Molecular pathology of colorectal cancer predisposing syndromes

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Als je nadenkt over het
mysterie van de scheppende
voortgang van de natuur, word
je overstelpt door het besef van
de begrenzingen van het
menselijk intellect.
(A.N. Whitehead)

Aan mijn ouders

Voor Francien en Herman

Contents

Aim and outline of this thesis

Each year, approximately eleven thousand new colorectal cancer (CRC) patients are registered in the Netherlands. Half of these patients will eventually die of this disease, especially those in whom metastasis to regional lymph-nodes or distant organs was present at the time of surgery. Consequently, it is of great importance to identify individuals with an increased risk for CRC. Timely colonoscopic surveillance offered to such individuals could lead to a reduction in the incidence of CRC and a reduction in overall mortality. A way to identify individuals at risk is to look at their family history in terms of the type of cancer and its presence in multiple family members combined with an early age of onset. The majority of families with highly penetrant syndromes will be identified on the basis of their clinical appearance.

Molecular tumor testing can be applied to direct germline gene testing as a cost effective approach in index patients of these families. Subsequently, these patients will be screened for the presence of a germline defect in the known high risk genes (*MLH1, PMS2, MSH2, MSH6,* or *MUTYH*). After identification of the underlying gene defect(s) causing a high risk of CRC, pre-symptomatic testing can be offered to these families, and screening options can be discussed in mutation carriers and individuals at risk who choose not to be tested. CRC families without identified mutations are due to either an undetected defect in known genes or the single high risk gene not yet having been identified as a target for mutations. Alternatively, the high risk for CRC could be the result of a combination of gene variations, with each contributing a low level of risk.

This thesis describes the search for molecular pathology tools that can play a role in identifying individuals with an increased risk for CRC based on their genetic makeup and it provides insight into the tumorigenesis of familial CRC.

The described work can roughly be divided into:

1) The use of reliable methods that are applicable for formalin-fixed paraffin-embedded (FFPE) tissues, which is of utmost importance since the majority of tumor tissue from familial CRC is only available as FFPE tissue.
2) Tumor profiling to guide genetic testing strategies and clinical genetic decision making, to gain insight into the tumorigenesis of familial CRC (including Lynch syndrome and *MUTYH*-associated polyposis), and to study the role of *CHEK2* and *PTPRJ*.

Chapter 1 provides a brief overview of colorectal tumorigenesis and a general introduction of the factors that determine the individual risk of CRC and inheritable CRC syndromes. The contribution of low level genetic risk factors and environmental factors in causing CRC are also discussed.

In **chapter 2** we evaluate the results of microsatellite instability (MSI) analysis in two groups of individuals suspected for Lynch syndrome: one that fulfills the Bethesda cri-

teria and a separate group that does not fulfill those criteria. Furthermore, we compare the results of immunohistochemical (IHC) staining and MSI analysis and assess the additional value of PMS2 staining.

In **chapter 3**, we compare genomic profiles using single nucleotide polymorphism (SNP) arrays in three groups of archival tumors that show a high frequency of microsatellite instability (MSI-high). In one group MSI-high is caused by a pathogenic mutation in one of the mismatch repair (MMR) genes, *MLH1*, *PMS2*, *MSH2,* and *MSH6* (23 patients). A second set of tumors consists of MSI-high carcinomas from patients with an unclassified variant (UV) in one of the MMR genes (8 patients). A third group contains sporadic colon carcinomas with microsatellite instability due to *MLH1* promoter hypermethylation (10 patients).

Chapter 4 describes the value of *KRAS2* somatic mutation analysis for identifying patients with (atypical) *MUTYH*-associated polyposis (MAP). FFPE tumor tissues were studied for *KRAS2* mutations followed by *MUTYH* hotspot analysis in normal FFPE materials.

In **chapter 5**, the patterns of genomic instability in MAP carcinomas are described. Twenty-six carcinomas of MAP patients were studied for ploidy, genome-wide copy number variations, and copy neutral loss of heterozygosity (cnLOH).

Chapter 6 describes a large family in which gene defects of *MUTYH* and *MSH6* co-segregate. In particular, we studied the tumors in a family branch with combinations of defects.

In **chapters 7** and **8**, we studied the individual effect of the cancer susceptibility alleles (*PTPRJ**1176 A>C and *CHEK2**1100delC) in individuals with familial clustering of CRC.

Chapter 9 contains concluding remarks and a discussion of the future implications of this study.

Chapter 10 summarizes the work described in this thesis.

Chapter 11 summarizes the work described in this thesis in Dutch, contains the curriculum vitae and the list of additional publications.

List of abbreviations

AFAP	attenuated FAP
BER	base excision repair
CD	Cowden disease
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
cnLOH	copy neutral loss of heterozygosity
CRC	colorectal cancer
FAP	familial adenomatous polyposis
FFPE	formalin-fixed paraffin-embedded
GWA	genome-wide association
HPPS	hyperplastic polyposis
IHC	immunohistochemistry
JPS	Juvenile polyposis syndrome
LOH	loss of heterozygosity
MAP	*MUTYH*-associated polyposis
MINT	methylated in tumors
MMR	mismatch repair
MSI-high	microsatellite unstable
MSI or MIN	microsatellite instability
MSS	microsatellite stable
MTS	Muir Torre syndrome
PAH	polycyclic aromatic hydrocarbons
PJS	Peutz-Jeghers syndrome
SNP	single nucleotide polymorphism
TS	Turcot syndrome
UV	unclassified variant

CHAPTER 1

General introduction

Colorectal cancer (CRC) is the second most common cause of death due to malignancy in the Western world. In the Netherlands, approximately 11,000 new cases of CRC are now diagnosed each year, and the lifetime risk of developing CRC in the general population is about 5%. The cause of CRC is multifactorial, involving high risk and low risk genetic factors as well as environmental factors including lifestyle [1-5]. The spectrum of CRC can be divided into two main groups: sporadic CRC and familial CRC (Figure 1). The majority of patients develop CRC on an apparently sporadic basis and are the sole family member with CRC (65-90% of all patients). Affected individuals develop carcinomas mostly at relatively advanced ages (mean age of 70 years) [6,7]. Approximately 10-35% of all cases show familiar clustering of CRC [8], and only a proportion can be explained by known highly penetrant syndromes such as Lynch syndrome, familial adenomatous polyposis (FAP), Peutz-Jeghers syndrome (PJS), Juvenile polyposis syndrome (JPS), Cowden disease (CD), and *MUTYH*-associated polyposis (MAP). The majority of these syndromes are caused by autosomal dominant genetically inherited risk factors. Thus far, only one syndrome (MAP) shows an autosomal recessive mode of inheritance.

For individuals from unexplained families with clustering of CRC, the lifetime risk for developing CRC compared to the general population is increased more than twofold when these individuals have an affected first degree relative. This risk is increased more than threefold when the first degree relative is younger than 50 [9-11]. Some of the currently unexplained familial risk could be due to yet unidentified high-penetrant genetic risk factors. Another explanation for a large proportion of this familial clustering could be the combination of several low-penetrant cancer susceptibility alleles [12].

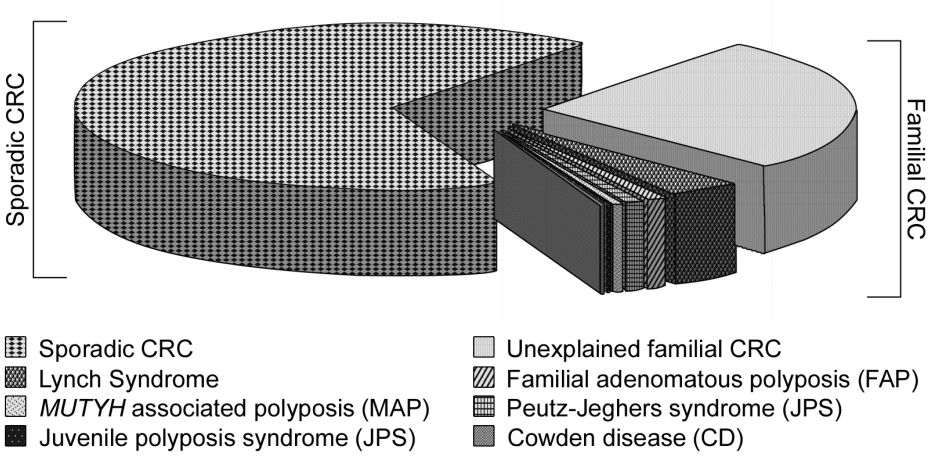

Figure 1. Spectrum of colorectal cancer (CRC)

Colorectal cancer can be divided into two main groups: sporadic CRC (65-90% of all patients) and familial CRC (10-35% of all patients). Up to 5% of CRC can be explained by these hereditary syndromes: Lynch syndrome, *MUTYH*-associated polyposis (MAP), familial adenomatous polyposis (FAP), Peutz-Jeghers syndrome (PJS), Juvenile polyposis syndrome (JPS), and Cowden disease (CD).

