overlapping regions in the *PMS2* promoter rich in CpG sites (although not fulfilling the classic CpG island definition[133]) and of an *MLH1*-promoter region critical for gene silencing was evaluated by a real-time polymerase chain reaction (PCR) approach and confirmed by methylation-specific PCR (see supplementary table 1 in the appendix). The analysis of melting curves after real-time PCR and of specific bands in agarose gel electrophoresis after methylation-specific PCR permitted a reliable differentiation between methylated and unmethylated alleles.

Statistical Analysis

Descriptive statistical analysis used analysis of variance, χ^2 tests, *t* tests, and Fisher exact tests. Logistic regression was used to analyze the variables associated with MMR-deficient tumors and to evaluate their independent effects.

RESULTS

The clinical characteristics of the study population are listed in Table 1. Based on the absence of MMR proteins, as judged by immunohistochemistry, 139 CRCs (13.2%) were classified as MMR deficient. These tumors occurred significantly more frequently in women ($P < .001$) and in the proximal colon ($P < .001$) and showed lower frequency of lymph node involvement ($P < .001$) and degree of cell differentiation ($P < .001$) than MMR-proficient CRCs.

Logistic regression of MMR status on these variables confirmed their independent effects (footnote in Table 1).

Table 1   **Characteristics of Patients and CRCs in Relation to Tumor MMR Status**

Characteristics	All cases	MMR-proficient CRC	MMR-deficient CRC	Difference (95% CI)	p^a
Patients no. (%)	1048 (100)	909 (86.7)	139 (13.2)		
Age at presentation, y					
Mean ($\pm$SD)	69.7 (11.9)	69.7 (11.4)	69.9 (14.9)		0.82
Median		71	71	74	
Range	23 – 94	23 – 93	27 – 94		
Sex, no. (%)					
Male	609 (58.1)	547 (60.2)	62 (44.6)	15.6 (6.8 – 24.3)	< .001
Female	439 (41.9)	362 (39.8)	77 (55.4)		
Site of Tumor, no. (%)					
Cecum	127 (12.1)	92 (10.1)	35 (25.2)		<.00005
Ascending colon	134 (12.8)	84 (9.2)	50 (36.0)		
Hepatic flexure	34 (3.2)	25 (2.8)	9 (6.5)		
Transverse colon	48 (4.6)	40 (4.4)	8 (5.8)		
Splenic flexure	21 (2.0)	19 (2.1)	2 (1.4)		
Descending colon	36 (3.4)	29 (3.2)	7 (5.0)		
Sigmoid colon	343 (32.7)	329 (36.2)	14 (10.1)		
Rectum	305 (29.1)	291 (32.0)	14 (10.1)		
Proximal to splenic flexure	343 (32.7)	241 (26.5)	102 (73.4)	46.9 (39.0 – 54.8)	<.00005
Distal to splenic flexure	705 (67.3)	668 (73.5)	37 (26.6)		
Pathologic classification of the primary tumor, no. (%)^b					
T1	70 (6.7)	63 (6.9)	7 (5.0)		
T2	163 (15.6)	146 (16.1)	17 (12.2)		
T3	636 (60.7)	544 (59.8)	92 (66.2)		
T4	179 (17.1)	156 (17.2)	23 (16.5)		
Pathologic classification of the regional lymph nodes, no. (%)^b					
N0	573 (54.7)	479 (52.7)	94 (67.6)		0.003
N1	266 (25.4)	244 (26.8)	22 (15.8)		
N2	209 (19.9)	186 (20.5)	23 (16.5)		
No lymph node involvement	573 (54.7)	479 (52.7)	94 (67.6)	14.9 (6.1 – 23.8)	<.001
Lymph node involvement	475 (45.3)	430 (47.3)	45 (32.3)		
Tumor grade, no. (%)					
Well differentiated (G1)	27 (2.6)	25 (2.8)	2 (1.4)	23.6 (16.5 – 30.7)	<.00005^c
Moderately differentiated (G2)	806 (76.9)	726 (79.9)	80 (57.5)		
Poorly differentiated (G3)	215 (20.5)	158 (17.4)	57 (41.0)		

CI, confidence interval.
^{a} Comparison between MMR-proficient and -deficient CRCs (t and χ2 tests). Logistic regression: the odds ratio was 1.6 (95%CI, 1.1 – 2.4) for women versus men, 7.1 (95%CI, 4.7 – 10.9 for proximal versus distal colon, 3.1 (95%CI, 2.0 – 4.8) for no lymph node involvement and 3.4 (95% CI, 2.2 – 5.2) for poorly versus well plus moderately differentiated tumors

[b] TNM and tumor grade classification according to Sobin LH, Wittekin DH, TNM claffication of malignant tumors, 6th ed. New York,: Willey-Liss, 2002. Only T and N stages are reported because they are histologically confirmed in all cases.
[c] Comparison between well plus moderately differentiated and poorly differentiated tumors

Table 2   Characteristics of Patients With MMR-Deficient Tumors

| | | CRCs not expressing | | | | | | | | | | | |
| | | MLH1 | | | PMS2 | | | MSH2 | | | MSH6 | | |
	All cases	Total	AC or BG[a]	Sporadic[a]	Total	AC or BG[a]	Sporadic[a]	Total	AC or BG[a]	Sporadic[a]	Total	AC or BG[a]	Sporadic[a]
Patients, no.(%)	139	103 (74)	28 (29)	68 (71)	16 (11.5)	12 (75)	4 (25)	15 (10.8)	9 (82)	2 (18)	5 (3.6)	3 (75)	1 (25)
Age at presentation, y													
Mean ($\pm$SD)	69.9 (14.9)	72.6 (14.1)	57.9 (15.7)	78.5 (8.2)	62.8 (14.7)	59.4 (15.4)	73.0 (5.0)	62.5 (17.2)	52 (12.7)	69.0 (7.1)	60.4 (5.1)	62.3 (6.1)	58
Median	74	76	57	78	61.5	57	74.5	64	53	69	58	61	
Range	27-94	27-94	27-91	52-94	42-82	42-82	66-77	34-87	34-71	64-74	57-69	57-69	
Sex, no.(%)													
Male	62 (45)	39 (38)	13 (46)	25 (37)	14 (88)	10 (83)	4 (100)	7 (47)	5 (56)	1 (50)	2 (40)	0	1 (100)
Female	77 (55)	64 (62)	15 (54)	43 (63)	2 (12)	2 (17)	0	8 (53)	4 (44)	1 (50)	3 (60)	3 (100)	0
Site of tumor, no.(%)													
Cecum	35 (25)	31 (30)	6 (21)	23 (34)	1 (6)	1 (8)	0	3 (20)	1 (11)	1 (50)	0	0	0
Ascending colon	50 (36)	35 (34)	6 (21)	28 (41)	7 (44)	3 (25)	4 (100)	6 (40)	4 (44)	0	2 (40)	2 (67)	0
Hepatic flexure	9 (6)	8 (8)	2 (7)	6 (9)	1 (6)	1 (8)	0	0	0	0	0	0	0
Transverse colon	8 (6)	7 (7)	1 (4)	6 (9)	1 (6)	1 (8)	0	0	0	0	0	0	0
Splenic flexure	2 (1)	0	0	0	2 (13)	2 (17)	0	0	0	0	0	0	0
Descending colon	7 (5)	4 (4)	3 (11)	0	2 (13)	2 (17)	0	1 (7)	1 (11)	0	0	0	0
Sigmoid colon	14 (10)	11 (11)	5 (18)	3 (4)	1 (6)	1 (8)	0	1 (7)	1 (11)	0	1 (20)	1 (33)	0
Rectum	14 (10)	7 (7)	5 (18)	2 (3)	1 (6)	1 (8)	0	4 (27)	2 (22)	1 (50)	2 (40)	0	1 (100)
Proximal to splenic flexure	102 (73)	81 (79)	15 (54)	63 (93)	10 (63)	6 (50)	4 (100)	9 (60)	5 (56)	1 (50)	2 (40)	2 (67)	0
Distal to splenic flexure	37 (27)	22 (21)	13 (46)	5 (7)	6 (37)	6 (50)	0	6 (40)	4 (44)	1 (50)	3 (60)	1 (33)	1 (100)
Pathological classification of the primary tumor, no. (%)[b]													
T1	7 (5)	5 (5)	3 (11)	1 (2)	2 (13)	1 (8)	1 (25)	0	0	0	0	0	0
T2	17 (12)	13 (13)	3 (11)	9 (13)	1 (6)	1 (8)	0	3 (20)	2 (22)	0	0	0	0
T3	92 (66)	68 (66)	18 (64)	45 (66)	10 (63)	8 (67)	2 (50)	10 (67)	6 (67)	2 (100)	4 (80)	2 (67)	1 (100)
T4	23 (17)	17 (16)	4 (14)	13 (19)	3 (19)	2 (17)	1 (25)	2 (13)	1 (11)	0	1 (20)	1 (33)	0

| | | CRCs not expressing | | | | | | | | | | | |
| | | MLH1 | | | PMS2 | | | MSH2 | | | MSH6 | | |
	All cases	Total	AC or BG[a]	Sporadic[a]	Total	AC or BG[a]	Sporadic[a]	Total	AC or BG[a]	Sporadic[a]	Total	AC or BG[a]	Sporadic[a]
Pathological classification of the regional lymph nodes, no. (%)[b]													
N0	94 (68)	67 (65)	18 (64)	44 (65)	14 (88)	10 (83)	4 (100)	11 (73)	6 (67)	2 (100)	2 (40)	1 (33)	1 (100)
N1	22 (16)	20 (19)	5 (18)	13 (19)	1 (6)	1 (8)	0	0	0	0	1 (20)	0	0
N2	23 (17)	16 (15)	5 (18)	11 (16)	1 (6)	1 (8)	0	4 (27)	3 (33)	0	2 (40)	2 (67)	0
No lymph node involvement	94 (68)	67 (65)	18 (64)	44 (65)	14 (88)	10 (83)	4 (100)	11 (73)	6 (67)	2 (100)	2 (40)	1 (33)	1 (100)
Lymph node involvement	45 (32)	36 (35)	10 (36)	24 (36)	2 (12)	2 (17)	0	4 (27)	3 (33)	0	3 (60)	2 (67)	0
Tumor grade, no. (%)													
Well differentiated (G1)	2 (1)	2 (2)	1 (4)	1 (1)	0	0	0	0	0	0	0	0	0
Moderately differentiated (G2)	80 (56)	55 (53)	22 (79)	29 (43)	12 (75)	8 (67)	4 (100)	9 (60)	6 (67)	2 (100)	4 (80)	2 (67)	1 (100)
Poorly differentiated (G3)	57 (41)	46 (45)	5 (18)	38 (56)	4 (25)	4 (33)	0	6 (40)	3 (33)	0	1 (20)	1 (33)	0
MSI[c]													
Unstable	127 (92)[d]	99 (96)	27 (96)	66 (97)	15 (100)[d]	11 (100)	4 (100)	12 (80)	7 (78)	2 (100)	1 (20)	1 (33)	0
Stable	11 (8)	4 (4)	1 (4)	2 (3)	0	0	0	3 (20)	2 (22)	0	4 (80)	2 (67)	1 (100)
Promotor methylation[e]													
Present	84 (82)		14 (50)	65 (96)	0	0	0						
Absent	19 (18)		14 (50)	3 (4)	15 (100)[d]	11 (100)	4 (100)						

[a] Percentage refers to the number of patients whose family pedigree was available (MSH2, 11; MSH6, 4; MLH1, 96; PMS2, 16; for details see supplementary Table 2). Sporadic CRCs were separated from those fulfilling the AC for HNPCC diagnosis or BG for MSI testing (for definitions, see Patients and Methods).

[b] TNM and tumor grade classification according to Sobin LH, Wittekind CH. TNM classification of malignant tumours. 6th ed. New York: Wiley-Liss, 2002. Only T and N stages are reported because they are histologically confirmed in all cases.

[c] MSI as detected by BAT26 analysis, except for PMS2 cases where dinucleotide repeats flanking *PMS2* on chromosome 7 were also investigated.

[d] In one case, DNA was not suitable for analysis

[e] Only *MLH1* or *PMS2* promoter were analyzed in MLH1-deficient or PMS2-deficient CRCs, respectively
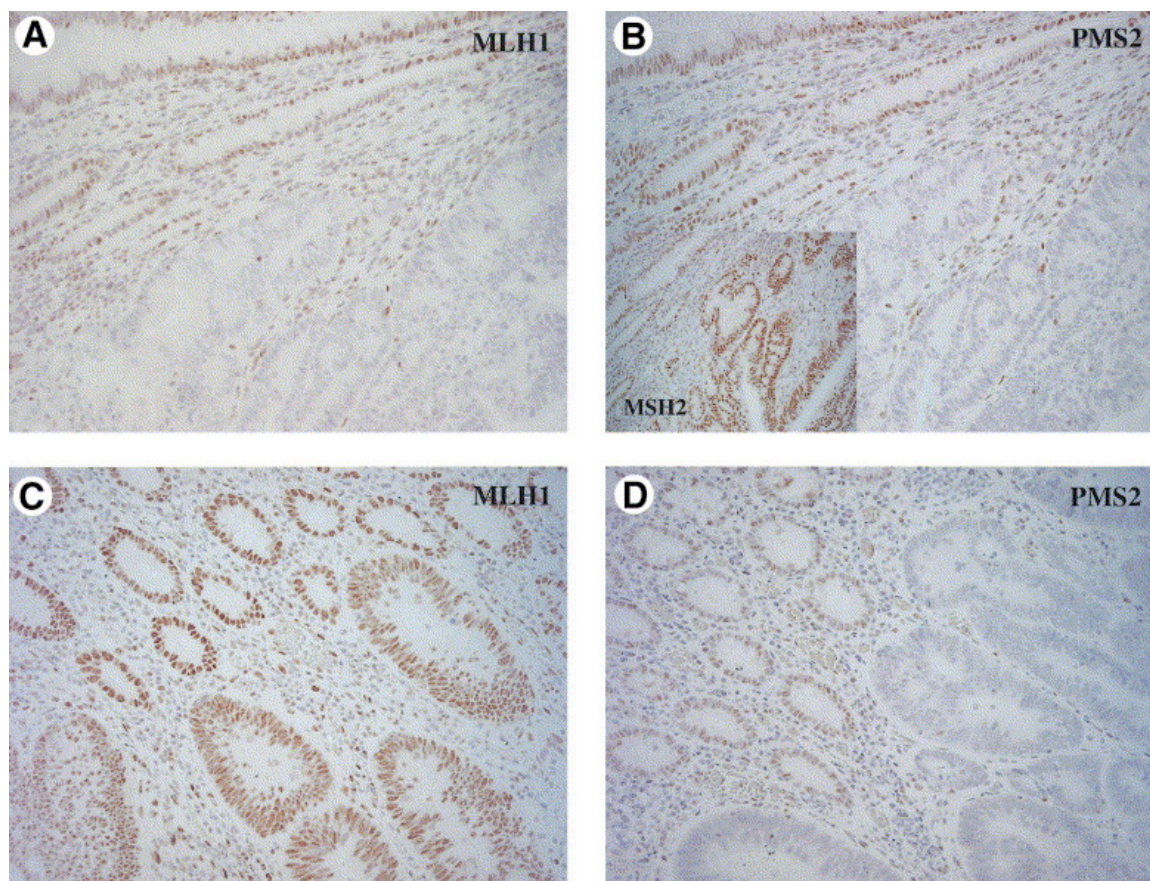
Figure 1   **Immunohistochemical staining of colorectal tumors for MMR proteins.**
(A) MLH1 is absent from tumor tissue, but normal crypts (upper part of the picture) and proliferating stromal cells express this protein normally.
(B) The same tumor does not express PMS2, because this protein is unstable in the absence of MLH1. However, other MMR proteins are expressed normally, as shown for MSH2 (inset). (C) The dysplastic crypts on the right side of this tumor express MLH1 levels similar to the normal crypts on the left; (D) however, the dysplastic crypts are deficient in PMS2.

Table 2 lists the characteristics of patients presenting with MMR-deficient tumors. A total of 103 of the MMR-deficient CRCs (74%) lacked MLH1 expression and consequently also PMS2 (see the introduction). These tumors are referred to as "MLH1 deficient." PMS2 was lacking in 16 MMR-deficient CRCs (11.5%) in which MLH1 was expressed normally (Figure 1). The latter tumor category is referred to as "PMS2 deficient." MSH2 was not detected in the 15 tumors (10.8%) in which MSH6 was also undetectable ("MSH2-deficient" category), whereas MSH6 was lacking in 5 tumors (3.6%) that expressed MSH2 normally ("MSH6-deficient" tumors). For the sake of consistency, cases not fulfilling the AC or BG were designated as sporadic in this table (see Patients and Methods for definition of AC and BG; as reported in the footnote to Table 2, 127 of the 139 pedigrees were available). The vast majority of sporadic tumors were deficient in MLH1. These tumors represented about half of the

entire group of MMR-deficient tumors. MLH1-deficient CRCs appear to have a higher average age of onset as compared with the other 3 groups (P=.004). However, this is due to the very late onset of sporadic MLH1-deficient tumors (P<.001, as compared with nonsporadic MLH1-deficient tumors). In contrast to CRCs not expressing MSH2, MSH6, and MLH1, the majority of PMS2-deficient tumors occurred in men (P=.003). The preferential occurrence of MMR-deficient tumors in the right colon was evident in all 4 groups, particularly in sporadic, MLH1-deficient CRCs (P<.001, as compared with the nonsporadic counterpart). No relevant differences among the 4 groups were found with regard to other variables; however, sporadic, MLH1-deficient CRCs were more often poorly differentiated than nonsporadic ones (P<.001). A detailed list of the MMR-deficient cases is reported in Supplementary Table 2 (see appendix). The clinical and genetic evaluation of MLH1-, MSH2-, and MSH6-deficient cases is under way. Few of the MMR-deficient tumors identified in this study were shown to originate from patients whose families are already on the Swiss Familial CRC Registry and in which the germline mutations are known. The results of this study will be the subject of another report.

As shown in table 2, 92% (127 of 138; in one case, the DNA was not suitable for analysis) of MMR-deficient tumors displayed MSI at the BAT26 locus, which is generally considered one of the most sensitive and reliable markers of MSI. The fact that the BAT26 marker was stable in 4 of 5 MSH6-deficient CRCs was not surprising due to the functional redundancy between MSH6 and MSH3 (see the introduction). The latter protein was indeed expressed in all 5 MSH6-negative cases (data not shown). Eighty-four of 103 MLH1-deficient tumors (82%) showed MLH1 promoter hypermethylation (Table 2), which was more frequent in sporadic cancers (P < .001), as anticipated (see the introduction). However, promoter hypermethylation was also present in half of the MLH1-deficient cases fulfilling AC or BG (4 and 10 patients, respectively; details in supplementary table 2, see appendix). This could represent either a mechanism of somatic inactivation of the wild-type allele in a tumor carrying a germline mutation or a germline epimutation[134]. It is noteworthy that analysis of *MLH1*-promoter methylation by real-time PCR proved to be as reliable as methylation-specific PCR (Supplementary Table 1; see appendix).

The immunohistochemical screening identified 16 PMS2-deficient tumors (Table 3). As shown in figure 2 (top panels), inactivation of both *PMS2* alleles in the tumor appeared to be a very early event, occurring already in small benign

adenomas. Because PMS2 was invariably expressed in normal tissues of the affected individuals, the presence of biallelic germline *PMS2* alterations could be excluded. In 5 patients, the tumors presented before the age of 50 years; in 3 patients, the tumors presented between 50 and 60 years of age. Two additional patients with CRC in their late 70s had had a previous CRC ~20 years earlier. Previous or synchronous adenomatous polyps were a common finding, and 4 patients were also affected with prostate, brain, or skin cancers. Fourteen patients (87.5%) were men. Ten tumors (62.5%) were located in the proximal colon, and only 2 (12.5%) showed lymph node involvement. Some of the previous or synchronous tumors of the index patients were available for immunohistochemistry and were also found to be lacking PMS2 expression (Table 3 and Figure 2). The cancer spectrum in relatives included CRCs (mean age, 48 years; range, 31–60 years) and cancers of other organs, often at earlier-than-average age of onset. Finally, BG were fulfilled in 12 of 16 cases. PMS2-deficient tumors showed MSI at BAT26 and at the dinucleotide markers used for LOH evaluation at the *PMS2* locus (Table 3). The same markers were stable in leukocyte-derived DNA. The LOH analysis was heavily affected by widespread MSI; however, it was clearly present in 6 tumors and neither excluded nor proven in the remaining cases. The *PMS2* promoter was unmethylated in all cases (Table 2). Using the approach described in Patients and Methods, we have been able to identify *PMS2* germline mutations in 6 patients (Table 3). All 6 are highly likely to be linked to CRC predisposition, because they lead to premature termination of PMS2 synthesis either as a result of frameshifts (4 insertions and one deletion) or a point mutation (Gln → stop). Because all of the truncated proteins would lack the MLH1-interacting domain, which is required for PMS2 stabilization, they would be rapidly degraded. Moreover, it is possible that the mutant messenger RNA will be degraded by nonsense-mediated decay. No germline mutations have to date been identified in the remaining cases, but mutations in *MLH1* were not found, thus excluding categorically the possibility that PMS2 deficiency might be secondary to defects of MLH1.

Table 3   Characteristics of Patients With CRCs Not Expressing PMS2

Patient no.	Age (y)	Sex	Site	Stage [a]	Grade [a]	MSI [b]	LOH	PMS2 Germline Mutation [c] f	Other tumors Organ	Other tumors Histology	Other tumors Age (y)	Tumors in family members Relative	Tumors in family members Organ	Tumors in family members Age (y)	AC and BG
64501	79	M	D	T3N0M0	2	P	NV[d]	NV[e]	Colon	Carcinoma	57				BG2
									Colon	Adenoma[f]	79				
53072	57	M	A	T3N0M0	2	P	LP	NF				Sister	Colon	31	BG4
												Sister	Pancreas	60	
												Brother	Lymphoma	47	
61263	73	M	A	T3N0M0	2	P	LP	NF							
66543	77	F	T	T4N0M0	2	P	NV[d]	Exon 11: 1828insA	Colon	Adenoma[f]	77	Sister	Breast	35	BG4
												Sister	Liver	77	
												Daughter	Uterus	42	
66732	46	M	D	T3N0M0	2	P	NV[d]	Exon 11: 1828insA	Colon	Adenoma[f]	46				BG1
52557	77	M	A	T1N0M0	2	P	NV[d]	NF	Colon	Adenoma[g]	76				
54832	42	M	A	T3N0M0	2	P	LP	Exon 11: 1828insA							BG1
53989	57	M	SF	T2N0M0	2	P	NV[d]	Exon 10: 1018delA	Prostate	Carcinoma[f]	55	Brother	Pancreas	39	BG4
61162	82	M	C	T3N0M0	3	P	NV[d]	NF	Skin	Squamos cell carcinoma[f]	75	Father	Stomach	50	BG4
65950	43	M	R	T3N0M0	3	P	LP	NF				Mother	Brain	58	BG1
												Uncle	Bladder	54	
												Uncle	Lung	68	
59519	78	M	SF	T3N0M0	3	P	NV[d]	NF	Colon	Carcinoma[f]	59				BG2
									Colon	Adenoma[g]	78				
11318	46	F	S	T3N0M0	2	P	NV[d]	NF				Aunt	Colon	60	BG1
20498	76	M	A	T3N0M0	2	P	LP	NF	Prostate	Carcinoma[f]	68	Father	Prostate	64	
												Mother	Breast	40	
16655	66	M	A	T4N0M0	2	P	NV[d]	Exon 11: 1828insA	Colon	Adenoma[g]	66	Brother	Lung	70	
												Brother	Stomach	65	
5194	57	M	HF	T4N2M1	3	P	LP	NV[e]	Colon	Adenoma[f]	57	Grandfather	Colon	55	BG3
27499	49	M	A	T1N0M0	2	ND	ND	Exon 6: 703C>T (Q235X)	Brain	[i]	9	Sister	Colon	41	
									Colon	Adenoma[g]	49	Grandfather	Colon	55	

M, male; F, female; D, descending colon; A, ascending; T, transversum; SF, splenic flexure; C, cecum; R, rectum; S, sigmoid colon; HF, hepatic flexure; P, present: in all cases MSI was present in both BAT26 and in dinucleotides except for tumors 52557 and 11318, where only the latter repeats were found unstable; ND, DNA extracted from microdissection was not suitable for MSI and LOH analyses; NV, not verifiable; LP, likely present; NF, not found with this approach.

[a] TNM and tumor grade classification according to Sobin LH, Wittekind CH. TNM classification of malignant tumors. 6th ed. New York: Wiley-Liss, 2002.
[b] MSI at BAT26 and dinucleotide repeats on chromosome 7p22.
[c] After sequencing all exons and intron-exon boundaries.
[d] Because of widespread MSI.
[e] Because blood was not available (patients deceased).
[f] This tumor does not express PMS2.
[g] This tumor expresses PMS2. The only case in which the lack of PMS2 expression did not affect the entire tumor.
[i] Histology was not available, because the tumor was treated with radiotherapy.
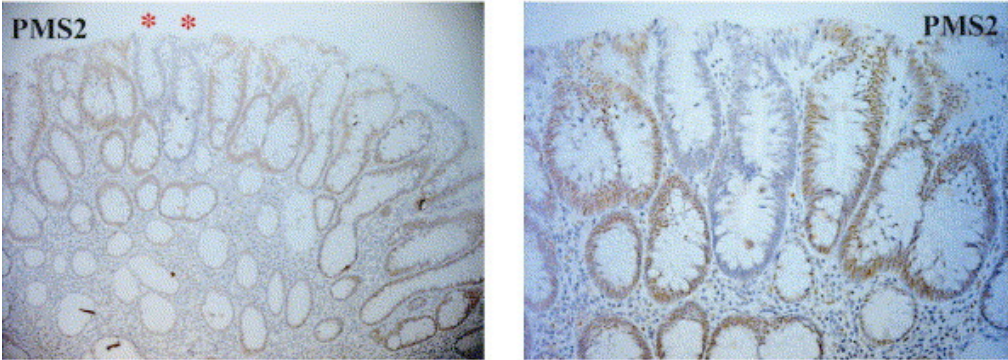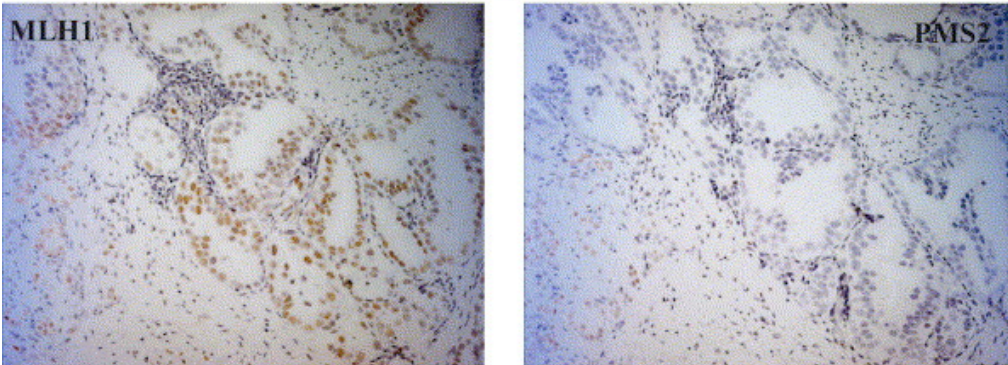
Figure 2   Immunohistochemical staining of a colon adenoma and extracolonic cancers for the MMR proteins MLH1 and PMS2. PMS2 is not expressed in the 2 mildly dysplastic crypts of a 2-mm benign colon adenoma (upper panels, asterisks; higher magnification is shown in the right panel; case 66732 of Table 3), in a prostate carcinoma (middle panels; case 20498), and in a squamous cell carcinoma (lower panels; case 61162). Note that, in the latter 2 samples, PMS2 is expressed in nonneoplastic tissues and MLH1 is expressed in both normal tissue and tumor tissue.

DISCUSSION

Immunohistochemical analysis of a large, consecutive series of unselected CRCs revealed that 13.2% were MMR deficient. Due to the biochemical properties of the MMR proteins (see the introduction), the use of antibodies against all 4 polypeptides of the 2 principal MMR heterodimeric complexes not only increased the accuracy of the immunostaining in detecting MMR-deficient cancers but also permitted the identification of tumors with a primary alteration of PMS2 (Figure 1). In this respect, our work differs from the other screening studies described to date, which used selection procedures that enriched for subjects with a clear family history of CRC or omitted PMS2 analysis[111,135-140].

Although immunohistochemistry is an extremely reliable tool for the detection of mutations that result in truncation and/or degradation of the antigen, it cannot distinguish between cells expressing variants of proteins carrying missense mutations that inactivate, but do not destabilize, the protein and cells expressing wild-type polypeptides. Correspondingly, we cannot exclude the possibility that a finite number of the analyzed CRCs harbored such *MMR* gene mutations. However, this number is expected to be very low, because all AC- or BG-fulfilling CRCs with MSI were accounted for by the lack of one of the 4 MMR proteins. In addition, in the Swiss Familial CRC Registry, most of the disease-associated *MSH2* and *MLH1* germline missense mutations were characterized either by the lack of protein staining in tumor cells or, more rarely, by a weak and diffuse (cytoplasmic and nuclear) antigen staining (manuscript in preparation). This does not apply to sporadic tumors, where silencing of MLH1 expression and subsequent degradation of PMS2 make the immunohistochemical analysis straightforward.

Thus, immunohistochemical analysis of MMR protein expression in CRCs should be encouraged, especially considering the implications of such screening for the follow-up and treatment of MMR-deficient tumors[141]. The frequency of MLH1-deficient sporadic CRCs is expected to increase worldwide because of population aging in developed and developing countries. In familial CRCs, immunohistochemistry helps identify the mutated gene and encourages the use of alternative procedures of mutational analysis[142-144] in cases in which standard methods fail.

The most striking finding of our study concerns the unexpectedly high frequency of PMS2-deficient CRCs (1.5%), which was similar to that of tumors

lacking MSH2 (1.4%). The fact that our patients did not belong to HNPCC families as defined by AC and were not believed by the physicians to be individuals at risk for CRC seemed to argue against their carrying germline *PMS2* mutations. However, several findings suggested that these cases differed from sporadic CRCs (Table 3). Many were diagnosed in patients in their fifth or sixth decade of life, some patients were also affected with extracolonic PMS2-deficient tumors, and several relatives were identified with CRCs or cancers in other organs at earlier-than-average age of onset. The preponderance of *PMS2* alterations in men is peculiar but needs confirmation in a larger series of patients. In contrast, the prevalent location in the proximal colon and the low frequency of lymph node involvement are characteristics of all MMR-deficient CRCs. Most importantly, our data suggest that most PMS2-deficient CRCs can be identified, at least as MMR-deficient tumors, if the revised BG were applied regularly. We also excluded methylation of the *PMS2* promoter that represents an epigenetic mechanism of gene inactivation generally associated with sporadic cancers, such as those deficient in MLH1. Taken together, this evidence points to germline *PMS2* defects. Indeed, a detailed analysis of the *PMS2* gene identified germline mutations in leukocyte DNA of 6 patients and somatic LOH in several tumors despite the genetic "noise" caused by the numerous *PMS2* pseudogenes on chromosome 7[121,145] and by MSI (see Patients and Methods). We are currently implementing modified protocols for the identification of germline mutations or deletions, with the hope of uncovering other genetic alterations. Interestingly, we identified 4 subjects carrying the same germline alteration. Although the individuals are unrelated, we wanted to find out whether we might be dealing with a founder mutation. Unfortunately, the haplotype analysis results were inconclusive to date (data not shown). Even if there is a founder effect, this should not affect the overall frequency of PMS2-deficient CRCs relative to that of other MMR-deficient tumors, because founder mutations have also been reported in MSH2 and MLH1[146-148].