Tumorigenesis of colorectal carcinomas

Accumulated genetic and epigenetic changes underlie the development of neoplasia of the colon. This multistep process leads to the transformation of normal colonic epithelium to colon adenocarcinoma. During this process, somatic mutations accumulate and determine the final phenotypic characteristics of the colorectal tumor [13].

Genetic instability

In CRC, there are two classic genetic pathways that direct tumorigenesis: chromosomal instability (CIN) and microsatellite instability (MSI or MIN), as depicted in Figure 2.

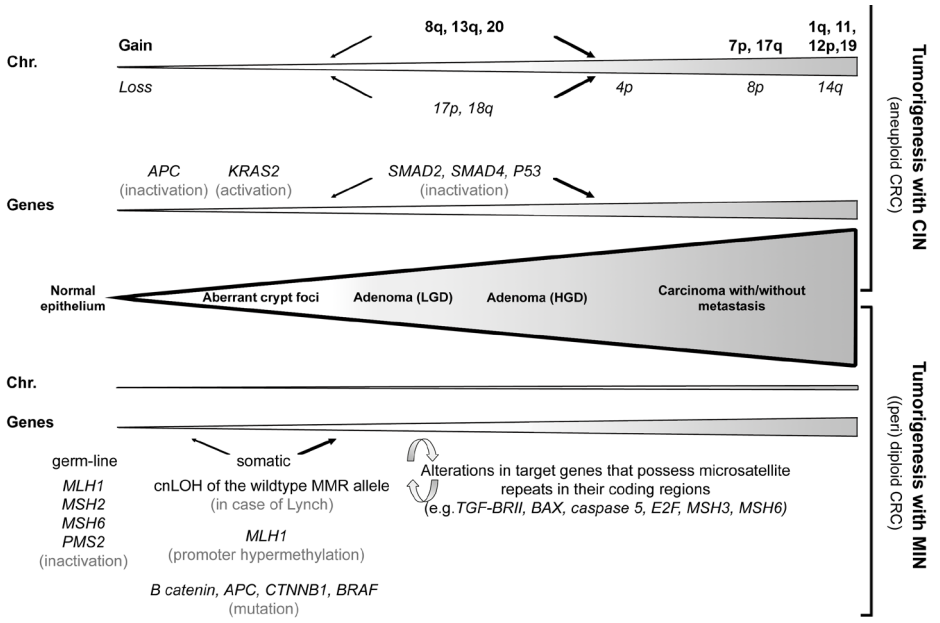

Figure 2. Stepwise progression from normal epithelium to cancer with metastasis (modified "Vogelgram").
Classic alterations in CIN tumors (upper element of the scheme) vs. MIN tumors (lower element of the scheme) during tumor progression are depicted. Adenomas are stratified according to architectural changes and presence of dysplasia (low vs. high grade dysplasia). In situ carcinomas are now considered to be high grade dysplastic.
Abbreviations: Chr., chromosome; CRC, colorectal cancer; LGD, low grade dysplasia; HGD, high grade dysplasia; cnLOH, copy neutral loss of heterozygosity.

CIN

CIN is a predominant pathway characterized by chromosomal copynumber variation including chromosomal gains, physical losses, and copy neutral loss of heterozygosity (cnLOH). These tumors show aneuploidy, which is the equivalent of a gross amount of CIN. In general, carcinomas with CIN present with losses of chromosomes 17p and

18q, and gains at 8q, 13q, and 20 that occur at early stages during the transition from adenoma to carcinoma, whereas loss of 4p is associated with transition from Dukes' A to B-D. Chromosomal loss of 8p and gains of 7p and 17q are reported to be associated with the transition from primary carcinoma to local and distal metastases. Loss of 14q and gains of 1q, 11, 12p, and 19 are considered to be late events [14,15].

The mechanism that underlies CIN in human cancers is not completely understood. In 1989, Shackney *et al*. proposed a conceptual model based on the observations that cancer cells can spontaneously double their chromosome number, followed by subsequent chromosomal losses and gains [16]. Specific mutations or gene silencing have also been suggested to be the direct or indirect cause of CIN [17]. This means that a variety of defects can underlie CIN such as the dysfunction of proteins involved in mitosis (microtubule, centromere and centrosome), chromosome breakage, and failure of cell cycle checkpoints. The following genes have been suggested to cause CIN in colorectal cancer: the mitotic checkpoint genes *BUB1* and *BUBR1* [18], the aurora kinases, which are essential for cell proliferation [19], adenomatous polyposis coli (*APC*), which has a crucial role in the Wnt/Wingless pathway [20], and the general tumor suppressor *FBXW7/CDC4* [21]. Additional genes associated with a CIN phenotype of CRC are *KRAS2* on chromosome 12 (12p12.1), which is involved in both cell cycle regulation and cellular adhesion, *SMAD4* on chromosome 18 (18q21.1), which is a tumor suppressor gene critical for transmitting signals from transforming growth factor-ß (*TGFß1*) on chromosome 19 (19q13.1), and *TP53* on chromosome 17 (17p13.1), which is an important player in a variety of cellular signaling pathways [13,22].

MIN

The second pathway is MIN or MSI, which is characterized by tumor cells with small deletions and insertions in coding and non-coding stretches of short repetitive DNA sequences distributed throughout the genome. Accumulation of these mutations leads to frameshifts within coding sequences and the subsequent inactivation of genes, thereby contributing to tumor development and progression [23-25]. These tumors are diploid or near-diploid [26]. MSI results from a defective mismatch repair (MMR) system, in which both alleles of an MMR gene (*MLH1*, *MSH2*, *MSH6,* and *PMS2*) are nonfunctional and lack the ability to repair DNA replication mismatches in the cells. However, in leukocyte DNA, low levels of MSI have been identified in *MLH1* and *MSH2* mutation carriers before tumor diagnosis. One explanation might be that these low levels of MSI reflect the presence of phenotypically normal MSI (-/+) cells; another possible explanation is the presence of circulating MSI (-/-) cells that have a complete loss of the MMR gene [27].

Epigenetic gene silencing

DNA methylation is present throughout the majority of the genome and is maintained in relatively stable patterns that are established during development [28]. Approximately 70% of CpG dinucleotides are methylated. There are regions in the genome that contain higher proportions of CpG dinucleotides called CpG islands, which are 0.2-3.0 kb-long sequences and by definition are composed of greater than 50% cytosines/guanines. They are present in the 5′ region of approximately 50-60% of genes and are normally maintained in an unmethylated state. In cancers, many of these CpG islands become aberrantly methylated, and this aberrant methylation can be accompanied by transcriptional repression. The silencing of multiple genes by DNA methylation can lead to tumorigenesis.

Methylation

Changes in DNA methylation in CRC involve simultaneous global demethylation, increased DNA-methyltransferases expression, and *de novo* methylation of CpG islands. Tumors can be classified into three distinct groups based on their CpG island methylation phenotype (CIMP) status: CIMP1, CIMP2, and CIMP negative. The CIMP1 subset is characterized by hypermethylation at *MLH1*, *Timp3*, methylated in tumors 1 (*MINT1*), and *RIZ1*. Furthermore, this subset presents with a high incidence of MSI, and *BRAF* is frequently mutated (V600E). The CIMP2 subset shows hypermethylation of *MINT27, MINT2, MINT31,* and *Megalin*, along with a high rate of *KRAS2* mutations. CIMP negative cases have a high frequency of *P53* mutations [29,30].

High genetic risk for colorectal cancer

The first high risk genetic factor predisposing to CRC, a defect of the *APC* gene, was identified in 1991 [31,32]. Subsequently, other gene defects leading to CRC syndromes were described; these were mostly autosomal dominant syndromes, but one autosomal recessive syndrome was also identified. These syndromes can be divided into non-polyposis and polyposis syndromes, the latter of which presents with a multitude of either adenomatous, hamartomatous, or hyperplastic polyps.