Our immunohistochemical data show that PMS2 and MSH2 deficiencies occur with similar frequencies and that both defects are linked with germline mutations. How is it possible then that mutations in *MSH2* cause HNPCC as defined by AC, whereas those in *PMS2* apparently do not? One possible explanation is that MLH1/PMS1 and/or MLH1/MLH3 heterodimers partially compensate for the loss of MLH1/PMS2, similar to the partial functional redundancy of MSH2/MSH3 and

MSH2/MSH6 (see the introduction). However, we have found that MLH1/PMS1 heterodimer does not contribute to MMR *in vitro*[114], whereas the contribution of MLH1/MLH3 to MMR in vitro is only marginal (Cannavó et al., manuscript in preparation). Furthermore, MSI was not detected in the intestinal mucosa of *Pms1-/-* mice[149] and in fibroblasts of *Mlh3-/-* mice[150], which were found to be free of morbid cancers in the first 14 and 9 months of life, respectively.

A second explanation might lie in the role of MMR proteins in the response of mammalian cells to DNA-damaging agents[151]. Functional MMR seems to be necessary for activating cell cycle checkpoints and cell death on exposure to methylating agents and cisplatin, and it might be anticipated that absence of DNA damage signaling might favor the survival and thus also transformation of MMR-deficient cells. If PMS2 involvement in this process were to be smaller than that of MSH2 and MLH1, PMS2-deficient cells might not have the same growth advantage as those mutated in *MSH2* or *MLH1*. However, this hypothesis is not supported by experimental evidence showing that PMS2-deficient cells are as tolerant to killing by the methylating agent N-methylN'-nitro-N-nitrosoguanidine as cells deficient in MLH1[131].

An alternative, third explanation lies in the finding that chromosome 7, which houses the *PMS2* gene, accommodates also numerous *PMS2* pseudogenes[121,132,145,152-154]. The existence of these pseudogenes has been documented to interfere with sequence analysis of the *PMS2* locus[121,145]. However, they might also serve as a homologous sequence pool for recombination. Thus, assuming that the initial frequency of *PMS2* germline mutations might be similar to that of *MLH1*, gene conversion events between *PMS2* and its pseudogenes might result in the reversion of some mutations[155], such that the overall mutation frequency might decline and resemble that seen at the *MSH2* gene. Were such events to occur in the germline, this might help explain why germline mutations in *PMS2* are apparently not inherited in an autosomal dominant manner, like the other *MMR* gene aberrations. In somatic cells, gene conversion might mitigate the severity of the *PMS2* defects compared with *MSH2* or *MLH1*.

Our data suggest that PMS2-deficient CRCs associated with heterozygous germline alterations in the *PMS2* gene will not be found in HNPCC families as defined by AC. However, clinical and pathologic data clearly differentiated this group of patients from those with sporadic CRCs. We believe that the main reasons why

PMS2-deficient tumors went undetected for so long are the following: (1) the screening focused on subjects belonging to families with an obvious history of CRC, (2) PMS2 staining was not included in many screening studies based on the unfounded credence in a minor role of PMS2 in MMR, and (3) mutation detection was complicated by the presence of *PMS2* pseudogenes. We have noticed, however, that immunostaining for PMS2 has been recently included in the screening of selected populations[111,137,139,140,145]. In support of our data, Nakagawa et al.[145] identified 6 PMS2-deficient CRCs, all in patients without a clear family history of cancer but presenting with tumors with MSI in which mutations in *MSH2*, *MSH6*, and *MLH1* were previously excluded. Heterozygous germline alterations were detected in 2 of these patients only after using diploid-to-haploid conversion technique[142].

In conclusion, our study shows that about 1.5% of CRCs, the second most frequent cancer in humans, are associated with a genetic defect of the *MMR* gene *PMS2*. This translates to about 2200 newly diagnosed cases of CRC per year in the United States. Thus, many apparently sporadic CRCs with MSI, in which alterations in other *MMR* genes are not detected, might be associated with genetic alterations in *PMS2*. It should be remembered that *PMS2* defects could also be found in tumors from other organs that display MSI. The characterization of the genetic mechanisms underlying *PMS2* defects and the phenotypes of this subset of tumors are currently the subject of intense study.

Addendum:
cDNA analysis and MLPA investigation of colorectal cancer patients with PMS2 deficiency in their tumors

INTRODUCTION

In the previous part of this thesis the mutation analysis in a cohort of 1048 colorectal cancers with immunohistochemical loss of a MMR protein has been described. In this study we were able to identify six patients with germline mutations in the *PMS2* gene, but there are still 10 patients with loss of PMS2 in the tumor but without any detectable germline mutation.

One problem in analyzing genes located on chromosome 7 is the presence of pseudogenes, which complicate mutation analysis. Hillier showed that about 45% of all genes on chromosome 7 are actually pseudogenes[156]. The *PMS2* gene is located on chromosome 7 and 15 pseudogenes have been described of the 5' part of *PMS2* and 1 nearly 100% homologous pseudogene of the 3' part[121]. Only exon 6 – 8 and exon 10 of the *PMS2* gene represent pseudogene free regions.

Another possibility why mutations could not have been detected is the presence of large genomic rearrangements like large deletions or duplications. The recently introduced MLPA technique allows the identification of such gene copy number variations.

In order to identify germline mutations in *PMS2* in the remaining 10 patients, cDNA analysis and the MLPA technique have been applied.

PATIENTS AND METHODS

Patients

Out of 1048 tumors collected from patients who underwent surgery in five Swiss hospitals, sixteen showed immunohistochemically loss of PMS2 (described in the PMS2 study). Using conventional DNA screening methods germline mutations in the *PMS2* gene have been detected in six patients. In order to detect the reason of the loss of PMS2 in the remaining ten patients additional methods as cDNA analysis and MLPA investigation were applied.

Mutation analysis using mRNA

To avoid co-amplification of pseudogenes cDNA of *PMS2* was analyzed in two overlapping segments. The reverse primer of segment 1 and the forward primer of segment 2 is located in the pseudogene-free region of exon 10 with subsegments amplified in nested PCRs (S1n, S1A, S1B, S2A, S2B, S2C) (Figure 1). The primers for the nested PCRs are located at crossovers from one exon to the other. All primers and reaction conditions are reported in the appendix.
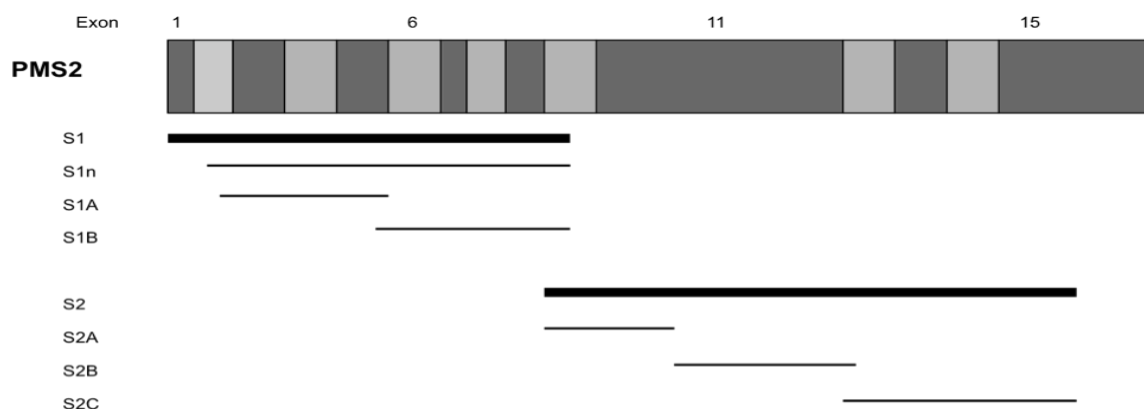
Figure 1: **Schematic delineation of different primers used for *PMS2* cDNA analysis**

MLPA analysis

For the detection of aberrant copy numbers in the *PMS2* gene in constitutional, leukocyte-derived DNA, the SALSA P008 MSH6/PMS2 test MLPA kit (MRC Holland, Amsterdam, the Netherlands) was used[157]. The kit contains probes for the 15 exons of *PMS2* as well as several probes located on different chromosomes as controls (see appendix for probemix). DNA samples from ten healthy probands were used to confirm the sensitivity and specificity of the method. All reactions were carried out according to the manufacturer's protocol. Fragment analysis was done on an ABI 310 capillary sequencer and results were analyzed using the Genescan and Genotyper software (Applied Biosystems) to identify the specific amplicons representing the respective exons and control loci. Peak areas and heights were then exported to a Microsoft Excel spreadsheet and calculations were done according to the method described by Taylor et al.[158]. An average dosage quotient close to 0.01 is expected for individuals with two copies, whereas values close to -0.50 indicate loss and close to 0.50 duplication of one copy.

RESULTS

cDNA analysis

The cDNA of ten patients with loss of PMS2 in their tumors was screened for mutations in two overlapping segments to avoid co-amplification of the pseudogenes present for *PMS2*. The result of the screening is depicted in Table 1.

After sequencing a frameshift was detected in segment 1 in six patients (Patients No. 5, 6, 8, 11, 12, 13). This frameshift is due to a deletion of 54 basepairs at the end of exon 4 (c.301_353del53) (Figure 2), which leads to a stop codon after 120 amino acids (p.Gln100fs120X). To confirm this frameshift, mutation-specific primers were designed comprising the respective location. The forward primer was located at the exon2/3 and the reverse primer at the exon 5/6 crossover. Using these primers the PCR product was smaller and thus, sequencing reaction revealed easier and better results (for primer sequences and PCR conditions see appendix).

In addition to this frameshift three SNPs in Segment 2 were detected: c.1621A>G (rs41534544) in all patients except of patient no. 4, c.2324A>G (rs17420802) in patient no. 4, and c.1408C>T (rs1805321) in patients no. 1, 9, 10, 13 and 14.

Table 1   **Summary of sequencing results of different cDNA segments of *PMS2***

Patient No.	cDNA Segment 1		cDNA Segment 2		
	S1 A	S1 B	S2 A	S2 B	S2 C
1	n.a.	n.a.	SNP c.1408C>T; p.Pro469Ser	SNP c.1621A>G; p.Lys540Glu	wildtype
2	no cDNA				
3	no cDNA				
4	wildtype	wildtype	wildtype	wildtype	SNP c.2324A>G; p.Lys775Ser
5	c.301_353del53	n.a.	wildtype	SNP c.1621A>G; p.Lys540Glu	wildtype
6	c.301_353del53	n.a.	wildtype	SNP c.1621A>G; p.Lys540Glu	wildtype
7	no cDNA				
8	c.301_353del53	wildtype	wildtype	SNP c.1621A>G; p.Lys540Glu	wildtype
9	n.a.	n.a.	SNP c.1408C>T; p.Pro469Ser	SNP c.1621A>G; p.Lys540Glu	wildtype
10	n.a.	n.a.	SNP c.1408C>T; p.Pro469Ser	SNP c.1621A>G; p.Lys540Glu	wildtype
11	c.301_353del53	n.a.	wildtype	SNP c.1621A>G; p.Lys540Glu	wildtype
12	c.301_353del53	n.a.	wildtype	SNP c.1621A>G; p.Lys540Glu	wildtype
13	c.301_353del53	wildtype	SNP c.1408C>T; p.Pro469Ser	SNP c.1621A>G; p.Lys540Glu	wildtype
14	wildtype	n.a.	SNP c.1408C>T; p.Pro469Ser	SNP c.1621A>G; p.Lys540Glu	wildtype

n.a.   not amplified
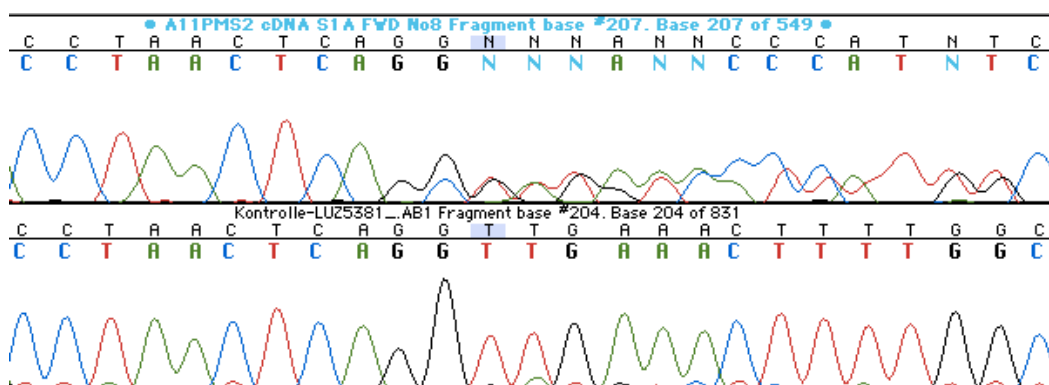
Patient No. 8

wildtype control

Figure 2   **Chromatogram of cDNA sequencing of Segment 1A from patient no. 8 displaying mutation c.301_353del53 compared to a wildtype control.**

MLPA analysis

Ten patients without identified pathogenic germline mutation in *PMS2* were screened for large genomic rearrangements like large deletions or insertions using the MLPA assay. The results of this investigation are depicted in table 2. One problem applying the MLPA assay was the relatively high standard deviation. Results with standard deviation values of more than 0.20 should be interpreted carefully (e.g. patient no. 3 and patient no. 12). In patient no. 4 a mean value of -0.68 indicated the loss of exon 15. In patient no. 9 a similar result was observed with possible additional loss of exon 13 (mean value -0.44). The mean value of -0.54 pointed to deletion of exon 8 in patient no. 10. Exon 8 seemed also to be deleted in patient no. 11 as well as exons 13 and 15 (mean values -0.55, -0.54 and -0.76 respectively).

Using the MLPA kit it was not possible to detect deletions in positive controls from Hendriks et al. (data not shown).

Table 2   **MLPA results of 10 patients showing the mean dosage quotient and the standard deviation (SD)**

Patient No. 3				Patient No. 4				Patient No. 7		
PMS2 Exon	**Mean**	**SD**		**PMS2 Exon**	**Mean**	**SD**		**PMS2 Exon**	**Mean**	**SD**
1	0.35	0.33		1	-0.06	0.14		1	-0.02	0.19
2	0.28	0.33		2	0.07	0.14		2	-0.16	0.19
5	0.28	0.33		5	0.05	0.14		5	-0.14	0.19
6	0.23	0.33		6	-0.16	0.14		6	-0.09	0.19
7	0.21	0.33		7	-0.02	0.14		7	0.23	0.19
8	-0.30	0.33		8	-0.09	0.14		8	0.19	0.19
9	-0.19	0.33		9	-0.05	0.14		9	0.12	0.19
10	-0.11	0.33		10	-0.06	0.14		10	-0.14	0.19
11	-0.03	0.33		11	-0.03	0.14		11	-0.30	0.19
12	-0.12	0.33		12	0.09	0.14		12	0.23	0.19
13	-0.22	0.33		13	-0.37	0.14		13	-0.10	0.19
14	-0.28	0.33		14	-0.29	0.14		14	-0.27	0.19
15	-0.41	0.33		15	-0.68	0.14		15	-0.32	0.19

Patient No.8				Patient No.9				Patient No.10		
PMS2 Exon	**Mean**	**SD**		**PMS2 Exon**	**Mean**	**SD**		**PMS2 Exon**	**Mean**	**SD**
1	-0.02	0.14		1	0.12	0.10		1	0.15	0.17
2	0.28	0.14		2	0.13	0.10		2	0.23	0.17
5	0.20	0.14		5	0.16	0.10		5	0.13	0.17
6	-0.04	0.14		6	-0.02	0.10		6	-0.03	0.17
7	-0.14	0.14		7	-0.08	0.10		7	-0.08	0.17
8	-0.50	0.14		8	-0.004	0.10		8	-0.55	0.17
9	0.002	0.14		9	-0.0002	0.10		9	-0.09	0.17
10	-0.15	0.14		10	-0.15	0.10		10	-0.08	0.17
11	-0.11	0.14		11	-0.11	0.10		11	-0.21	0.17
12	0.08	0.14		12	0.03	0.10		12	-0.17	0.17
13	-0.16	0.14		13	-0.44	0.10		13	-0.18	0.17
14	0.26	0.14		14	-0.08	0.10		14	-0.16	0.17
15	0.04	0.14		15	-0.69	0.10		15	-0.14	0.17

Patient No.11				Patient No.12		
PMS2 Exon	**Mean**	**SD**		**PMS2 Exon**	**Mean**	**SD**
1	0.22	0.18		1	0.25	0.23
2	0.36	0.18		2	0.33	0.23
5	0.29	0.18		5	0.28	0.23
6	0.04	0.18		6	0.11	0.23
7	-0.15	0.18		7	-0.11	0.23
8	-0.55	0.18		8	-0.08	0.23
9	-0.08	0.18		9	-0.13	0.23
10	-0.27	0.18		10	-0.18	0.23
11	-0.24	0.18		11	-0.33	0.23
12	-0.29	0.18		12	-0.37	0.23
13	-0.54	0.18		13	-0.35	0.23
14	-0.28	0.18		14	-0.06	0.23
15	-0.76	0.18		15	-0.52	0.23

DISCUSSION

In ten patients without identifiable germline *PMS2* mutations described in Chapter 4 of this thesis, cDNA analysis and MLPA investigation were carried out to detect additional *PMS2* germline mutations.

Analysis of *PMS2* is complicated by the presence of several pseudogenes. De Vos et al. have described the pseudogene of *PMS2* in detail: There are 15 pseudogenes of the 5' part (exon 1 – 5) and one nearly 100% homologous pseudogene of the 3' part of the gene (exon 9, 11 – 15)[121]. This means that only exons 6, 7, 8 and 10 are of the *PMS2* gene are free of pseudogenes. To avoid co-amplification of pseudogenes cDNA of *PMS2* was analyzed in two overlapping segments using primers located in peudogene-free exons. Amplification of the two segments turned out to be quite difficult and conditions had to be optimized carefully. In addition, it was necessary to reamplify both segments in nested PCRs and in "subsegments" (S1A, S1B, S2A, S2B and S2C). A frameshift was detected in segment 1 (c.301_353del53) which leads to a stop codon after 120 amino acids (p.Gln100fs120X). Surprisingly the same mutation occurs in six patients (patients no. 5, 6, 8, 11, 12, 13) which raises the question for pathogenicity of the alteration. In general, mutations leading to a premature stop codon and therefore to truncation of the protein can be regarded as pathogenic. Another point why the detected mutation can be regarded as pathogenic is the fact that it occurs only in CRC patients and not in tested healthy persons. But it is very uncommon that the same mutation is detected in six unrelated patients. In addition, we find it in the cDNA of two patients carrying germline mutations in *PMS2* (patients no. 6 and 13). Patient no. 6 carries the "first" germline mutation in the pseudogene-free exon 6, whereas patient no. 13 displays an exon 11 alteration.

So the question for pathogenicity of p.Gln100fs120X remains open. Possibly, an alternative spliced region of the gene has been detected. The control of splicing requires precise recognitions of cis-regulatory elements in exons and their surrounding introns by the splicing machinery. Either mutations of these cis-regulatory elements, or differential expression or activation of trans-acting factors that recognize these elements can change the default splicing pattern of a gene, leading to alternative splicing[159]. Using an alternative splice site predictor[160] showed a putative splice site at the detected frameshift start. Thus, we detected a possible alternative spliced region, which is not described until date.

Another possibility is that mutations in *PMS2* are inherited in a recessive manner. So, both mutations in patients no. 6 and 13 are detected whereas the second mutation in the remaining 4 patients has to be identified.

To clarify the question regarding pathogenicity of p.Gln100fs120X additional cDNAs of healthy persons should be analyzed. The possible pathogenic meaning of the alteration would be strengthened although it is present in six patients if it doesn't occur in healthy persons. Another possibility to analyze the *PMS2* gene avoiding co-amplification of pseudogenes is the application of long-range PCR[161].

For the detection of aberrant copy numbers in the *PMS2* gene, the recently introduced MLPA technique from MRC Holland was applied. The first problem in using the SALSA P008 MSH6/PMS2 kit was the appearance of high standard deviation values. Using DNA obtained by the salting out method revealed better results than DNA purified with a DNA purification kit. Possibly, reagents in the applied DNA cleaning kit interfere with reagents in the MLPA kit and lead to a high standard deviation. But although unpurified DNA was used in two patients (patients no. 3 and 12) the standard deviation was still too high (0.33 and 0.23, respectively), so the MLPA results could not be interpreted reliably. Looking at the results, mostly the same exons appeared to be deleted (exons 8, 13 and 15). It is very unlikely, however, that these exons are lost separately in the same patients (no. 9 and 11).

To test the reliability of the MLPA kit, we checked DNA samples of Hendriks et al.[110] for the presence of deletions. They identified large genomic deletions by Southern blot and provided us their samples to have positive controls. But the MLPA kit did not detect the deletions (data not shown), which advises great caution in using this kit for *PMS2*. Although the annealing points for the probes are selected carefully pseudogenes may interfere and complicate MLPA analysis. And indeed, the following warning is part of the probemix description: "PMS2 exon 13-14-15 probes have been reported to provide variable results by two users. Many sequences related to this exon 13-15 region are present in the genome. Results obtained with these probes should be treated with caution or be disregarded."

6. MSH3 - a novel susceptibility gene for
hereditary nonpolyposis colorectal cancer (HNPCC)?

INTRODUCTION

The autosomal dominantly inherited colorectal cancer predisposition HNPCC (hereditary nonpolyposis colorectal cancer) accounts for approximately 5% of all colorectal cancers[162]. The disease is caused by germline mutations in DNA mismatch repair (MMR) genes, predominantly *MLH1* and *MSH2*. The primary function of the MMR system is to eliminate single-base mismatches and insertion-deletion loops that may arise during DNA replication[163,164]. At least six different MMR proteins are required. *MSH2* protein forms a heterodimer with either *MSH6* or *MSH3* to recognize the mismatch and recruits a second protein-heterodimer consisting of *MLH1* and *PMS2* coordinating the interplay between the mismatch recognition complex and other proteins necessary for MMR[165]. While the *MSH2* and *MSH6* dimer (the hMutSα complex) recognizes base-base mismatches and single base loops, the *MSH2* and *MSH3* dimer (the hMutSβ complex) recognizes insertion/deletion loops of more than one base[166]. It has been shown that MSH3 and MSH6 are partially redundant in MSH2-dependent mismatch repair, whereby the substrate specificity of the repair process may be dictated by interaction of MSH2 with either MSH3 or MSH6[167].

The majority of MMR germline mutations have been identified in *MLH1* and *MSH2* (ca. 90%) and a small fraction accounts for mutations in *MSH6* (ca. 10%) and *PMS2* (less than 5%) (International Collaborative Group on HNPCC, http://www.n-fdht.nl). According to the human gene mutation database (http://www.hgmd.cf.ac.uk/ac/index.php) and to literature search (http://www.ncbi.nlm.nih.gov/sites/entrez) no germline mutations in *MSH3* have been described so far.

Here we present a patient carrying a germline missense mutation in *MSH3* and the assessment of the putative pathogenic role of the alteration in adenoma and colorectal cancer development.

MATERIAL & METHODS

Subject

Following colonoscopy because of blood in the stool the 1958 born male patient (patient ID: 2341) was diagnosed of two adenomas located in the distal colon. One of these adenomas contained a carcinoma *in situ* at the base. Detailed family history indicated a putative cancer predisposition. His father had developed a colon carcinoma at the age of 74 years and his grandfather died of oesophageal cancer at the age of 42 years. The mother and four sisters of the patient were investigated by colonoscopy without any findings (Figure 1). Written informed consent was obtained from the patient and the family members tested.
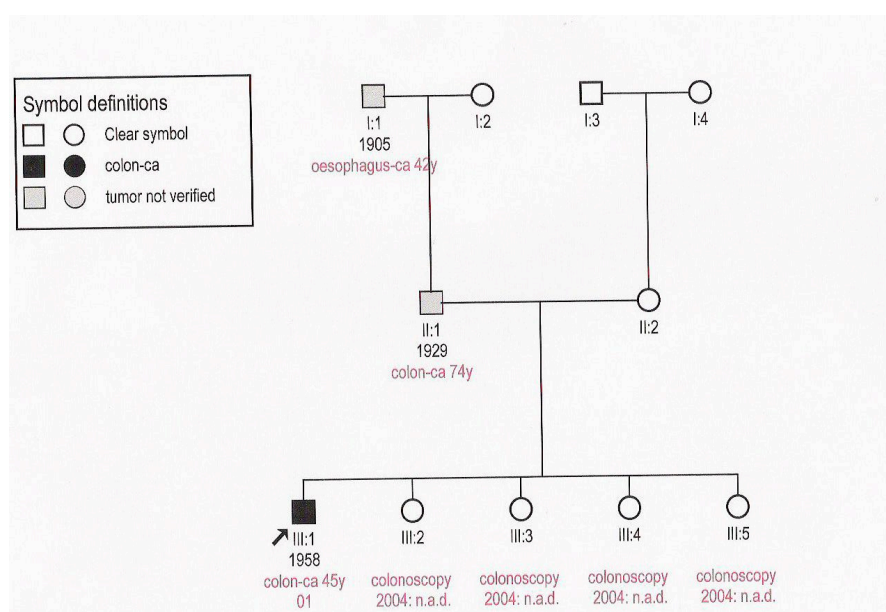
Figure 1: **Pedigree of family 2341**

Analysis of microsatellite instability and immunohistochemistry

A panel of five microsatellite loci (BAT25, BAT26, BAT40, D5S346 and Mycl-1) was used to assess microsatellite instability[47]. In addition three tetra- (MSH2_TAAA, MSH2_TTTA and MSH2_TTTG) and three penta-nucleotide markers (4A, D17S2227 and D17S2230) were applied to assess microsatellite instability. The presence or absence of 5 MMR proteins (MLH1, MSH2, PMS2, MSH6 and MSH3) in the tumor was examined by standard immunohistochemical techniques[168] at the Institute of Molecular Cancer Research in Zurich, Switzerland (Dr. G. Marra).

RNA extraction from leukocytes and first-strand cDNA synthesis

RNA extraction from blood-derived leukocytes was isolated using a combined approach of the TRIZOL®Reagent (Invitrogen) and RNeasy Mini Kit according to the

manufacturer's protocol (QIAGEN, Basel, Switzerland). First-strand cDNA synthesis was carried out applying the Omniscript Reverse Transcriptase protocol (QIAGEN, Basel, Switzerland).

DNA extraction from peripheral blood and tumor tissue

DNA from peripheral blood was isolated applying the salting-out method described by Miller et al.[102]. DNA from formalin-fixed tumor tissue blocks was extracted using the QIAamp tissue kit according to the manufacturer's protocol (QIAGEN, Basel, Switzerland).

MSH3 gene mutation analysis

Due to the coding sequence of 3414 base pairs of *MSH3* primers for six overlapping segments of cDNA were designed to analyze the gene (GenBank accession number NM_002439.2). PCR products were directly sequenced using the BigDye Terminator Cycle Sequencing Kit and analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Rotkreuz, Switzerland).

Restriction enzyme digest with PfoI

The minor allele frequency for a single nucleotide polymorphism (SNP) to be considered common is usually above 1% (www.hapmap.org). To test if the identified missense mutation p.Arg795Trp represents a SNP 100 healthy persons were investigated by restriction digest with PfoI. The enzyme cuts the PCR product only if the DNA carries the wildtype sequence of *MSH3* and produces two bands on a 2% agarose gel of 129 and 113 bp length.

Analysis of loss of heterozygosity

Loss of heterozygosity (LOH) was investigated using the following *MSH3* flanking polymorphic microsatellite markers: D5S424, D5S641, D5S428 and D5S346. LOH was scored at any informative marker if the area under one allelic peak in the tumor was reduced by >50%[169]. In addition direct sequencing of exon 18 was carried out.

RESULTS

A 46 years old male patient (patient ID: 2341/01) was diagnosed of a large adenoma with a carcinoma *in situ* at the base. Because of a putative predisposition to colorectal cancer tumor tissue was investigated for the presence or absence of five MMR proteins (MLH1, MSH2, PMS2, MSH6 and MSH3) by immunohistochemistry. This investigation revealed exclusively loss of MSH3 whereas the four other MMR

proteins were present. Therefore the patient's blood and tumor tissue were referred to our research group.

Firstly, DNA from formalin-fixed colorectal cancer tissue was investigated for the presence of MSI using the recommended NCI panel of microsatellite markers[54]. None of the applied markers were found to display novel alleles, corresponding to a microsatellite-stable status (MSS). In addition to the recommended markers, MSI analysis was carried out using three tetra- and three penta-nucleotide markers, which were also found to be MSS.

Secondly, due the immunohistochemically proven loss of MSH3, leukocyte derived cDNA was investigated for mutations in the *MSH3* gene. Bidirectional sequencing of the *MSH3* gene identified a novel missense mutation in exon 18, c.2383C>T, which results in an amino acid change from arginine to tryptophan (p.Arg795Trp). This missense mutation was then confirmed by sequencing of exon 18 of leukocyte derived DNA (Figure 2a). Interestingly, the father of the patient who had actually developed colorectal cancer at age 74 years, did not carry the c.2383C>T missense mutation, whereas his at the age of 75 years still healthy mother did.

wildtype control

a) 2341/01 leukocyte derived DNA

b) 2341/01 tumor DNA
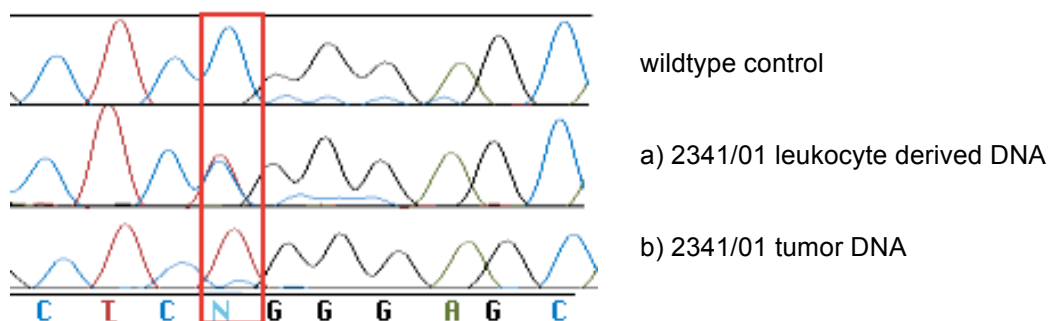
Figure 2: **Sequencing chromatograms displaying the identified missense mutation c.2383C>T in leukocyte (a) and tumor derived DNA (b) compared to a wildtype control**

The missense mutation leads to the change from a basic-polar (arginine) to an aromatic-nonpolar (tryptophan) amino acid and suggests therefore modification of the protein function. To further investigate the pathogenicity of the missense mutations alignment of the respective amino acid from different species was carried out. It was found that the missense mutation is located in a conserved region of the MSH3 protein comparing different vertebrates (dog, mouse, rat, chicken, and others) (Figure 3). In MutS of *E. coli* and in *S. cerevisiae*, however, another amino acid is located at the respective position (glycine and lysine, respectively). The amino acid

change is located in the MutS_III domain of the protein, which is responsible for the binding of dsDNA. This suggests that an alteration in this domain impedes mismatch repair proficiency.
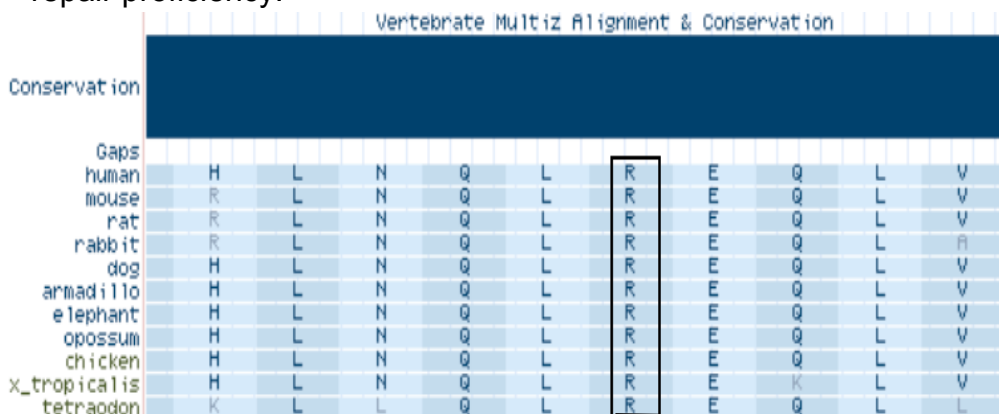
Figure 3: **Vertebrate alignment of p.Arg795Trp**

Furthermore, 200 alleles of 100 healthy probands were tested for the presence of c.2383C>T using the restriction enzyme PfoI which cuts the DNA in the presence of the wildtype sequence**.** All of the tested DNAs were digested, so the allele frequency for p.Arg795Trp is less than 1%. Therefore the criteria for a single nucleotide polymorphism (SNP) indicating a minor allele frequency of more than 1% is not met.