Autosomal dominant inheritable CRC without polyps

Lynch syndrome MIM No 114500

The most common hereditary CRC syndrome, which accounts for 1–6% of all CRC cases, is Lynch syndrome [33]. Lynch syndrome, formerly known as Hereditary Non Polyposis Colorectal Carcinoma (HNPCC) is characterized by an increased risk of early-onset

CRC and other cancers, including tumors of the endometrium, stomach, small intestine, hepatobiliary system, kidney, ureter, brain, and ovary [34-37]. Whether breast and prostate cancers are integral tumors of Lynch syndrome is still a matter of debate [38,39]. The increased risk for malignancy in Lynch syndrome is caused by a mutation in the MMR genes: *MLH1* (chr. 3 [3p21.3]), *MSH2* (chr. 2 [2p22-p21]), *MSH6* (chr. 2 [2p16]), and *PMS2* (chr. 7 [7p22.2]) [40-45]. Germline mutations in *MLH1* and *MSH2* comprise more than 90% of all known MMR mutations in Lynch syndrome [46], while germline mutations in *MSH6* account for 5–10% of all mutations [47,48]. Heterozygous truncating germline mutations in *PMS2* also play a role in a small subset of Lynch syndrome families [49].

Mutations in DNA MMR genes result in a failure to repair errors in repetitive sequences that occur during DNA replication. A heterodimer of MSH2 and MSH6 recognizes single nucleotide mismatches, insertion and deletion loops (IDL's), whereas a heterodimer of MSH2 and MSH3 recognizes IDL's in the absence of MSH6 [50]. The heterodimer of MLH1 and PMS2 mediates cross talk between mismatch recognition and the actual repair complex [51]. In the absence of PMS2, the MLH3 protein is the remaining protein for forming a heterodimer with MLH1 [52]. The failure to repair errors in repetitive sequences by one of the MMR genes leads to MSI in the tumor, which is the molecular hallmark of Lynch syndrome [23,53-55]. In 1997, at an NCI workshop, clinical guidelines (Bethesda criteria) were proposed for individuals with CRC suspected for Lynch syndrome [56]. In 2004, these criteria were revised [7]. Patients who fulfill these criteria concerning family history, cancer type and the presence of cancer in multiple family members in combination with an early age of onset are eligible for additional analysis of tumor materials. The presence or absence of MSI is determined by a PCR-based analysis, and protein expression of the MMR enzymes is analyzed with immunohistochemical (IHC) techniques. Based on the results, eligibility for mutation analysis of the MMR genes is determined [55]. The result of the IHC pinpoints the MMR gene most likely to be mutated [57]. This type of tumor pre-analysis makes MMR germline mutation screening less time consuming and expensive.

However, in an undefined percentage of the cases analyzed for mutations in one of the MMR genes, variants of unknown clinical significance, so-called unclassified variants (UVs), are identified. Clinically, the uncertainty regarding the contribution of a MMR-UV to the risk of developing cancer is a major problem. While carriers of a pathogenic MMR-mutation are at increased risk for developing cancer, those with an MMR-UV could also represent rare variants without increased risk of cancer. For pathogenic MMR carriers, clinical geneticists offer pre-symptomatic testing for the detection of neoplasia at an early stage. For patients carrying an MMR-UV with unproven pathogenicity, offering pre-symptomatic testing is difficult. Ten criteria are used to obtain insight into the pathogenicity of MMR gene variants: *de novo* appearance of a mutation, segregation of the UV with pedigrees, absence of the UV in control individuals, a change in amino acid

polarity charge or size in the encoded peptide, occurrence of the amino acid change in a domain that is evolutionarily conserved between species and/or shared between proteins belonging to the same protein family, loss of the non-mutated allele in tumor material of the patient, absence of IHC staining for the corresponding protein in tumor material, presence of MSI in tumor material of the patients, effect of the mutation on MMR capacity in functional assays, and previous inclusion of the mutation in disease-specific mutation databases [58].

Two variations of Lynch syndrome

1. Muir Torre Syndrome (MTS) MIM No 0158320

MTS is a rare inherited syndrome that is considered a part of Lynch syndrome. Patients present with a sebaceous gland tumor (adenoma and carcinoma), keratoacanthoma, and at least one visceral malignancy [59].

2. Turcot syndrome (TS) MIM No 276300

TS is a rare syndrome that is considered a part of Lynch syndrome and familial adenomatous polyposis (FAP). TS is classically referred to as the combination of colorectal polyposis and primary tumors of the central nervous system (glioblastoma, astrocytoma, or spongioblastoma) [60].

Autosomal dominant inheritable CRC with adenomatous polyps

Familial adenomatous polyposis (FAP) MIM No 175000

Approximately 1% of CRCs are caused by FAP. The syndrome is characterized by the presence >100 adenomatous polyps of the colon and small intestine in the later stages [61]. Patients have a risk of virtually 100% of developing colon cancer at a mean age of 40 years if the colon is not removed at an early stage of life [62]. The colorectum is not the only organ at risk for tumors; the risk of cancer of the duodenum, thyroid, pancreas, liver (hepatoblastoma), and central nervous system is also increased [63,64]. Furthermore, there is a risk for desmoïd disease especially in specific genotypes, and this is often triggered by previous abdominal surgeries such as colectomy or caesarian sectioning. The increased risk for malignancy in FAP is caused by a mutation in the *APC* gene located on chromosome 5 (5q21-22). Ten to 25% of these cases occur de novo [31,32,65,66].

Attenuated FAP (AFAP)

Attenuated FAP is a phenotypic variant of FAP, characterized by the presence of fewer than 100 polyps, a later age of onset, and mutations that predominantly occur in the 5′ and 3′ ends and in exon 9 of the *APC* gene [32,67,68].

Autosomal dominant inheritable CRC with hamartomatous polyps

Peutz-Jeghers syndrome (PJS) MIM No 175200

Less than 1% of CRCs are due to PJS. Patients with PJS have hamartomas predominately in the small intestine and fewer polyps in the colon and stomach [69]. The hallmarks of the disease are melanin spots on the lips and buccal mucosa, observed in 95% of patients. The lifetime risk of developing cancer is as high as 85% [70]. Patients also have an elevated risk for tumors of the breast, ovary, uterus, cervix, lung, and testis [70]. In 30-80% of all PJS cases, there is a germline mutation in the nuclear serine threonine kinase gene (*STK11*) on chromosome 19 (19p13.3) [70,71].

Juvenile polyposis syndrome (JPS) MIM No 174900

The population incidence of JPS is even lower than that of PJS. JPS is characterized by multiple hamartomatous polyps of the gastrointestinal tract predominantly affecting the colorectal region. Most individuals with JPS have some polyps by 16 years of age. The lifetime risk of gastrointestinal cancers in families with JPS is as high as 60%. Most of this increased risk is attributed to colon cancer, but gastric, duodenal, and pancreatic tumors have also been reported. A mutation in *SMAD4* on chromosome 18q21.1 is found in 15-30% of individuals affected with JPS. About 20-40% of individuals have mutations in the *BMPR1A* gene located on chromosome 10 (10q22.3) [2].

Cowden disease (CD) MIM No 158350

The number of individuals affected with CD is also very low. CD differs from both PJS and JPS in that polyposis is not the defining feature. Rather, most cases are ascertained because of distinctive mucocutaneous lesions, benign and malignant thyroid and breast disease, and macrocephaly. The onset of clinical manifestations of CD in patients may be diagnosed as early as 4 years or as late as 75 years of age [72]. Approximately 80% of patients with CD have a mutation in the *PTEN* tumor suppressor gene on chromosome 10 (10q23.3) [73].