Sequencing and LOH analysis of the patient's tumor showed that p.Arg795Trp is present in a hemizygous state (Figure 2b). The three markers surrounding the *MSH3* locus as well as marker D5S346 and D5S428 encompassing the *APC* locus on chromosome 5 indicated consistent loss of the wildtype allele (Figure 5).

Marker	Location	Ratio	Interpretation
D5S1981	5p15.33	1,06	no LOH
D5S406	5p15.32	1,01	no LOH
D5S407	5q11.2	1,04	no LOH
D5S424	5q13.3	0,08	LOH
MSH3	5q14.1		
D5S641	5q14.2	7,55	LOH
D5S428	5q14.3	2,29	LOH
APC	5q22.2		
D5S346	5q22.2	5,8	LOH
D5S400	5q34	5,2	LOH
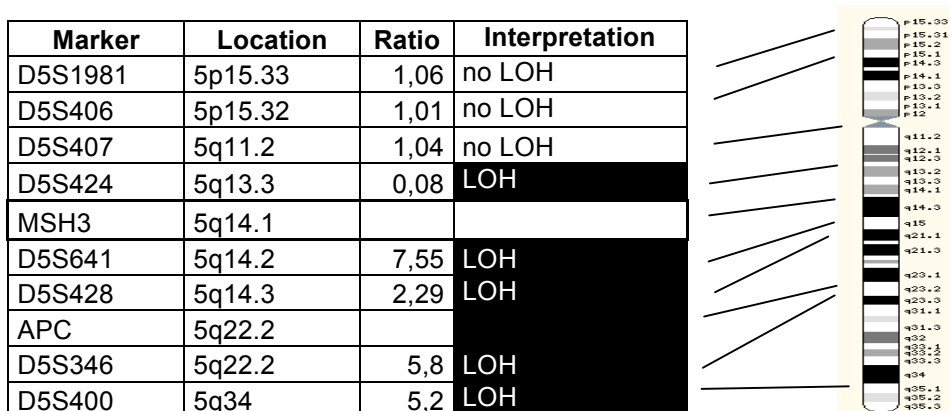
Figure 5: **Results of LOH analysis with 8 different markers on chromosome 5**

DISCUSSION

In this study we present a 46 years old patient whose tumor showed exclusively loss of MSH3. Sequencing analysis of leukocyte derived DNA from the patient revealed a *MSH3* germline missense mutation. With regard to published data the overall detection rate for pathogenic germline mutations in *MSH3* appears to be very low. Ohmiya et. al[170] detected a missense variant (c.Pro2043Ser) in a conserved region of the mouse *MSH3* homologue. But no further investigation with regard to pathogenicity of this variant was carried out. Whereas nonsense, frameshift and splice site mutations can be easily interpreted as pathogenic, pathogenicity of missense mutations is often difficult to determine. To be able to counsel patients with a missense mutation regarding the risk of colorectal cancer, it is necessary to understand the functional effect of a given missense variant on MMR proficiency[171].

Criteria defining the pathogenic nature of mutations in general and in particular of mutations associated with HNPCC have been reported in several publications[172-174]. The identified missense mutation c.2383C>T (p.Arg795Trp) was checked for pathogenicity using these criteria:

Criteria to define the pathogenic nature of MMR gene variants

1. De novo appearance of a mutation:

    p.Arg795Trp is not a de novo mutation because the mutation was detected also in the mother of our index patient.

2. Segregation of the mutation with pedigrees:

    The mutation was detected in the index patient and in his healthy mother.

3. Absence of the mutation in control individuals:

    In none of 100 healthy persons tested for p.Arg795Trp the missense variant was present.

4. A change of amino acid polarity charge or size in the encoded peptide:

    Polarity changed because of the replacement of the non-polar wildtype amino acid arginine by the polar amino acid tryptophan.

5. Occurrence of the amino acid change in a domain which is evolutionarily conserved between species and/or shared between proteins belonging to the same protein family:

    Looking at different species (chimp, dog, mouse, rat, chicken, fugu, zebrafish) we found that p.Arg795Trp is located in a conserved region. Furthermore, the

alteration is located in the MutS_III domain of the MSH3 protein. This domain is responsible for DNA binding in DNA mismatch repair.

6. Loss of the non-mutated allele in tumor material of the patient (loss of heterozygosity, LOH):

    Sequencing and LOH analysis of tumor DNA using markers located around the *MSH3* locus indicate loss of the wildtype allele.

7. Absence of immunohistochemical staining for the corresponding protein in tumor material:

    The immunohistochemical analysis of the patient's tumor revealed loss of MSH3.

8. Presence of MSI in tumor material of the patients:

    MSI was not detected in the tumor using 11 different markers. This could be explained by the partially redundancy of MSH6 and MSH3[175,176].

9. Effect of the mutation on MMR capacity in functional assays:

    In collaboration with S. Ollila und M. Nystrom, Helsinki, Finland this approach is currently investigated.

10. Previous inclusion of the mutation in disease-specific mutation databases:

    The mutation has not been reported so far.

Taken together, the available data support a pathogenic role of the missense variant p.Arg795Trp.

Looking at the patient's mother, she's still healthy by the age of 75 years although she carries the mutation. This may be explained by a possibly low penetrance of *MSH3* mutations. The MSH6 and MSH3 proteins are shown to be functionally redundant, so that MutSα can partially compensate the function of MutSβ[175,177,178]. This redundancy could explain the lower penetrance of *MSH3* mutations. In addition, the genetic background[179] and the effect of possible modifier genes could also play a role.

LOH-analysis showed that chromosome 5q is completely deleted in the tumor of our patient. As mentioned MSH3 is located on 5q but also APC can be found on the long arm of chromosome 5. Possibly, the loss of APC is therefore the major driving force in tumor formation.

Taken together, the alignment of *MSH3* from different species, looking at allele frequency of the observed missense mutation, the appearance of the mutation in a homozygous state in the tumor and the loss of the wildtype allele suggest the possible pathogenic meaning of p.Arg795Trp.

To finally determine the functional effect of p.Arg795Trp on MMR proficiency of MSH3 functional analysis of this alteration, however, is necessary.

7. GENERAL DISCUSSION

This thesis has focused on genotype-phenotype correlations and rare susceptibility genes in two hereditary colorectal cancer syndromes, familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). In particular, the aims were to survey predicted genotype-phenotype correlations and to establish the contribution of the comparatively rare mutated genes *MYH* to FAP and *PMS2* as well as *MSH3* to HNPCC.

All investigations aim ultimately to aid clinicians in selecting colorectal cancer patients for optimal genetic testing and to provide them guidelines and an overview for the best surveillance and prevention strategies and genetic counselling schemes.

The most important clinical step regarding the diagnosis of a hereditary colorectal cancer syndrome is a compilation of a thorough family cancer history[162]. The focus should be on identifying cancer of all types and sites, the family member's age at onset of cancer, any associations with phenotypic features that may be related to cancers such as colonic adenomas, and documentation of pathological finding whenever possible. This information will frequently identify a hereditary colorectal cancer predisposition in a family. Molecular genetic testing may then provide verification of the diagnosis. Once a pathogenic germline alteration has been identified, at risk family members should be informed about the possibility of predictive genetic testing which has been shown to reduce morbidity and mortality[180,181].

Pre- and post-test genetic counselling is of high importance for the patient as well as for his/her family members. They should be informed on the details for surveillance and management and the necessity for genetic testing[182,183]. Mutation-positive subjects can be advised on appropriate prophylactic measures, such as endoscopy and surgery in FAP or colonoscopic surveillance in HNPCC. Mutation-negative subjects and their children need no further increased evaluation.

Familial adenomatous polyposis (FAP) and *MYH* associated polyposis (MAP):
In the first study (page 15) of this thesis genotype-phenotype correlations were assessed investigating 101 index patients with the clinical diagnosis of FAP. Genotype-phenotype correlations, linking the site of the germline mutation with the

severity of the disease, have been reported by several research groups: Severe polyposis with thousands of colorectal polyps has been associated with *APC* germline mutations between codons 1240 and 1464[19]. In contrast, patients carrying mutations at the extreme 5' end (codons 1-177)[20-23], the alternatively spliced exon 9 (codons 312-412)[24-26], and the 3' end (codons >1580)[23,27,28] presented more often with attenuated polyposis. In addition, certain extracolonic disease manifestations have been correlated with the site of the germline mutation, e.g. alterations between codons 1403 and 1578 with desmoid tumours[29,30].

In contrast to reported genotype-phenotype correlations, nearly half of our *APC* mutation carriers with alterations in the classical region actually displayed an attenuated polyposis phenotype*.* In addition, four (57%) out of seven 5' *APC* mutation carriers in our consecutive series actually presented with severe polyposis coli displaying hundreds to thousands of colorectal polyps. Extracolonic disease manifestations were evenly distributed among patients with mutations in the "classical FAP" or the "AFAP region". With regard to upper gastrointestinal adenomas, desmoids and osteomas, 59% (10 out of 17) of patients actually carried mutations outside the regions correlated with these manifestations.

In our study population of *APC* mutation carriers, no genotype-phenotype correlations with regard to polyp number or extracolonic disease manifestations could be established. These data challenge the prevailing view on genotype-phenotype correlations and advise great caution when basing clinical management decisions for an individual patient on the site of the *APC* germline mutation. These findings were confirmed in a second study[85].

This second study (page 27) reports phenotypic differences among AFAP families, both between and within kindreds with mutations in each of the three AFAP-associated regions of APC. We have found that patients with germline *APC* mutations in the 5' and 3' regions of the gene or the alternatively spliced region of exon 9 have a highly variable large-bowel phenotype, in that the number of colorectal adenomas varies from almost none to the hundreds or thousands of lesions found in classical FAP[15].

We investigated the somatic *APC* mutation spectrum in AFAP patients with 3'-mutations and compared them with the other AFAP-associated regions of APC. The study had found that 'three hits' at *APC* often occur in AFAP adenomas (Figure 1). In

such polyps, the 'third hit' appears to be required for the initiation of tumorigenesis. In conclusion, the phenotypic and somatic molecular heterogeneity in AFAP means that clinical management of patients with AFAP associated mutations must be empirical. A more accurate prediction of phenotype may eventually be possible when additional genetic and environmental factors, such as modifier genes, that influence colorectal cancer susceptibility and disease severity are identified.
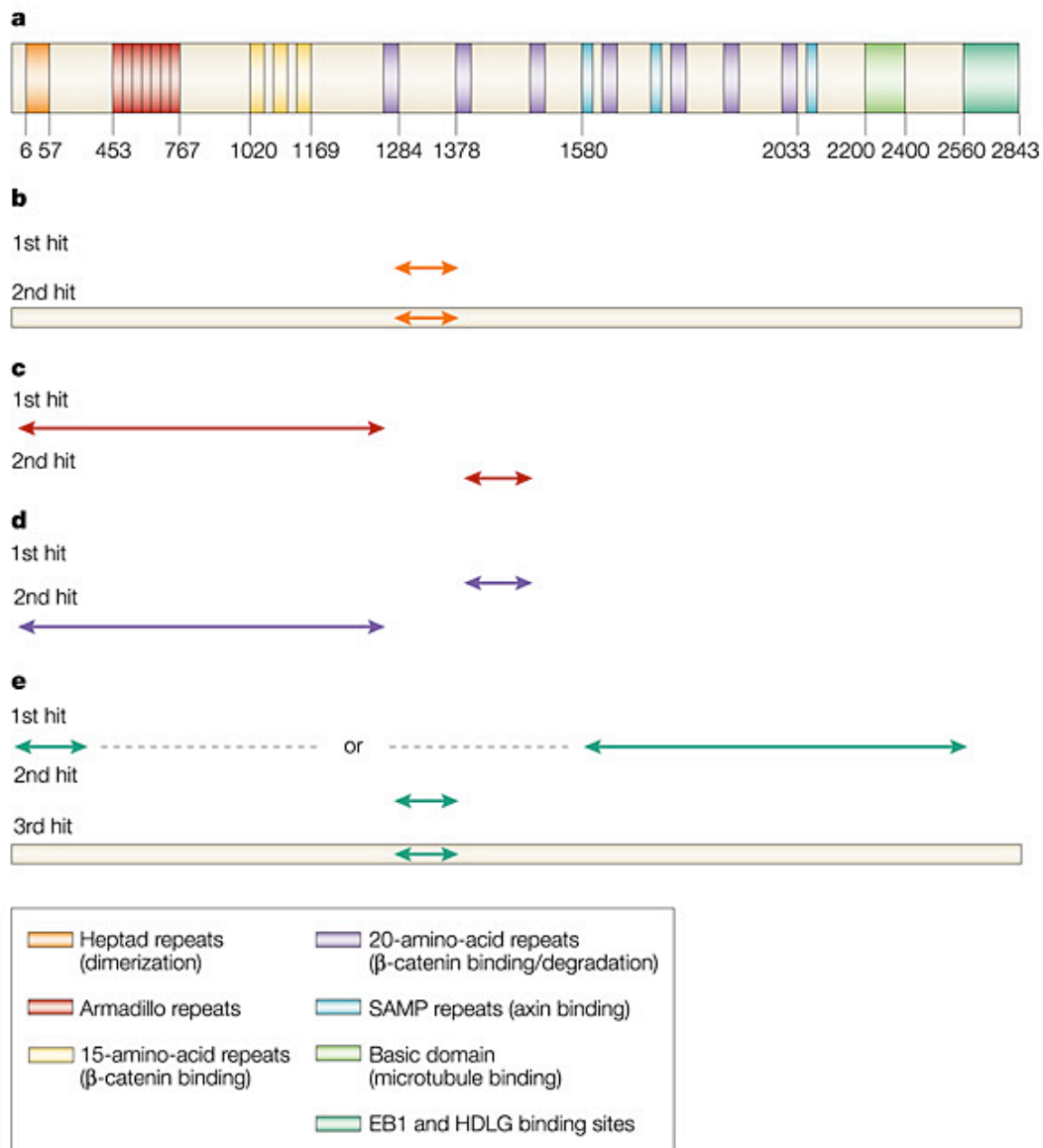
Figure 1 **A simplified model of the associations between the first hit and the second hit at APC in colorectal tumors[184]. a) In colorectal tumors the "first hit" and "second hit" are interdependent. The following patterns are seen: b) "first hits" between codons 1284 and 1378 and LOH as "second hit"; c) truncating "first hits" before codon 1284 and truncating "second hit" between codons 1378 and 1580; d) as for c) but in reverse order; e) in AFAP patients sometimes a "second hit'"by truncation close to codons 1284–1378 and a 'third hit' by allelic loss of the inherited mutant allele occur.**

This thesis reports in the third study (page 49) on the assessment of frequency of *MYH* mutation carriers in 79 unrelated Swiss patients. Despite extensive genetic testing, in about 20-50% of FAP patients worldwide no germline *APC* mutation can be identified. About 50% of these so-called *APC*-negative patients display a multiple colorectal adenoma phenotype with less than 100 colorectal polyps at a later age of onset[75,99,185]. In addition, extracolonic manifestations are less frequently observed. There are several reasons for the failure in identifying *APC* germline mutations in FAP patients:

- A combination of several different screening techniques would result in a better detection rate than only one individual method.
- Other genes may be responsible for the development of FAP or may lead to a similar clinical phenotype[186,187].

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]. In this study we assessed the frequency of *MYH* mutation carriers in 79 Swiss polyposis patients and investigated them for phenotypic differences between biallelic, monoallelic mutation carriers and *APC/MYH* mutation-negative patients. 9% of patients were found to harbor biallelic (n = 7) and 11% monoallelic (n = 9) germline mutations in the base excision repair gene *MYH.* 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. Colorectal cancer was significantly more frequent in biallelic as compared to monoallelic mutation carriers or those without *MYH* alterations but with regard to other phenotypic properties (age of onset, extracolonic disease manifestations), it is virtually impossible to discriminate biallelic from monoallelic *MYH* mutation carriers and *MYH* mutation-negative polyposis patients.

Based on our results, we suggest that *MYH* mutation screening should be offered to individuals who fulfill all of the following criteria: (i) presence of classical or attenuated polyposis, (ii) absence of an *APC* germline mutation, and (iii) a family history compatible with an autosomal recessive mode of inheritance.

Hereditary non-polyposis colorectal cancer (HNPCC):

HNPCC is caused by germline mutations in DNA mismatch repair (MMR) genes. The majority of MMR germline mutations have been identified in *MLH1* and *MSH2* (ca. 90%) and a small fraction accounts for mutations in *MSH6* (ca. 10%). To date, heterozygous germline mutations in *PMS2* have not been reported in HNPCC families as defined by Amsterdam criteria.

The fourth study (page 59) reports the analysis of PMS2 expression along with that of its heterodimeric partner MLH1 in a large series of unselected CRCs. This approach, supported by the analysis of the *PMS2* gene, allowed us to identify patients affected by cancers with a primary defect of PMS2 (i.e. not secondary to an MLH1 defect) and to describe their phenotype. The most striking finding of our study concerns the unexpectedly high frequency of PMS2-deficient CRCs (1.5%), which was similar to that of tumors lacking MSH2 (1.4%). Looking at the phenotype of germline *PMS2* mutation carriers, many of them were diagnosed the fifth or sixth decade of life, some were also affected with extracolonic PMS2-deficient tumors, and several relatives were identified with CRCs or cancers in other organs at earlier-than-average age of onset. It has to be mentioned, that several pseudogenes located on chromosome 7 can interfere with the mutation analysis of *PMS2* [121,132,145,152-154].

With regard to clinical management, PMS2 defects should be remembered as a reason for colorectal cancer and thus, should be taken into account of genetic testing.

The fifth part of this thesis (page 87) deals with apparently rare findings in HNPCC: mutations in the MMR gene *MSH3.* To date, no germline mutation in *MSH3* has been described. This study reports the case of a 46 years old colorectal cancer patient with immunohistochemically proven loss of MSH3 in the adenocarcinoma. Screening *MSH3* for germline mutations, a missense mutation c.2383C>T was identified. The fact that this variant does not occur in 100 healthy persons, that alignment of the respective gene in several species showed that the variant is located in a conserved region, and that tumor DNA sequencing and LOH analysis showed loss of the wildtype allele suggest that the missense mutation c.2383C>T may indeed be pathogenic albeit at a low penetrance level as explained by the functional redundancy of MutSα and MutSβ.

In summary, germline mutations in *MSH3* appear to be very rare but nevertheless geneticists and clinicians should bear this gene in mind when examining familial colorectal cancer patients where no apparent defects in the common MMR genes have been found.

Taken together, in hereditary colorectal cancer syndromes genetic testing is of great importance for the patients and their families. Once the patients' familial risk is determined a complex program of cancer surveillance and management has to be undertaken[188-190].

To identify germline mutations in the respective genes well established methods like the protein truncation test, sequencing analysis, immunohistochemistry, and microsatellite instability analysis are methods of choice. In hereditary cases without identified germline alterations the recently introduced MLPA assay may help to detect gene copy number changes. And finally, cDNA analysis can be applied for large genes or genes where pseudogenes impede mutation analysis. The use of these molecular genetic testing methods allows early identification of at-risk family members, improves diagnostic certainty and reduces the need for costly screening procedures like colonoscopies in those family members not carrying the inherited disease-causing mutation. Identification of the responsible germline mutation will help to improve prevention and risk assessment in a family with a hereditary cancer syndrome.

Advances in technology in cancer screening, identification of biological markers of cancer susceptibility and specific germline testing is necessary to help physicians in management of patients with hereditary colorectal cancer syndromes. In addition, molecular genetic research has to be intensified in searching for new mutations, screening novel, rarely mutated or modifier genes and assessing genotype-phenotype correlation where possible in these heterogeneous disorders.

Looking for target genes Sjöblom et al.[3] described in their huge study the systematic analysis of 13023 in 11 breast and 11 colorectal cancers. They identified 189 genes (average 11 per tumor) that were mutated at significant frequency. The vast majority of these genes were not known to be genetically altered in tumors. This study shows that to date only the tip of the iceberg is known about genes involved in cancer. But whole genome microarrays contain a great potential to identify new genes responsible for hereditary colorectal cancer syndromes. This technology

promises to monitor the whole genome on a single chip, so researchers can investigate thousands of potential CRC genes simultaneously. Recently, Albert el al. published a highly efficient and cost-effective method for capturing targeted regions of the genome via NimbleChip microarrays in preparation for high-throughput 454 Sequencing. The technology, called "sequence capture", enables fast and accurate enrichment of thousands of selected genomic regions, such as segments of chromosomes or all genes or exons. The study demonstrates that the sequence capture process is simpler, more accurate, more efficient and more cost-effective than the multiplex PCR that was previously used to prepare genomic samples for sequencing[191].

8. APPENDIX

GENERAL INTRODUCTION

Amsterdam criteria I and II

Amsterdam criteria I

- three or more relatives with colorectal cancer (CRC)
- one affected patient should be a first-degree relative of the other two
- CRC should involve at least two generations; at least one case of CRC should be diagnosed before the age of 50 years

Amsterdam criteria II

- three or more relatives with HNPCC-associated cancer
- one affected patient should be a first-degree relative of the other two
- two or more successive generations should be affected
- FAP should be excluded; Tumors should be verified by pathological examination

Bethesda guidelines

Bethesda guidelines

- Individuals with cancer in families that fulfill the Amsterdam Criteria
- Individuals with two HNPCC-related cancers
- Individuals with CRC and a firs-degree relative with CRC and/or HNPCC related extracolonic cancer and/or colorectal adenoma; one of the cancers diagnosed before the age of 45 years and the adenoma before the age of 40 years
- Individuals with CRC or endometrial cancer diagnosed before the age of 45 years
- Individuals with right-sided CRC with an undifferentiated pattern diagnosed before age of 45 years

Revised Bethesda guidelines

- Individuals diagnosed with CRC before the age of 50 years
- CRC or other HNPCC-associated tumors regardless of age
- CRC with a high-MSI morphology diagnosed before the age of 60 years

- CRC with one or more first-degree relatives with CRC or other HNPCC-related tumors, one cancer diagnosed before the age of 50 years including adenoma diagnosed before the age of 40 years
- CRC with two or more first- or second degree relatives with CRC or other HNPCC-related tumor, regardless of age

Stages of Colorectal Cancer

Colon and rectal cancer are staged according to how far they have spread through the walls of the colon and rectum and whether they have spread to other parts of the body. This staging process allows doctors to determine the best treatments for the particular cancer. It also allows them to determine if the cancer is getting better with treatment or not responding.

Stage 0

Stage 0 cancer of the colon is very early cancer. The cancer is found only in the innermost lining of the colon.

Dukes A colon cancer

The cancer has spread beyond the innermost lining of the colon to the second and third layers and involves the inside wall of the colon. The cancer has not spread to the outer wall of the colon or outside the colon.

Dukes B colon cancer

tends through the muscular wall of the colon, but there is no cancer in the lymph nodes (small structures that are found throughout the body that produce and store cells that fight infection).

Dukes C colon cancer

The cancer has spread outside the colon to one or more lymph nodes (small structures that are found throughout the body that produce and store cells that fight infection).

Dukes D colon cancer

The cancer has spread outside the colon to other parts of the body, such as the liver or the lungs. The tumor can be any size and may or may not include affected lymph nodes (small structures that are found throughout the body that produce and store cells that fight infection).

AFAP STUDY

Supplementary Table 1

Somatic *APC* mutations and allelic loss in tumours from AFAP patients with 5' germline mutations. All tumours with mutation or LOH are shown.

Patient ID	Germline mutation	Somatic mutation	Type of somatic change	20 AARs in somatic mutant allele	LOH
DFAP 48	141FS	1309FS	3927del5bp	1	NL
AVC.III.2	Q163X	1305FS	3914delC	1	NL
AVC.III.2	Q163X	1309FS	3927del5bp	1	NL
554.iii.2	179FS	Q1378X	4132C>T	1	NL
554.iii.2	170FS	1398FS	4192delAG	2	NL
554.iii.2	170FS	1439FS	4316delC	2	NL
554.iv.2	170FS	1462FS	4386delGA	2	NL
AFX MK	G125X	S1356X	4067C>G	1	LOH mut
AVC.III.S	Q163X	1309FS	3927del5bp	1	LOH mut

FS = frameshift; LOH = loss of heterozygosity; wt = germline wild-type allele; mut = germline mutant allele, where this assignment was possible

Supplementary Table 2

Somatic *APC* mutations and allelic loss in 79 tumours from AFAP patients with germline mutations in the alternatively spliced region of exon 9.

Patient ID	Somatic mutation(s)	Type(s) of somatic change(s)	20AARs in somatic mutant allele(s)	LOH
673.iii.3	K792X	2374A>G	0	NL
578.iv.4	1335FS	4004del31bp	1	NL
1571.ii.2	1462FS	4386delGA	2	NL
578.iv.1	1505FS	4514dup7bp	2	NL
578.iv.1	K1469X	4405C>T	2	NL
578.iv.4	1518FSwt	4552delA	3	NL
578.iv.7	E1265X; 1462FS	3793G>T; 4386del4bp	0 ; 2	NL
578.FPL	1462FS mut	4386delGA	2	NL
578.iv.7	1462FS mut	4386delGA	2	NL
578.iii.9	1372FS ; 1554 FS	4114delG; 4661insA	1 ; 3	NL
578.iv.4	1372FS ; 1554 FS	4117delC; 4661insA	1 ; 3	NL
578.AA	1554FS mut	4661insA	3	NL
578.FPL	1554FS mut	4661insA	3	NL
578.FPL	1554FS mut	4661insA	3	NL
578.FPL	1554FS mut	4661insA	3	NL
578.iii.9	1554FS mut	4661insA	3	NL
578.iii.9	1554FS mut	4661insA	3	NL
578.iii.9	1554FS mut	4661insA	3	NL
578.iii.9	1554FS mut	4661insA	3	NL
578.iii.9	1554FS mut	4661insA	3	NL
578.iv.4	1530FS mut	4661insA	3	NL
578.iv.4	1554FS mut	4661insA	3	NL
578.iv.4	1530FS mut	4661insA	3	NL

Patient ID	Somatic mutation(s)	Type(s) of somatic change(s)	20AARs in somatic mutant allele(s)	LOH
578.iv.4	1554FS mut	4661insA	3	NL
587.iv.7	1554FS mut	4661insA	3	NL
587.iv.7	1554FS mut	4661insA	3	NL
587.iv.7	1554FS mut	4661insA	3	NL
587.iv.7	1554FS mut	4661insA	3	NL
1571.ii.2	1554FS	4661insA	3	NL
1571.ii.2	1537FS	4611delAG	3	NL
578.iv.1	1554FS	4661insA	3	NL
578.iv.1	1554FS	4661insA	3	NL
578.iv.1	1554FS	4661insA	3	NL
578.iv.1	1554FS	4661insA	3	NL
578.iv.1	1554FS	4661insA	3	NL
578.iv.1	1554FS	4661insA	3	NL
578.iv.4	1554FS	4661insA	3	NL
DFAP 81	1554FS	4661insA	3	NL
578.iv.1	none detected			LOH wt
578.iv.1	none detected			LOH wt
578.iv.1	none detected			LOH wt
578.iii.9	1554FS mut	4661insA	3	LOH wt
578.iii.9	1554FS mut	4661insA	3	LOH wt
578.iv.7	1554FS mut	4661insA	3	LOH wt
578.iv.7	1554FS mut	4661insA	3	LOH wt
578.iv.1	1554FS mut	4661insA	3	LOH wt
578.iv.7	1554FS mut	4661insA	3	LOH wt
578.FPL	1394FS	4182delTA	1	LOH mut
578.iii.9	E1286X	3856G>T	1	LOH mut
578.iii.9	S1315X	3944C>T	1	LOH mut

See Supplementary Table 1 for abbreviations.

Supplementary Table 3

Somatic *APC* mutations and allelic loss in 126 adenomas from AFAP patients with 3' germline mutations.

Patient ID	Germline mutation	Somatic mutation(s)	Type(s) of somatic change(s)	20AARs in somatic mutant allele(s)	LOH
MD2976	1919FS	R283X	847C>T	0	NL
MD2976	1919FS	R283X	847C>T	0	NL
MD2976	1919FS	R283X	847C>T	0	NL
77-11	1943FS	E1374X	4120G>T	1	NL
DW20284	2078FS	Q1338X	4012C>T	1	NL
J42424	2078FS	1300FS	3902insC	1	NL
L12562	2078FS	Q1338X	4012C>T	1	NL
110.2.vi	2078FS	1354FS	4061delTT	1	NL
01/266	1738FS	1441FS	4323delA	2	NL
01/266	1738FS	1441FS	4323delA	2	NL
01/266	1738FS	1441FS	4323delA	2	NL

Patient ID	Germline mutation	Somatic mutation(s)	Type(s) of somatic change(s)	20AARs in somatic mutant allele(s)	LOH
01/266	1738FS	1441FS	4323delA	2	NL
01/266	1738FS	1441FS	4323delA	2	NL
01/266	1738FS	1441FS	4323delA	2	NL
2333/3	1919FS	1398FS	4192delAG	2	NL
2233/3	1919FS	1407FS	4219delAG	2	NL
2233/3	1919FS	R1435X	4303A>T	2	NL
77-11	1943FS	1490FS	4468insG	2	NL
77-11	1943FS	Q1406X	4216C>T	2	NL
110.2.vi	2078FS	1403FS	4209insCT	2	NL
MD2976	1919FS	1129FS wt; 1554 FS mut	3387delT; 4661insA	0;3	NL
344-44	1597FS	1554FS	4661insA	3	NL
01/266	1738FS	1576FS	4726delG	3	NL
77-11	1943FS	1579FS	4709del6bp	3	NL
2233/3	1919FS	none detected			LOH wt
2233/3	1919FS	none detected			LOH wt
2233/3	1919FS	none detected			LOH wt
77-11	1943FS	none detected			LOH wt
77-11	1943FS	none detected			LOH wt
77-11	1943FS	none detected			LOH wt
77-11	1943FS	none detected			LOH wt
77-12	1943FS	none detected			LOH wt
77-12	1943FS	none detected			LOH wt
77-40	1943FS	none detected			LOH wt
1460/6	1982FS	none detected			LOH wt
1460/88	1982FS	none detected			LOH wt
1624/04	1982FS	none detected			LOH wt
77-11	1943FS	1462FS mut	4386delGA	2	LOH wt
77-11	1943FS	1429FS mut	4286delA	2	LOH wt
77-11	1943FS	1234FS	37ins8bp	0	LOH mut
77-11	1943FS	none detected			LOH mut
77-11	1943FS	none detected			LOH mut
1460/6	1982FS	none detected			LOH mut
1460/42	1982FS	none detected			LOH mut
1460/42	1982FS	none detected			LOH mut
1460/42	1982FS	none detected			LOH mut
1624/04	1982FS	none detected			LOH mut
J42424	2078FS	none detected			LOH mut
J42424	2078FS	none detected			LOH mut
1460/42	1982FS	1357FS	4069delGG	1	LOH mut
1489/10	1982FS	1354FS	4060delAA	1	LOH mut
1624/04	1982FS	E1322X	3964G>T	1	LOH mut
01/266	1738FS	1431FS	4294delC	2	LOH mut
MD2976	1919FS	1493FS	4479delG	2	LOH mut

See Supplementary Table 1 for abbreviations.