Autosomal recessive inheritable CRC with adenomatous polyps

MUTYH-associated polyposis (MAP) MIN No 608456

In 2002, the autosomal recessive syndrome MAP was described [74]. MAP patients develop between 10-500 polyps at a mean age of approximately 50 years [75-77]. The increased risk for malignancy in this syndrome is caused by bi-allelic germline *MUTYH* mutations. *MUTYH*, located on chromosome 1 (1p34.3-p32.1), is an important cellular player in the base-repair (BER) system, which is a multi-step process that repairs frequently occurring 8-oxo-guanine (8-oxoG) DNA lesions formed upon oxidative DNA damage. A

bi-allelic germline *MUTYH* mutation predisposes carriers to somatic G>T transversions in *APC* and *KRAS2*, which are involved in the tumorigenesis of CRC. These G>T transversions seem to occur mostly at GAA sequences in *APC* [74,78]. In *KRAS*, an c.34G>T mutation is found in up to 60% of the MAP carcinomas and is infrequent in sporadic CRC [79,80]. Although MUTYH deficiency triggers carcinogenesis by G>T transversions, the exact role of MUTYH deficiency in tumor progression in MAP patients is still unknown. In the Netherlands, clinical geneticists advise diagnostic testing for *MUTYH* germline mutations based on family history, the number of adenomas, and age at diagnosis. *MUTYH* will be analyzed in patients with 10 to 100 adenomas at ages under 70 years, whereas Lynch syndrome could also be considered in CRC patients with a history of <10 adenomas. In patients with classic polyposis (>100 adenomas), germline *APC* mutations (FAP) can be excluded prior to *MUTYH* testing [81]. Previously, in large cohorts of CRC patients (with or without polyps), approximately 1% of MAP patients were found to be bi-allelic , some of whom were without polyps [82,83]. Although no *MUTYH* mutation carriers were detected in other cohorts of patients with fewer than 10 polyps [84], the question remains as to the prevalence of the (bi-allelic) *MUTYH* mutations in familial CRC cases with <10 polyps, with or without concomitant CRC.

Low-penetrance cancer susceptibility alleles

To identify cancer susceptibility alleles, studies have been performed both in mice and humans. In mouse models, at least 100 cancer susceptibility alleles have been identified in different cancer models [85,86]. Since the completion of the human genome and the HapMap projects [87], DNA sequences have become available, as well as numerous naturally presenting polymorphic genetic variants that may determine individual susceptibility to cancer. There are most likely up to hundreds of these low-penetrance cancer susceptibility alleles, with each contributing only a small proportion of the total genetic component of risk [88].

In humans, two types of approaches are used to identify low-penetrance cancer susceptibility alleles: a candidate gene approach and, more recently, genome-wide association (GWA) studies. The latter is performed by genotyping using so-called "tagged" and non-synonymous coding single nucleotide polymorphisms (SNPs) in groups of individuals affected with CRC versus controls. This GWA approach is based on the common disease-common variant theory. After identification of possible cancer susceptibility alleles with this latter approach, the significance of these alleles is determined in well-characterized patient cohorts with different ethnicities. In the end, it remains to be determined if identified alleles can be helpful in predicting the risk of CRC [85].

Carefully designed studies with sufficient statistical power may identify possible low-penetrance cancer susceptibility alleles in unexplained familial CRC cases. In these studies, enrolled patients need to be well characterized together with affected relatives and controls and stratified by ethnicity, gender, and tumor localization. Furthermore, relevant dietary and lifestyle habits should be taken into account.

Two meta-analyses of published data on the candidate gene approach were described in 2002, and a summary of genes with significant associations are shown in Table 1 [3,89].

Table 1. CRC susceptibility alleles and common variants described in the literature.

Candidate gene approach	**SNP**	**Chromosome**	**Gene**	**Reference**
gene involved in the folate pathway		1p36.3	*MTHFR**	[3,89]
gene involved in the proton pump inhibitor pathway	rs1801725/rs1042636/rs1801726	3q13	*CASR*	[90]
gene involved in the Wnt pathway	rs1801155	5q21-q22	*APC*	[89]
gene involved in metabolic pathways		8p22	*NAT2* (phenotype)	[3]
gene involved in the Wnt pathway	rs7903146	10q25.3	*TCF7L2*	[91]
oncogene		11p15.5	*HRAS1*	[3,89]
gene involved in alcohol metabolism		12q24.2	*ALDH2*	[3]
tumor suppressor gene		17p13.1	*TP53* (intron 3) *	[3]
gene involved in metabolic pathways		22q11.23	*GSTT1*	[3]
Association approach	SNP	Chromosome	Gene	Reference
	rs16892766	8q23.3	*eIF3f*	[95]
	rs6983267	8q24		[92,93,94,95]
	rs10505477	8q24		[96,97]
	rs719725	9p24		[96]
	rs1075668	10p14		[95]
	rs3802842	11q23.1		[94]
	rs4779584	15q13.3	*CRAC1(HMPS)*	[100]
gene involved in the TGFB pathway	rs4939827/rs12953717/ rs4464148	18q21	*SMAD7*	[94,95,99]

* decreased risk

One study of candidate genes published after 2002 reported the association between three *CASR* gene variants and the risk for colorectal adenoma [90], and a second study reported that the T allele of rs7903146 in *TCF7L2* gives an increased risk of CRC [91]. GWA studies identified a CRC susceptibility allele (rs6983267) on chromosome 8q24 [92-95]. In a case-unaffected sibling analysis, the risk estimate for the associations between this SNP and CRC was modest; however, the high frequency suggests that it is an important

cancer susceptibility allele. In this study, rs10505477 in 8q24 was also significantly associated with CRC [96]. Gruber *et al*. performed a population-based case-control study of CRC in northern Israel and found that rs10505477 potentially accounts for 14% of the analyzed CRC cases [97]. Li *et al*. also confirmed the association identified between rs6983267 on chromosome 8q24 and CRC in a population-based case-control study [98]. Tomlinson *et al*. performed a GWA study and identified association of rs10795668 located at 10p14 and rs16892766 at 8q23.3 [95]. In other studies, common alleles in a known gene were determined to be associated with CRC. The association between rs4939827 of *SMAD7* and CRC was reported to be highly significant in GWA studies [94,95,99]. Two additional *SMAD7* alleles, rs12953717 and rs4464148, also displayed association [99]. Jeager *et al*. used a different approach; they mapped a high-penetrance gene (*CRAC1*) associated with CRC [100] and searched for a low-penetrance variant in this gene. Rs4779584 turned out to be strongly associated with increased CRC risk [100], and these results were confirmed in a GWA study [95]. The CRC susceptibility alleles identified with GWA studies and potential associated genes are summarized in Table 1.

Environmental factors

It has been proposed that environmental factors characterized by a Western lifestyle are closely related to the risk of CRC [101,102]. In this introduction, we subdivide lifestyle into four categories: alcohol consumption, smoking, diet, and obesity. Although the last two categories show some overlap, the mechanisms that might lead to cancer in obese patients are different from those that lead to CRC due to a moderate but unhealthy diet.

Alcohol

High alcohol consumption has been weakly related to an increased CRC risk [101]. Kim *et al*. reviewed several studies; a meta-analysis of five cohort studies and 22 case-control studies published from 1996 to 1989 showed a weak positive association [103]. A second analysis, which combined eight prospective cohort studies from Western countries, reported a 16% increase in the risk of CRC among people consuming at least 30 g (4 units) of alcohol per day [104]. The total ethanol intake, irrespective of the type of drink, is likely to be related to the association between alcohol consumption and CRC risk [105]. The underlying mechanism of the association might be explained by the role of alcohol in the folate pathway. Alcohol functions as a folate antagonist, thereby weakening folate absorption, increasing folate excretion, and decreasing its hepatic uptake [106,107].

Smoking

The associations between smoking and colorectal carcinomas turned out to be inconsistent. Nevertheless, long-term heavy smoking increases the risk for colorectal adenomas by two- to threefold [108]. Furthermore, Ji *et al*. observed a stronger association between current smoking and hyperplastic polyps than with adenomatous polyps [109]. The association between smoking and colorectal tumors is expected to be linked to different genotoxic compounds that are formed by the burning of tobacco products. These compounds include carcinogenic polycyclic aromatic hydrocarbons (PAH), aromatic amines, and N-nitrosamines. N-nitrosamines are known to induce G:C>A:T transitions. Benzo[a]pyrene (B[a]P), a PAH indicator, was found to induce G:C>T:A transversions [110]. Interestingly, microsatellite unstable (MSI-high) carcinomas are elevated in smokers [111]. Cigarette smoking also appears to increase the risk of Lynch syndrome-associated colorectal tumors [112].

Diet

A higher intake of red meat, possibly in association with high temperature cooking, has been suggested to increase the risk for CRC [113,114]. On the other hand, higher intakes of vegetables, particularly raw and green vegetables, have been associated with a reduced risk of CRC [101,115,116]. Such reduced risk of CRC is suggested to be related to the folate pathway. Folate is one of the main micronutrients in vegetables and appears to be of great importance in the synthesis and regeneration of S-adenosylmethionine (SAM), which is an important methyl donor for DNA synthesis. Although published information on the exact effect of folate deficiency on DNA methylation is inconsistent, DNA methylation is an important epigenetic determinant in gene expression and the maintenance of DNA integrity and stability. As mentioned before in this introduction, dysregulation and aberrant patterns of DNA methylation are involved in colorectal carcinogenesis [117,118]. Another hypothesis for this reduced risk of CRC is the anti-inflammatory and anti-neoplastic properties of salicylic acid found in a wide range of fruit, vegetables, herbs, and spices [119]. Of note is that patients treated with aspirin, a non-steroidal anti-inflammatory drug (NSAID), the principal metabolite of which is salicylic acid, seem to have a lower risk of CRC [120].

Obesity

The ratio of energy intake to energy expenditure must be in balance to maintain a healthy body weight. A positive energy balance leads to weight gain, and a person with a body mass index (BMI) of 30 kg/m^2 or more is classified as obese [International Obesity Taskforce. http://www.iotf.org, accessed 2005].

Diverse epidemiological studies have consistently demonstrated a positive relationship between increased body size (energy balance) and colorectal malignancy,

as reviewed in 2006 by Gunter *et al*. [121]. Different mechanisms are proposed to link energy balance and CRC. Biomarkers of these mechanisms are growth factors (IGF-1, IGFBP-3), insulin resistance (insulin, d-peptide, HbA1c), chronic inflammation (IL-6, CRP, TNF-alpha), and steroid hormones (estrogen, progesterone, SHBG). The relationship between these mechanisms and potential body-size susceptibility loci may in the future give insight into mechanisms underlying the pathogenesis of obesity. Physical activity compensates for an excess of energy intake and acts to maintain energy balance. An inverse relationship between physical activity and CRC risk has been demonstrated in the literature [122].

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CHAPTER 2

Microsatellite instability, immunohistochemistry, and additional PMS2 staining in suspected hereditary nonpolyposis colorectal cancer

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Microsatellite Instability, Immunohistochemistry, and Additional PMS2 Staining in Suspected Hereditary Nonpolyposis Colorectal Cancer

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ABSTRACT

Purpose: **Immunohistochemistry (IHC) and microsatellite instability (MSI) analysis can be used to identify patients with a possible DNA mismatch repair defect [hereditary nonpolyposis colorectal carcinoma (HNPCC)]. The Bethesda criteria have been proposed to select families for determination of MSI. The aims of this study were to assess the yield of MSI analysis in families suspected for HNPCC, to compare the results of immunohistochemical staining and MSI analysis, and to assess the additional value of PMS2 staining.**

Experimental Design: **Clinical data and tumors were collected from 725 individuals from 631 families with suspected HNPCC. MSI analysis was performed using eight markers including the 5 National Cancer Institute markers. Four immunohistochemical staining antibodies were used (MLH1, MSH2, MSH6 and PMS2).**

Results: **A MSI-H (tumors with instability for >30% of the markers) phenotype in colorectal cancers (CRCs) was observed in 21–49% of families that met the various Bethesda criteria. In families with three cases of CRC diagnosed at age > 50 years, families with a solitary case of CRC diagnosed between ages 45 and 50 years, and families with one CRC case and a first-degree relative with a HNPCC-related cancer, one diagnosed between ages 45 and 50 years (all Bethesda-negative families), the yield of MSI-H was 10–26%. Immunohistochemical staining confirmed the MSI results in 93% of the cases. With IHC, adding PMS2 staining led to the identification of an additional 23% of subjects with an *hMLH1* germ-line mutation (35 carriers were tested).**

Conclusions: **The Bethesda guidelines for MSI analysis should include families with three or more cases of CRC diagnosed at age > 50 years. The age at diagnosis of CRC in the original guidelines should be raised to 50 years. Routine IHC diagnostics for HNPCC should include PMS2 staining.**

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of death due to malignancy in the Western world. The cause of CRC is multifactorial, involving genetic and environmental factors (1). The most common hereditary colorectal carcinoma syndrome is hereditary nonpolyposis colorectal carcinoma (HNPCC), which accounts for 1–6% of all CRC cases (2). HNPCC is an autosomal dominant disease characterized by an increased risk of early-onset CRC and other cancers, including tumors of the endometrium, stomach, small intestine, hepatobiliary system, kidney, ureter, brain, and ovary (3–6). In up to 15–25% of all cases of CRC, clustering of this type of tumor is observed in the family (7). The role of environmental or genetic factors in these cases is largely unknown.

The increased risk for malignancy in HNPCC is caused by a mutation in one of the following DNA mismatch repair (MMR) genes: *MLH1*; *MSH2*; *MSH6*; *PMS1*; and *PMS2* (8–10). Germ-line mutations of *MLH1* and *MSH2* account for >90% of all known MMR mutations in HNPCC (11), and germ-line mutations of *MSH6* account for 5–10% of all known MMR mutations in HNPCC, whereas mutations of other genes are rare (10, 12).

Mutations in DNA MMR genes result in a failure to repair errors in repetitive sequences that occur during DNA replication. This failure leads to microsatellite instability (MSI) of the tumor, which is the hallmark of HNPCC (13–16).

Due to the heterogeneity of the mutation spectrum of the MMR genes, screening for mutations is both time-consuming and expensive. In addition to family history, MSI analysis and immunohistochemistry (IHC) can both be used to identify families eligible for mutation analysis of the MMR genes (2, 17). In 1997, the Bethesda criteria were proposed to select families for MSI testing. In the present study, we evaluated the yield of MSI analysis in families categorized according to these criteria. We also evaluated MSI in other subsets of families that do not meet these criteria.

Previous studies from numerous groups, including ours

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(Refs. 18, 19 and the references herein) have shown that immunohistochemical analysis using antibodies against the MLH1, MSH2, and MSH6 proteins is another sensitive method to identify carriers of MMR gene mutations. Because the PMS2 protein forms a heterodimer with the MLH1 protein, it might be expected that absence of the MLH1 protein due to a germ-line mutation also leads to loss of the PMS2 protein caused by abrogation of the total protein complex (20). In the present study, we compared the results of immunohistochemical staining with the outcome of MSI analysis and evaluated the additional value of IHC using PMS2 staining.

PATIENTS AND METHODS

Patients. We used the database of colorectal tumors from the unit molecular diagnostics of the pathology department from the Leiden University Medical Center, the Netherlands. This database contains colorectal tumors ($n = 771$) sent to our department for MSI analysis from different medical genetic centers and laboratories in the Netherlands between November 1999 and December 2002. For 46 patients, it was impossible to perform MSI analysis because the obtained formalin-fixed, paraffin-embedded material was not sufficient, or MSI analysis was redundant due to the fact that the segregating mutation was already known in the family. This resulted in MSI analysis of 725 tumors from individuals with CRC of 631 families with clustering of CRC or with a solitary patient with CRC at a young age. Retrospectively, we scored the available complete pedigrees (528 pedigrees were enclosed with the request for MSI analysis from the medical genetic centers; from 103 subjects, only a fragmentary pedigree was sent) according to the Bethesda and additional criteria (Table 1), and we performed immunohistochemical staining (MLH1, MSH2, and, subsequently, MSH6 and PMS2). Finally, we had 528 tumors with complete information from the pedigree and MSI analysis, 330 tumors with results on MLH1 and PMS2 staining (including 35 tumors from patients with a *hMLH1* mutation), and 284 tumors with interpretable results on both MSI and IHC (four proteins). The reason for the major decrease in the number of patients was that in this retrospective series, not all samples were still available for additional staining. From 84 families, we had tumor material of at least two relatives.

DNA Isolation. Genomic DNA of normal and tumor tissue was isolated from the paraffin-embedded material by taking tissue punches (diameter, 0.6 mm) with a tissue microarrayer (Beecher) from tumor and normal areas selected on the basis of a HE-stained slide. Using the Chelex extraction method, DNA was isolated from three punches, resuspended in 96 μl of PK-1 lysis buffer [50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20, and 0.1 mg/ml gelatin] containing 5% Chelex beads (Bio-Rad, Hercules, CA) and 5 μl of proteinase K (10 mg/ml), and incubated for 12 h at 56°C. The suspension was incubated at 100°C for 10 min and centrifuged at 13,000 rpm for 10 min, and the supernatant containing the DNA was carefully transferred to a new tube.