PMS2 STUDY

Supplementary Table 1

Primers and Reaction Conditions for Analysis of MSI, LOH, Germline *PMS2* Mutations and Promoter Methylation Status

Primers for analysis of BAT 26*

Sense Primer	Antisense Primer
5'-TGACTACTTTTGACTTCAGCC-3'	5'-AACCATTCAACATTTTTAACCC-3'

Primers for LOH analysis**

Marker location	Forward Primer	Reverse Primer
d7s531	5'-GTCCTGCCCCTCTGTCAGT-3'	5'-TGGAAGACACCAGCTTTAGGA-3'
d7s517	5'-TGGAGAAGCCATGTGAGT-3'	5'-AGCTGTAATTAGTTGCTGGTTTGA-3'
d7s511	5'-ACTTGCTTGAGCCCAGG-3'	5'-AGTGATCTGCCCACCGT-3'
d7s2478	5'-GTGCTCCGCCATTTCTGTAT-3'	5'-CTGCAGCCAAAATGATCTGC-3'
d7s2201	5'-AGTTCAACCTGGGCAACATA-3'	5'-TCAAGCCAAGGCATTTTCTA-3'
d7s481	5'-CACCCCCATATTAATTTTATTCTTGT-3'	5'-TTTTTACCAGACTATTAAATCAGCAA-3'
d7s2553	5'-TTGAGAGGTGGGGACT-3'	5'-CATGTTTTTATGCTTTAACTACATT-3'
d7s2514	5'-CATCAGTTGTTAAACTTTGCCAT-3'	5'-CAACCAGCCGTCATCTT-3'

Primers for PMS2 sequencing***

Exon	Sense Primer	Antisense Primer	Product size	Tm (°C)	MgCl$_2$ (mM)
1	5'-AGCACAACGTCGAAAGCAG-3'	5'-AGAGGGGACACCGGAAGACT-3'	162	60	1.5
2	5'-TGTTTCTTGTAACTGATTTCTC-3'	5'-CTTAACTACAACAACATTCACAG-3'	224	54	2.5
3	5'-CTGATAGCATGGGTCCGTTT-3'	5'-TTGCATTTCCCAAGACAGTG-3'	225	60	2.5
4	5'-TCTTGGGAAATGCAAAAACA-3'	5'-AAGGGGTCAAGTGAGTGGAT-3'	248	50	1.5
5	5'-CCCAACATCATGGGTCTCTC-3'	5'-TGCTCATGTGCATTAACCAA-3'	289	60	1.5
6	5'-ACTTGAGCTGTGTAATTCC-3'	5'-CCCGCTATAATCACTAGAGC-3'	289	60	1.5
7	5'-GTCCACTCTGTCTTTATTAG-3'	5'-AGCTCTCAGGATAAAATGTTC-3'	204	60	1.5
8	5'-TCCCTTTCACTCTGGAATCCT-3'	5'-TCCACGTAAACTGCCTATTATCA-3'	236	60	1.5
9	5'-GGGGCTGGGAACATTTGTC-3'	5'-ATAGCAGAGCTGTAGAATTTC-3'	215	60	1.5

10	5'-TGAGACGCTGTCTGAAAATAATAA-3'	5'-AATAAGGAAACACATTAGCTAAAAGC-3'	300	60	2.5
11_1	5'-CAGGATAGTCCCTGACCCTCT-3'	5'-GGACGCCTTTGTCAGAGATG-3'	300	60	1.5
11_2	5'-GAAGGAGCCCTCTAGGACAGA-3'	5'-GCGAGATTAGTTGGCTGAGG-3'	388	60	1.5
11_3	5'-CGACTCTTTTTCAGATGTGGAC-3	5'-CTGCGCAACAGAGCAAGAC-3'	472	65	1.5
12	5'-AAAAGAAAGCGGGATGGCTA-3'	5'-CTCAAACTCCTGGCCTCTTG-3'	397	60	1.5
13	5'-TTGTTTTCATTTCATTTCTGCTG-3'	5'-CCACACCCAGCCGCTATAGTT-3'	216	55	1.5
14	5'-CGTGTTTGTCAAGTCATGGA-3'	5'-CTGAGACCTTCCTCGACTGC-3'	279	62	2.5
15	5'-CTACTAAAACGTTGAACCATTGTG-3'	5'-GCGCATGCAAACATAGAGAA-3'	384	55	1.5

Primers for the Analysis of the Methylation Status of the MLH1 Promoter by Real-Time PCR§

Sense Primer	Antisense Primer	Amplicon ^
5'-GAGTTTTTAAAAAIGAATTAATAGGAAGAG-3'	5'-TAAATACCAATCAAATTTCTCAACTCTA-3'	from -299 to - 152

Primers for the Analysis of the Methylation Status of the MLH1 Promoter by Methylation-Specific PCR (MSP)§§

Reaction	Sense Primer	Antisense Primer	Amplicon ^
Methylated Reaction (M)	5'-AACGAATTAATAGGAAGAGCGGATAGCG-3'	5'-CCTCCCTAAAACGACTACTACCCG-3'	from -288 to - 202
Unmethylated Reaction (U)	5'-TAAAAATGAATTAATAGGAAGAGTGGATAGTG-3'5'-AATCTCTTCATCCCTCCCTAAAACA-3'		from -292 to - 190

Primers for the Analysis of the Methylation Status of the PMS2 Promoter by Real-Time PCR¶

Amplicon	Sense Primer	Antisense Primer	Amplicon ^^
1st Amplicon	5'-GGTATGGTAGAATTAAAGTAAAAG-3	5'-AAAACCTAAACCAATCAAAACACA-3'	from -349 to - 193
2nd Amplicon	5'-GTGTGTTTTGATTGGTTTAGG-3'	5'-CTCCTAAACTCCCATTAACTA-3'	from -217 to – 68

Primers for the Analysis of the Methylation Status of the PMS2 Promoter by Methylation-Specific PCR (MSP) ¶¶

Reaction	Sense Primer	Antisense Primer	Amplicon ^^
Methylated Reaction (M)	5'-AGAGGCGCGTCGTTTTCGTG-3'	5'-CTCCGTCGTAACCTCTAACG-3'	from -391 to – 271
Unmethylated Reaction (U)	5'-GTAGGTGGGAAGTTTTATATGGAG-3'	5'-CCAATCTCCATCATAACCTCTAACA-3'	from -413 to - 266

*Amplifications were carried out using a reaction mix of 8μl of True Allele PCR Premix (Applied Biosystems), 5pM primers and 30-50 ng DNA. Thermal cycling conditions: 95°C for 12 min, 94°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec for 10 cycles followed by 89°C for 15 sec, 55°C for 15 sec and

72°C for 30 sec for 20 cycles. Fragment analysis was performed on an ABI 310 (Applied Biosystems) using 1µl PCR product in 20.5µl fromamide/GenScan350TAMRA-Mix. Data were analyzed with the Genotyper version 2.5 software.

** PCR was performed in an Eppendorf Mastercycler using a reaction mix of 9µl of True Allele PCR Premix (Applied Biosystems), 5pM primers and 20ng (genomic)/10 ng (tumor) DNA. Thermal cycling conditions: 94°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec for 13 cycles followed by 89°C for 15 sec., 55°C for 15 sec and 72°C for 30 sec for 23 cycles. Fragment analysis was performed on an ABI 310 (Applied Biosystems) using 1µl of PCR product in 24.6µl HiDi-formamide/Rox400-Mix. Data were analyzed with the Genotyper version 2.5 software.

*** PCR amplification of genomic DNA was carried out in a 35µl reaction containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 2.5mM each of the four dNTPs, 1.5/2.5mM MgCl2, 5pM of each primer, 20ng of DNA and 2.5U of Taq Polymerase. Thermal cycling conditions (in an Eppendorf Mastercycler) were as follows: 95°C for 30s, 50-65°C for 1.5 min, and 70°C for 1.5 min for 35 cycles. PCR products were sequenced (Big dye terminator kit, Applied Biosystems) and analyzed with an ABI 3700.

§ Real Time PCR was performed by the Roche LightCycler System using the QuantiTect SYBR Green Kit (Qiagen) according to the manufacturer's instructions, 0.5µM of each primer and 2µL of the bisulphite-treated DNA in 20µL final volume of reaction. PCR conditions: 95°C for 15 min, then 55 cycles (94°C for 15 sec, 51°C for 20 sec, 72°C for 10 sec), followed by a melting curve analysis step (65°C-95°C, 0.1°C/sec slope). The analysis of melting curves allowed us to distinguish between methylated and unmethylated alleles (see Supplemental Figure S1). ^From the ATG start site (GenBank accession no. U83845): this promoter region has been found to be critical for gene silencing[192]

§§ Reactions were carried out in a 25µL final volume, using 2µL of bisulfite-treated DNA, 1.25U ampliTaq Gold (Applied Biosystems) with final primer concentrations of 0.5µM for the methylated reaction (M) and 1µMf or the unmethylated reaction (U). After an initial step (95°C for 10 min), 37 cycles (95°C for 30 sec; 62°C (M) or 60°C (U) for 30 sec; 72°C for 30 sec) were performed and followed by a final step of elongation for 7 minutes at 72°C. Products were loaded on 1.8% agarose gels and stained with ethidium bromide (see Supplemental Figure SI). From the ATG start site (GenBank accession no. U83845).

¶ Real Time PCR was performed by the Roche LightCycler System using the QuantiTect SYBR Green Kit (Qiagen) according to the manufacturer's instructions, 0.5µM of each primer and 2µL of the bisulphite-treated DNA in 20µL final volume of reaction. PCR conditions: 95°C for 15 min, then 55 cycles for the 1st Amplicon (94 °C for 15 sec, 50°C for 20 sec, 72°C for 10 sec) or 45 cycles for the 2nd Amplicon (94 °C for 15 sec, 50°C for 20 sec, 72°C for 10 sec), followed by a melting curve analysis step (65°C-95°C, 0.1°C/sec slope). The analysis of melting curves allowed us to distinguish between methylated and unmethylated alleles (see Supplemental Figure S1). ^^ From the presumptive ATG starting codon (Gene-Bank accession number U24168).

¶¶ Reactions were carried out in a 25µL final volume, using 2µL of bisulfite-treated DNA, 1.25U ampliTaq Gold (Applied Biosystems) with final primer concentrations of 0.5µM. After an initial step (95°C for 10 min), 37 cycles (95°C for 30 sec; 58°C for 30 sec; 72°C for 30 sec) were performed and followed by a final step of elongation for 7 minutes at 72°C. Products were loaded on 1.8% agarose gels stained with ethidium bromide (see Supplemental Figure S1). ^^ From the presumptive ATG starting codon (Gene-Bank accession number U24168).

Supplementary Table 2
Characteristics of Patients with MMR-deficient CRCs

Tumor no.	Absent MMR protein*	BAT26	Promoter Methylation**	Age	Sex	Tumor site***	History of cancer¶
59255	MSH2	unstable		77	f	A	n.d.
69101	MSH2	unstable		64	m	R	sporadic
17676	MSH2	stable		81	f	R	n.d.
52012	MSH2	unstable		86	m	A	n.d.
51470	MSH2	unstable		39	m	C	BG1
52285	MSH2	unstable		65	f	S	BG5
50633	MSH2	unstable		71	m	R	AC
13617	MSH2	unstable		61	f	R	AC
4556	MSH2	stable		56	m	A	BG3
21269	MSH2	unstable		74	f	C	sporadic
11752	MSH2	stable		39	m	A	BG1
7429	MSH2	unstable		87	f	C	n.d.
6831	MSH2	unstable		50	f	D	AC
14685	MSH2	unstable		53	f	A	AC
7467	MSH2	unstable		34	m	A	AC
69770	MSH6	unstable		61	f	A	BG2&4
54013	MSH6	stable		69	f	S	BG5
21532	MSH6	stable		57	f	A	BG5
22577	MSH6	stable		57	m	R	n.d.
13894	MSH6	stable		58	m	R	sporadic
1081	MLH1	unstable	met	81	f	A	BG5
55121	MLH1	unstable	unmet	39	m	A	AC
53430	MLH1	unstable	met	81	m	C	sporadic
65453	MLH1	unstable	unmet	60	f	R	BG4
63887	MLH1	unstable	met	48	m	R	BG1
58560	MLH1	unstable	met	81	f	A	BG5
56727	MLH1	unstable	met	57	f	D	AC
55998	MLH1	unstable	unmet	27	m	T	BG1&5
63850	MLH1	unstable	unmet	55	f	C	BG4&5
69999	MLH1	unstable	met	72	f	HF	sporadic
67543	MLH1	unstable	met	76	m	A	sporadic
50005	MLH1	unstable	met	77	f	C	sporadic
53460	MLH1	unstable	met	88	f	A	sporadic
307	MLH1	unstable	met	73	m	A	sporadic
61282	MLH1	unstable	met	74	f	S	BG5
9549	MLH1	unstable	met	78	f	HF	sporadic
63585	MLH1	unstable	unmet	59	m	S	AC
60661	MLH1	unstable	met	94	f	S	sporadic
67255	MLH1	unstable	met	91	f	HF	AC
70511	MLH1	unstable	met	74	f	A	sporadic
23348	MLH1	unstable	met	85	f	A	sporadic
52458	MLH1	unstable	unmet	36	f	D	AC
50263	MLH1	unstable	met	79	f	A	sporadic
52201	MLH1	unstable	met	76	f	A	sporadic

Tumor no.	Absent MMR protein*	BAT26	Promoter Methylation**	Age	Sex	Tumor site***	History of cancer¶
55843	MLH1	unstable	met	87	f	C	sporadic
54631	MLH1	unstable	met	83	m	C	sporadic
60404	MLH1	unstable	met	69	m	A	BG5
61104	MLH1	unstable	met	71	f	A	sporadic
64041	MLH1	unstable	met	69	f	A	BG5
65509	MLH1	unstable	unmet	52	f	C	AC
66221	MLH1	unstable	unmet	69	m	R	AC
51385	MLH1	unstable	unmet	69	m	R	sporadic
52187	MLH1	unstable	met	83	f	T	sporadic
9251	MLH1	unstable	met	87	m	A	sporadic
2421	MLH1	unstable	met	70	f	C	sporadic
6534	MLH1	unstable	unmet	60	f	R	BG5
5941	MLH1	unstable	met	88	f	T	sporadic
6459	MLH1	unstable	met	86	m	A	sporadic
655	MLH1	unstable	met	84	f	A	sporadic
20140	MLH1	unstable	met	81	f	A	sporadic
12167	MLH1	stable	met	49	m	S	BG1
13458	MLH1	unstable	met	76	f	C	sporadic
11628	MLH1	unstable	unmet	42	m	S	AC
13297	MLH1	unstable	met	71	f	HF	sporadic
20719	MLH1	stable	met	69	f	C	sporadic
20079	MLH1	unstable	met	76	m	A	sporadic
19372	MLH1	unstable	met	78	m	S	sporadic
17325	MLH1	unstable	met	47	f	C	BG1
16165	MLH1	unstable	unmet	57	m	C	BG3
14895	MLH1	unstable	met	71	f	A	sporadic
14230	MLH1	unstable	met	77	f	A	sporadic
22857	MLH1	unstable	met	73	m	A	BG4&5
22643	MLH1	unstable	met	68	m	A	sporadic
12287	MLH1	unstable	unmet	56	m	C	sporadic
11656	MLH1	unstable	unmet	36	f	S	BG1
11779	MLH1	unstable	met	94	f	C	sporadic
14124	MLH1	unstable	met	78	m	C	AC
15600	MLH1	unstable	met	90	f	A	sporadic
17291	MLH1	unstable	met	80	m	R	sporadic
19604	MLH1	unstable	met	52	f	X	sporadic
4734	MLH1	unstable	met	67	m	T	sporadic
22752	MLH1	unstable	met	76	m	C	sporadic
22666	MLH1	unstable	met	84	f	C	sporadic
23697	MLH1	stable	met	79	f	D	n.d.
700	MLH1	unstable	met	49	f	D	AC
9658	MLH1	unstable	met	79	f	A	sporadic
22529	MLH1	unstable	met	78	f	C	sporadic
20758	MLH1	unstable	unmet	89	f	C	n.d.
6579	MLH1	unstable	met	76	f	HF	sporadic
9465	MLH1	unstable	met	74	m	T	sporadic
10570	MLH1	unstable	met	81	m	A	sporadic
15659	MLH1	unstable	met	79	f	A	sporadic