MSI Analysis. Eight microsatellite markers were evaluated [two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, and D17S250) recommended by the National Cancer Institute Workshop on MSI for Cancer Detection and Familial Predisposition (13), supplemented by three mononucleotide repeat markers (BAT40, MSH3, and MSH6)]. BAT40 is a very informative marker. The choice for MSH3 and MSH6 was initially for research purposes. Tumors were classified as (*a*) tumors with instability for >30% of the markers (MSI-H), (*b*) tumors with instability for <30% of the markers (MSI-L), and (*c*) tumors with no instability [microsatellite stability (MSS)]. We distinquished between MSI-L with instability of only a dinucleotide marker (MSI-Ld) and instability of only a mononucleotide marker (MSI-Lm).

IHC. Staining of MMR proteins was performed with anti-MLH1 (initially with clone 14; 1:75; Calbiogen, Cambridge USA, later supplemented and substituted by clone G168-728; 1:50; BD Biosciences, NJ), anti-PMS2 (clone A16-4; 1:50; BD Biosciences), anti-MSH2 (clone GB-12; 1:100; Oncogene Research Products, San Diego, CA), and anti-MSH6 (clone 44; 1:400; BD Biosciences). Immunohistochemical staining was performed on 4-μm-thick, formalin-fixed, paraffin-embedded tissue sections that were prepared on DAKO slides and dried overnight at 37°C. Next, tissue sections were deparaffinized three times in xylene for a total of 15 min and subsequently rehydrated. Antigen retrieval was done by boiling in 10 mM citrate buffer [pH 6.0 (MSH6 and MLH1), clone G168-728] or in 1 mM EDTA (MLH1, clone 14, PMS2 and MSH2) for 10 min using a microwave oven, after which the sections were cooled in this buffer for at least 1 h at room temperature. After rinsing in demiwater, the tissue sections were stained in a DAKO Techmate 500+ automated tissue stainer using the DAKO ChemMate System Kit Peroxidase/DAB K5011 (DAKO, Glostrup, Denmark). Briefly, in this system, slides were incubated with the primary antibody diluted in ChemMate Antibody diluent (DakoCytomation, Glostrup, Denmark) for 8 h at room temperature. Sections were automatically washed and incubated with ready-to-use biotinylated secondary antibody for 30 min and washed. Endogenous peroxidase was then blocked in peroxidase

Table 1 Explanation of used criteria

Bethesda	Criteria	
Positive	1	Fulfilling the Amsterdam II criteria
	2	Solitary patient with CRC[a] and a HNPCC-related cancer
	3	Patient with CRC and a FDR with a HNPCC-related cancer, one of the cancers diagnosed age < 45 yrs
	4	Solitary patient with CRC diagnosed at age < 45 yrs
Negative	5	Solitary patient with CRC diagnosed at age 45–50 yrs
	6	Patient with CRC and a FDR with a HNPCC-related cancer, one of the cancers diagnosed at age 45–50 yrs
	7	Late-onset family: patient with CRC and two FDRs with a HNPCC-related cancer, both cancers diagnosed at age > 50 yrs
	8	Patient with CRC and a FDR with a HNPCC-related cancer, both cancers diagnosed at age > 50 yrs

[a] CRC, colorectal cancer; HNPCC, hereditary nonpolyposis colorectal carcinoma; FDR, first-degree relative.

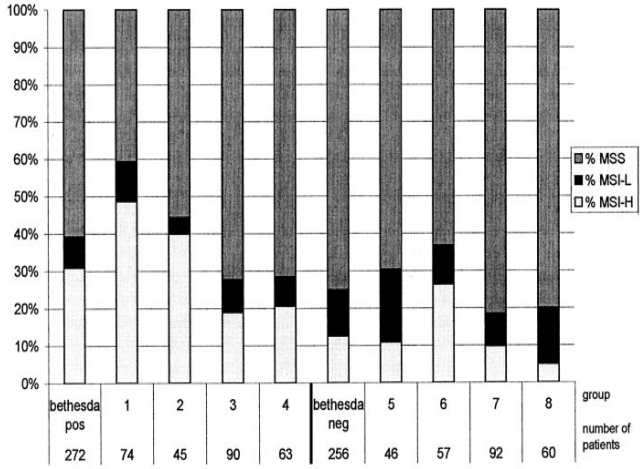

Fig. 1 Yield of MSI in several types of families (n = 528). See Table 1 for groups 1–8.

blocking solution for 7.5 min and washed and incubated with ready-to-use streptavidin-conjugated with peroxidase for 30 min. Sections were washed and developed with two-component hydrogen peroxide/diaminobenzidine for 15 min. The sections were then counterstained with hematoxylin for TechMate, dehydrated, cleared in xylene, and mounted with micromount. Microscopic analysis was done by a pathologist (H. M.). Tissue stroma and normal epithelium or lymph follicles served as positive internal controls when analyzing MLH1, PMS2, MSH2, and MSH6 expression. Expression of MLH1, PMS2, MSH2, and MSH6 was scored as positive (+), negative with a positive internal control (0/+), and doubtfully negative [when both tumor and internal control stain negative (0/0)], and when the internal control was stronger than the positive tumor cells, it was scored as +/++.

RESULTS

Yield of MSI in Subjects from Bethesda-Positive and -Negative Families. In the families that met the Bethesda criteria (Bethesda-positive group; n = 272), 84 tumors (31%) were MSI-H, 23 tumors (8%) were MSI-L, and 165 tumors (61%) showed MSS (Table 1; Fig. 1). In tumors from subjects from an Amsterdam-positive family (n = 74), the yield of MSI-H was 49%; for Bethesda 2 (n = 45), it was 40%; for Bethesda 3 (n = 90), the yield was 19%; and for Bethesda 4 (n = 63), it was 21%. The proportion of MSI-L tumors in these four groups was 11%, 4%, 9%, and 8%, respectively.

In the families that did not meet the criteria (Bethesda-negative group; n = 256), 32 colorectal tumors (12.5%) were MSI-H, 32 tumors (12.5%) were MSI-L, and 192 tumors (75%) showed MSS. We subdivided the Bethesda-negative families into four subgroups (Table 1, criteria 5–8). In Fig. 1, the yield of MSI for the different subgroups is shown.

IHC and MSI. Data on MSI analysis as well as immunohistochemical staining (four proteins) were available for 284 tumors. Among these 284 tumors (Table 2), 91 tumors showed MSI-H as well as abnormal staining, and 136 tumors showed MSS and normal protein expression, leading to concordant results in 93% (227 of 245) of the MSI-H and microsatellite stable tumors. In view of the remarks by Perucho (21) on the marker sets used for MSI, we subdivided MSI-L in MSI-Lm (instability of only a mononucleotide marker) and MSI-Ld (instability of only a dinucleotide marker). Although the number of tumors is small, we found a difference between both groups: 35% of tumors in the MSI-Lm group and 13% of tumors in the MSI-Ld group showed absence of at least one MMR protein (Table 2).

Subsequently, we evaluated the results of IHC in the Bethesda-negative groups (Table 3).

PMS2 Staining. To evaluate the additional value of PMS2 staining, we compared the results of MLH1 and PMS2 staining in 330 tumors (see "Patients and Methods"). Among these, 35 tumors were from *hMLH1* mutation carriers (25 tumors had been described previously, without staining for PMS2; Ref. 18). Thirty tumors were from subjects in whom a *hMLH1* mutation could not be detected, and 265 tumors were from subjects with an unknown mutation status of *hMLH1* (from these 265 tumors, 7 tumors were from carriers of a *hMSH2* germ-line mutation, and 10 were from subjects with a *hMSH6* germ-line mutation; Table 4). In Fig. 2, three staining examples are shown. In 292 tumors (88%), both stainings gave the same results. If MLH1 stained negative with a positive internal con-

Table 2 Comparing MSI[a] and IHC staining results

MSI	Normal expression	≥1 MMR protein absent	Concordance
MSI-H	12	91	88%
MSS	136	6	96%
MSI-Lm	20	11	
MSI-Ld	7	1	

[a] MSI, microsatellite instability; IHC, immunohistochemistry; MMR, mismatch repair; MSS, microsatellite stability; MSI-Lm, MSI-L with instability of only a mononucleotide marker; MSI-Ld, MSI-L with instability of only a dinucleotide marker.

Table 3 MSI[a] and IHC results of the Bethesda-negative groups

See Table 1 for explanation of the groups.

	Intact expression of all 4 proteins				Absent expression ≥ 1 protein			
	Group 5	Group 6	Group 7	Group 8	Group 5	Group 6	Group 7	Group 8
Total no. of cases	25	22	46	22	6	9	8	1
No. of MSS	22	20	45	20	0	0	0	0
No. of MSI-L	3	1	1	0	1[b]	1[c]	0	0
No. of MSI-H	0	1	0	2	5[d]	8[e]	8[f]	1[g]

[a] MSI, microsatellite instability; IHC, immunohistochemistry; MSS, microsatellite stability.
[b] Abrogation of MSH6.
[c] Abrogation of MSH6.
[d] Abrogation of MLH1, or PMS2, or MLH1/PMS2 (2 cases), or MSH2/MSH6.
[e] Abrogation of MLH1/MSH6, PMS2, MSH2, or MSH2/MSH6, or MSH6 (4 cases).
[f] Abrogation of MLH1/PMS2 (2 cases), or PMS2, or MSH2, or MSH6 (2 cases), MSH2/MSH6, or MSH2/MSH6/PMS2.
[g] Abrogation of MLH1/PMS2.

trol (0/+), PMS2 also stained 0/+ in 39 cases (93%). If MLH1 stained positive, PMS2 stained positive in 252 (92%) cases. Among these 252 cases is 1 carrier of a *hMLH1* mutation (unclassified variant; Table 5, case 8). In 19 tumors 7% (all MSI-H tumors), there was 0/+ staining of PMS2, whereas staining of MLH1 was positive (+, 16 tumors; +/++, 3 tumors). In the latter scoring (+/++), the internal control clearly stained more positive than the tumor nuclei (Fig. 2). Among these 19 patients were 8 *hMLH1* germ-line mutation carriers (Tables 4 and 5; cases 18, 19, and 26–31). In three cases, a possible *PMS2* defect was present, and in eight cases, the mutation status of *hMLH1* was not yet determined. Two tumors showed 0/+ MLH1 staining and positive staining for PMS2. One subject is a carrier of a *hMLH1* mutation (case 14, Table 5) and the mutation status of the second subject, although tested, is still unknown (case 1, Table 5). In 14 cases, the MLH1 staining was not interpretable (0/0) because of the absence of staining of normal tissue, whereas the PMS2 staining was interpretable in 13 of these 14 cases. In one case, both stainings were not interpretable (0.3%). Therefore, overall, PMS2 staining gave additional value in 32 of 330 tumors (10%).

When only evaluating the 35 tumors of *hMLH1* mutation carriers, 23 tumors showed absence of at least MLH1. In only 17 tumors (49%) was an MLH1-negative staining accompanied by normal MSH2 and MSH6 staining patterns. Eight of the 35 tumors showed absence of only PMS2 (all other three proteins showed positive expression). Therefore, an additional 23% of the subjects with an *hMLH1* germ-line mutation were identified solely with IHC.

Table 4 PMS2 and MLH1 staining

	PMS2			
hMLH1	0/+[a]	+	0/0	Total
hMLH1 mutation				
0/+[a]	21	1	1	23
+	5	1		6
+/++	3			3
0/0	3			3
No *hMLH1* mutation				
0/+	5			5
+	3	18	1	22
0/0		3		3
Mutation status unknown				
0/+	13	1		14
+	8	233	2	243
0/0	2	5	1	8
Total	63	262	5	330

[a] Staining results: +, nuclear staining; +/++, internal control is stronger than the positive tumor cells; 0/+, negative with a positive internal control; 0/0, tumor and internal control stain negative.

Disconcordant Results. When we evaluated all results in the whole database, there were 31 cases with remarkable combinations of results of the (pre-)screening tests (Table 5). Six cases (cases 2–7) are patients with microsatellite stable tumors in combination with abnormal IHC (see also Table 2). Cases 7–12 are patients with a germ-line MMR variant (two of them are considered to be true pathogenic mutations, and four are unclassified variants), but without evidence of instability. Cases 13–21 are patients, all with MSI-H tumors, but with an uncommon combination of absence of proteins. Then we noticed a group of patients (cases 15 and 21–25) without a mutation of *hMLH1, hMSH2,* or *hMSH6*, but with a MSI-H or MSI-L tumor and the absence of one or more proteins. The last group (cases 18, 19, and 26–31) has already been described in this article (see also Table 4).

Interfamilial Variety. We identified 84 families in which we assessed MSI in tumors from at least two relatives. We evaluated the phenotype in these tumors. In 69 families, the first tumor was microsatellite stable. The second tumor in these families showed MSI-H in 13 tumors (19%) and MSI-L in 12 tumors (17%). We evaluated whether we could find an explanation by evaluating the pedigree. We did not find a difference in family type between families with two microsatellite stable tumors and families with one microsatellite stable and one MSI-H or MSI-L tumor. Overall, in the 69 families in which the first tumor showed MSS, 24 tumors were located in the rectum. In the 25 families (mutation unknown) with two affected relatives tested, one relative with a microsatellite stable tumor and one with a MSI-H or MSI-L tumor (in total, 50 tumors), eight of the tumors first tested were located in the rectum.

DISCUSSION

Identification of families with HNPCC is extremely important because it makes it possible to target effective preventive measures that lead to a substantial reduction in CRC-related

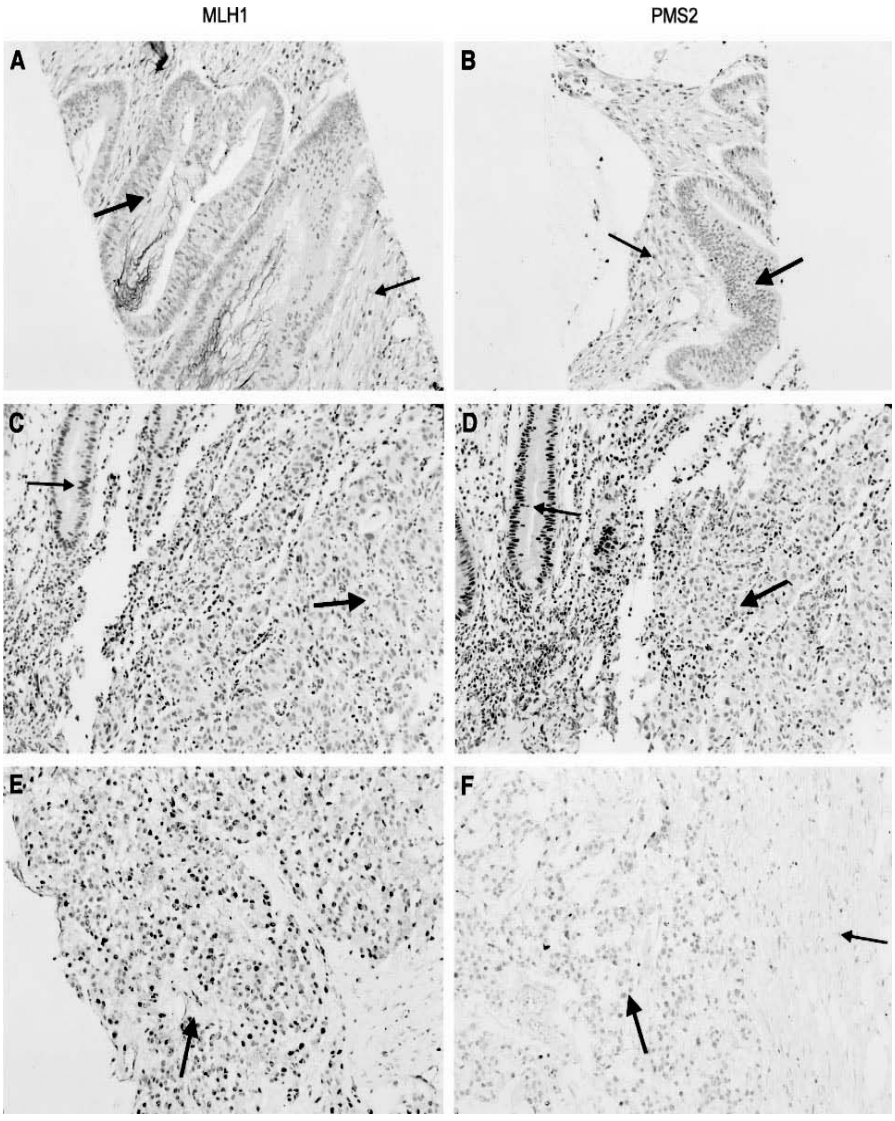

Fig. 2 Immunostaining with antibodies against MLH1 and PMS2. *Thick arrows* indicate tumor cells. *Thin arrows* indicate internal control cells, either stromal or epithelial. *A* and *B,* well-differentiated MSI-H colon carcinoma (*hMLH1* germ-line mutation, exon 16 delK618) with negative MLH1 and PMS2 staining in tumor nuclei, with retained staining of stromal cells. *C* and *D,* poorly differentiated MSI-H colon carcinoma (*hMLH1* germ-line mutation, exon 5 Q149X, 445C>T) with positive MLH1 staining but negative PMS2 staining in tumor nuclei, although there is stronger positivity for MLH1 in normal crypt cells than in tumor cells. *E* and *F,* poorly differentiated MSI-H colon carcinoma (*hMLH1* germ-line mutation exon 1, G6fsX25, 18_34del17) with retained MLH1, MSH2, and MSH6 staining but abrogated PMS2 staining.

mortality (22). In the present study, we evaluated the value of MSI analysis and immunohistochemical staining for the identification of HNPCC in a large series of families. A significant proportion of MSI-H tumors were detected not only in families that complied with the Bethesda criteria but also in families that met other specific criteria. In addition, we found that immunohistochemical staining (including staining for PMS2) and MSI analysis gave concordant results in 93% of the cases. IHC alone, including PMS2 staining, led to the identification of an additional 23% of subjects with an *hMLH1* germ-line mutation.