Tumor no.	Absent MMR protein*	BAT26	Promoter Methylation**	Age	Sex	Tumor site***	History of cancer¶
25013	MLH1	unstable	met	67	f	C	sporadic
6110	MLH1	unstable	met	78	f	A	sporadic
10335	MLH1	unstable	met	75	f	A	sporadic
15581	MLH1	unstable	met	64	f	S	n.d.
1398	MLH1	unstable	met	85	m	C	sporadic
5160	MLH1	unstable	met	91	m	A	sporadic
218	MLH1	unstable	met	76	f	A	sporadic
2019	MLH1	unstable	met	68	f	C	sporadic
2503	MLH1	unstable	met	80	m	HF	sporadic
7366	MLH1	unstable	met	72	m	HF	sporadic
12306	MLH1	unstable	met	86	f	C	sporadic
17296	MLH1	unstable	met	85	f	A	sporadic
17520	MLH1	unstable	met	88	f	C	sporadic
18573	MLH1	unstable	met	76	m	T	sporadic
19353	MLH1	unstable	met	68	f	A	sporadic
21492	MLH1	unstable	unmet	82	f	C	n.d.
18102	MLH1	unstable	unmet	79	m	S	sporadic
22391	MLH1	unstable	met	62	f	S	n.d.
27489	MLH1	unstable	unmet	48	m	HF	BG1
27300	MLH1	stable	met	83	f	C	sporadic
34746	MLH1	unstable	met	60	m	A	n.d.
32757	MLH1	unstable	met	93	f	C	sporadic
35096	MLH1	unstable	met	73	m	T	sporadic
37919	MLH1	unstable	met	84	f	A	sporadic
19698	MLH1	unstable	met	88	f	C	sporadic
1648	MLH1	unstable	met	78	m	C	sporadic
11768	MLH1	unstable	met	82	m	A	sporadic
13452	MLH1	unstable	met	67	m	C	BG5
13674	MLH1	unstable	met	89	f	C	sporadic
20938	MLH1	unstable	unmet	48	m	R	AC
64501	PMS2	unstable	unmet	79	m	D	BG2
53072	PMS2	unstable	unmet	57	m	A	BG4
61263	PMS2	unstable	unmet	73	m	A	sporadic
66543	PMS2	unstable	unmet	77	f	T	BG4
66732	PMS2	unstable	unmet	46	m	D	BG1
52557	PMS2	stable#	unmet	77	m	A	sporadic
54882	PMS2	unstable	unmet	42	m	A	BG1
53989	PMS2	unstable	unmet	57	m	SF	BG4
61162	PMS2	unstable	unmet	82	m	C	BG4
65950	PMS2	unstable	unmet	43	m	R	BG1
59519	PMS2	unstable	unmet	78	m	SF	BG2
11318	PMS2	Stable#	unmet	46	f	S	BG1
20498	PMS2	unstable	unmet	76	m	A	sporadic
16655	PMS2	unstable	unmet	66	m	A	sporadic
5194	PMS2	unstable	unmet	57	m	HF	BG3
27499	PMS2 (partial)	§	§	49	m	A	BG1&4

*Lack of expression as detected by immunohistochemistry
**Data from the analysis of the MLH1 promoter region -299 to -152 and from 2 regions of the PMS2 promoter (see Methods and Supplemental Table S1 for details)
***C cecum; A ascending; HF hepatic flexure; T transversum; SF splenic flexure; D descending colon; S sigmoid colon; R rectum
¶ AC: revised Amsterdam Criteria, BG: revised Bethesda Guidelines; n.d.=not determined (5 patients died; 2 patients: parents unknown; 5 patients: questionnaire not returned)
unstable in other markers
§ DNA extracted from microdissection not suitable for molecular analysis

Supplementary Figure 1

Methylation analysis of *MLH1* (a and b) and *PMS2* (c and d) gene promoters in colorectal cancers (CRCs) (see Methods in Supplemental Table S1)
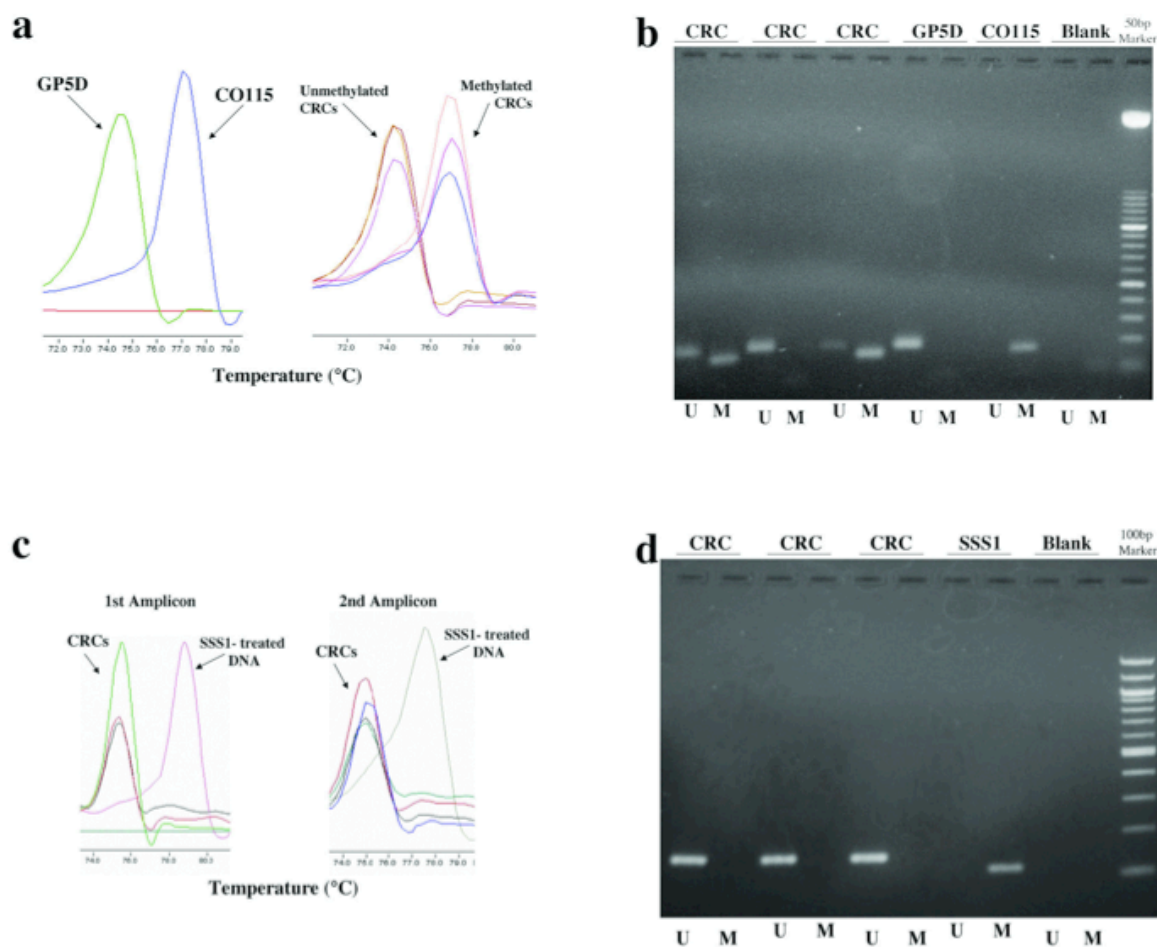
a) Fluorescence melting peaks for the *MLH1*-promoter fragment −299 to −152. The melting temperature of PCR products obtained from the bisulfite-treated DNA of the CRC cell lines GP5D and Co115 are shown as unmethylated and methylated controls, respectively.

b) Unmethylated (U) and Methylated (M) alleles as detected by Methylation Specific PCR (MSP). The presence of both alleles In 2 CRCs is due to contamination with DNA from stromal cells.

c) Fluorescence melting peaks for two overlapping fragments amplified in the *PMS2* gene promoter. Human genomic DNA treated with SSS1 methyltransferase (New England Biolabs) was used as positive control for methylated alleles.

d) All CRCs tested by MSP showed only *PMS2* unmethylated alleles (U).

ADDENDUM

PMS2 cDNA Primers

	FWD	REV	Product size	MgCl2-Konz.
Segment 1 (S1)	ATCGGGTGTTGCATC	CAAAATTTGCCTTTTATCTGGA	1048 bp	1,5
nested Segment 1 (S1n)	ATTTGCTCTGGGCAGGTG	CAAAATTTGCCTTTTATCTGGA	957 bp	1,5
Segment 1A (S1A)	ATTTGCTCTGGGCAGGTG	CCATTTTGGCATACTCCTTCTT	478 bp	1,5
Segment 1B (S1B)	AGGAATTTCAAAGGAATATTAAGAAGG	CAAAATTTGCCTTTTATCTGGA	521 bp	1,5
confirmation fs Ex4 (fsEx4)	GGTGCCACTAATATTGATCTAAAGC	GAGGCTTTGCAACTGCTTCT	570 bp	1,5
Segment 2 (S2)	TGTTACTCCAGATAAAAGGAAA	TTCCATACAGTGACTACGGTCAG	1604 bp	1,5
Segment2A (S2A)	TGTTACTCCAGATAAAAGGAAA	GGAGCTGGCCCGCATACTC	565 bp	1,5
Segment 2B (S2B)	ACGGACCCAGTGACCCTAC	GAGGTGCTATGAGCCTCTGC	744 bp	3,5
Segment 2C (S2C)	AAAAGAGATAAGTAAAACGATGTTTGC	TTCCATACAGTGACTACGGTCAG	614 bp	1,5

PCR conditions:

S1 + fsEx4:
94°C - 5 min
94°C - 1 min ⎤
60°C - 1 min ⎬30x
68°C - 3 min ⎦
68°C - 10 min
Hold 4°C

S1n + S1A + S1B:
94°C - 5 min
94°C - 1 min ⎤
55°C - 1 min ⎬36x
68°C - 3 min ⎦
68°C - 10 min
Hold 4°C

S2 + S2A + S2C:
94°C - 5 min
94°C - 1 min ⎤
58°C - 1 min ⎬ 36x
68°C - 3 min ⎦
68°C - 10 min
Hold 4°C

S2B:
94°C - 5 min
94°C - 1 min ⎤
60°C - 1 min ⎬35x
68°C - 3 min ⎦
68°C - 10 min
Hold 4°C

SALSA MLPA P008 MSH6 / PMS2 probemix

Length (nt)	SALSA MLPA probe	Gene	Chromosomal position
64-70-76-82*	DQ-control fragments		
88-92-96**	DD-control fragments		
130	0797-L0463	Control probe	Chr. 5q31
136	4249-L3604	TACSTD1	27 Kb before MSH2 exon 1
142	4243-L3598	MSH6	Exon 7
148	1685-L1265	MLH1	Exon 1
154	4661-L4043	PMS2	Exon 1
160	4244-L3599	MSH6	Exon 8
166	1686-L1266	MLH1	Exon 1
172	4245-L4021	MSH6	Exon 9
180	1176-L4022	PMS2	Exon 2
190	4248-L3603	TACSTD1	15 Kb before MSH2 exon 1
196	4246-L3601	MSH6	Exon 10
202	1245-L0793	MLH3	Exon 3
211	4676-L3352	MUTYH	Exon 14
220	2107-L0794	MLH3	Exon 10
229	1179-L0740	PMS2	Exon 5
238	1247-L0795	MSH3	Exon 1
247	1180-L0741	PMS2	Exon 6
256	1248-L0899	MSH3	Exon 8
265	1181-L0742	PMS2	Exon 7
274	1249-L0797	MSH3	Exon 20
283	1182-L0743	PMS2	Exon 8
292	4677-L3353	MUTYH	Exon 16
301	1183-L0744	PMS2	Exon 9
310	4322-L4024	MUTYH	Exon 1
319	1184-L0745	PMS2	Exon 10
328	1250-L0798	MSH6	Exon 1
337	1185-L0900	PMS2	Exon 11
346	4247-L3602	MSH6	Exon 2
355	2735-L2162	MSH2	Exon 1
364	1252-L0902	MSH6	Exon 4
373	4663-L6248	PMS2	Exon 13
382	1253-L0801	MSH6	Exon 6
391	1188-L0749	PMS2	Exon 14
401	4323-L1368	MUTYH	Exon 2
409	4664-L4046	PMS2	Exon 15
418	3964-L3351	MUTYH	Exon 3
427	1439-L0908	MSH6	Exon 3
436	4662-L4044	PMS2	Exon 12
445	1440-L0909	MSH6	Exon 5
454	1810-L0984	APC	Exon 7
463	4675-L0792	MLH3	Exon 2

* Not ligation-dependent, this indicates the amount of DNA used.

** Ligation-dependent, these fragments give a warning for incomplete DNA denaturation.

MSH3 study

PCR conditions for *MSH3* analysis

cDNA	Product size	MgCl2 -Conc. (mM)	Tm (°C)	Q- Solution	FWD-Primer 5'-3'	REV-Primer 5'-3'
S1	953 bp	1,5	60	+	CTTGCCCTGCCATGTCTC	TGCTGCAGTTTCAGTTTGCT
S1/S2	347 bp	1,5	60	-	CAGCAGCACAAAGATGCAGT	TCAACATTTACAGCATCATCCA
S2	999 bp	1,5	60	-	CAGACTGTTTGTTCATGTACGC	TCTATGTCGGGCAATTTACG
S3	910 bp	1,5	60	+	CTTCATTTGGGAGACGGAAG	CCCAGCAACACATCAATCAC
S3/S4	271 bp	1,5	60	-	GTCCTTGACTGCAGTGCTGA	TCTCTGAGTCCTCTGATAAATCTGT
S4	956 bp	1,5	60	+	CAAGGTCGCTAAGCAAGGAG	TGTACAGTTGGTATTTTTAATTCTCCA
genomic DNA						
Ex 18	241 bp	1,5	60	-	GTGATGGCATTTCGGATTTT	TTTTTCCAGTCTGTTTCTGATAGC
tumor DNA						
Ex 18	193 bp	1,5	55	-	GTGATGGCATTTCGGATTTT	TTGTAAACTCACTCTAGAAAATCAAGC

95°C - 3 min
93°C - 30 sec
55°/60°C - 45 sec } 35 Zyklen
72°C - 45 sec
72°C - 5 min
Hold 4°C

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10. CURRICULUM VITAE
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TALKS

Group evening Center of Biomedicine, Basel, February 2nd, 2006:

On genes rarely mutated in HNPCC: Germline alterations in *PMS2* and *MSH3*

Onco-Meeting ZLF, Basel, February 15th, 2006

Genetic characterization of colorectal cancer patients with loss of PMS2 expression in the tumor

Group evening Center of Biomedicine, Basel, February 6th, 2007:

Lack of genotype-phenotype correlations in a consecutive series of patients with familial adenomatous polyposis (FAP)

POSTER PRESENTATIONS

11. – 15. 06. 2004

European Conference of Human Genetics, Munich:

Molecular characterization of colorectal cancers with loss of PMS2 expression

14. – 17. 06. 2005

International Society for Gastrointestinal Hereditary Tumors (InSiGHT) Conference, Newcastle upon Tyne:

Identification of novel *APC* mutations and genotype-phenotype correlations in Swiss familial adenomatous polyposis (FAP) patients

06. – 09. 06. 2006

European Conference of Human Genetics, Amsterdam:

A 7 year survey on familial adenomatous polyposis patients in Switzerland: Identification of novel *APC* germline mutations and genotype-phenotype correlations

17. 10. 2006

Biovalley Life Science Day, Basel

MSH3 - a novel susceptibility gene for Hereditary Nonpolyposis Colorectal Cancer (HNPCC)?

PUBLICATIONS

K.Truninger*, M.Menigatti*, **J.Luz***, A. Russell, R. Haider, J.-O. Gebbers, F.
Bannwart, H. Yurtsever, J. Neuweiler, H.-M. Riehle, M.S. Cattaruzza,
K. Heinimann, P. Schaer, J. Jiricny, G. Marra
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negative Polyposis patients
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Thirlwell C, Howarth K, Jaeger E, Robinson J, Volikos E, Silver A, Kelly
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Declaration of independence

I declare that I wrote this thesis "Hereditary colorectal cancer: Assessment of genotype-phenotype correlations and analysis of rare susceptibility genes in familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) " with the help indicated and only handed it in to the faculty of science of the University of Basel and to no other faculty and no other university.