Several years ago, the Bethesda guidelines were developed for selection of families whose tumors should be tested for MSI. In the present study, we examined the validity of these criteria in relation to MSI status. Another important aim was to assess

Table 5 Special cases (see Table 1 for family diagnosis)

Case	Sex	Age at diagnosis (yrs)	Family type	Site of tumor	MSI[a]	IHC[b] MLH1	IIHC MSH2	IHC MSH6	IHC PMS2	Mutated gene[c]	Exon	Amino acid change	Nucleotide change
1	M	65	7	Rectum	Ld	0/+	+	+	+	?			
2	F	39	4	Sigmoid	S	+	+	0/+	+	?			
3	F	41	4	Cecum	S	+	+	0/+	+	no 6			
4	F	70	3	Colon	S	+	+	0/+	na	no 1/2/6			
5	M	40	4	Right colon	S	+	0/+	0/+	+	?			
6	M	51	7	Left colon	S	+	0/+	na	+	?			
7	M	56	1	Cecum	S	0/+	+	+	0/+	*MLH1*	8	R226Q	677G>A (splice donor)
8	M	42	4	Sigmoid	S	+	na	na	+	*MLH1*, UV		DelIVS13	500 bpdeletion
9	F	51	5	Left colon	S	na	na	na	na	*MSH2*, UV	15	S860L	2579C>T
10	M	65	5	Colon	S	+	+	+	+	*MSH6*, UV	4	S503C	1508C>G
11	M	34	4	Ascendens	S	na	na	na	na	*MSH6*, UV	5	T1102T	3306T>A
12	M	45		Right colon	S	na	na	na	na	*MSH6*	4	V907fsX	2719_2720delGT
13	M	71	1	Ascendens	H	0/+	+	0/+	na	(*MLH1*)	11	Q301X	901C>T
14	M	46		Cecum	H	0/+	+	0/+	+	*MLH1*	16	K618del	1852–1854del
15	M	40	4	Flexura lienalis	H	0/+	+	0/+	0/+	no 1/2/6			
16	F	47	3	Flexura hepatica	H	0/+	+	0/+	0/+	?			
17	M	74		Cecum	H	+	+	0/+	0/+	?			
18	F	38		Colon	H	+	+	0/+	0/+	*MLH1*, UV	10	R264C	
19	M	39	1	Cecum	H	+	+	0/+	0/+	*MLH1*, UV	10	R264C	
20	M	42		Flexura lienalis	H	0/0	+	0/0	0/+	(*MSH6*, UV)	IVS 9		3969_4002+51dup
21	M	74	7	Left colon	H	+	0/+	0/+	0/+	no 1/2/6			
22	M		7	Left colon	H	+	0/+	na	+	no 1/2/6			
23	M	53	1	Cecum	H	+	0/+	na	na	no 1/2/6			
24	F	49		Right colon	H	+	0/+	na	+	no 1/2/6			
25	M	35	4	Transversum	Lm	+	0/+	0/+	+	no 1/2/6			
26	M	39	1	Ascendens	H	+	+	0/+	0/+	*MLH1*	1	G6fsX25	18_34del17
27	M	39	1	Transversum	H	+	+	+	0/+	*MLH1*	16	K618del	1852–1854del
28	F	90	7	Transversum	H	+	+	+	0/+	*MLH1*, UV	3	S93G	277A>C
29	M	34	4	Cecum	H	+/++	+	na	0/+	*MLH1*, UV	2	S44F	131C>T
30	M	46	1	Colon	H	+/++	na	na	0/+	*MLH1*	5	Q149X	445C>T
31	F	43	1	Cecum	H	+/++	+	+	0/+	*MLH1*	5	Q149X	445C>T

[a] MSI, microsatellite instability; H, MSI-H; Lm, MSI-L with instability of only a mononucleotide marker; Ld, MSI-L with instability of only a dinucleotide marker; S, stability; MSS, microsatellite stability.

[b] IHC (immunohistochemistry). 0/0, tumor cell, no nuclear staining, internal control also absent; 0/+, no nuclear staining; +, nuclear staining; +/++, internal control more positive than tumor; na, not analyzed.

[c] Mutated gene: no 1/2/6, no mutation found in *hMLH1*/*hMSH2* or *hMSH6*; no 6, no mutation found in *hMSH6*; ?, mutation status not fully tested; (*MLH1*), relative is known with a *MLH1* mutation, in this case not tested; UV, unclassified variant.

whether other criteria should be added to identify more families with MSI-positive tumors.

The yield of MSI-H in our series of families that met the different Bethesda criteria varied from 19% to 49%. This is in agreement with the results of previous studies on the yield of MSI in such families (23, 24).

The families in our series who did not meet the Bethesda criteria comprised families with one CRC diagnosed between age 45 and 50 years (category 5); families with one case of CRC and a first-degree relative with a HNPCC-related cancer, one diagnosed between age 45 and 50 years (category 6); families with three or more CRC cases diagnosed at age > 50 years (category 7); and families with one CRC and a first-degree relative with a HNPCC-related cancer, both diagnosed at age > 50 years (category 8). In categories 5, 7, and 8, the yield of MSI-H tumors was relatively low. In categories 5 and 7, all MSI-H tumors showed absence of at least one protein (see earlier). It is remarkable that only 4 of 13 tumors in these two categories might be explained by MLH1 abrogation due to promoter methylation. Theoretically, hypermethylation of the other MMR genes is possible, but not yet known. In the families of category 6, the yield of MSI-H tumors was 26%, which is higher than the percentage of MSI-H tumors (~10%) reported for sporadic CRC (25), often due to MLH1 promoter methylation (16, 26). Based on these results, we suggest the extension of the Bethesda criteria with criteria that can identify these types of families (groups 5–7).

A few studies have shown that immunohistochemical staining of tumors using antibodies against the MMR proteins is a sensitive method to identify families eligible for mutation analysis (27–30). Most studies reported so far used antibodies against MLH1, MSH2, and MSH6. Rigau *et al.* (19) also included PMS2 antibodies. Because the PMS2 protein forms a heterodimer with the MLH1 protein, absence of the MLH1 protein due to a mutation also leads to loss of the PMS2 protein caused by abrogation of the total protein complex (20). Absence of PMS2 staining might therefore suggest the presence of a *hMLH1* or *PMS2* germline mutation or somatic abrogation of *hMLH1*.

When we compare the results of MLH1 and PMS2 staining, concordant results were observed in 88% of the cases. In the 35 tumors associated with a known *hMLH1* mutation, absence

of both MLH1 and PMS2 staining was observed in 21 tumors. In eight other tumors, staining for PMS2 was negative, whereas staining for MLH1 was positive. This finding means that by using staining for PMS2, significantly more *hMLH1* mutation carriers would have been identified. Rigau *et al.* (19) observed four cases with isolated loss of PMS2, and all were microsatellite stable. Rigau *et al.* (19) concluded that there is no need to include PMS2 in the panel of antibodies to be used when looking for MMR-deficient cases by IHC. The majority of their MSI-H tumors, however, most likely consisted of tumors with sporadic abrogation of MLH1, in which PMS2 staining is indeed not necessary. In our studied cases, the type of underlying mutation (missense mutation, in-frame deletion, or unclassified variant) may explain why the MLH1 protein was still intact in the nucleus, whereas the binding of PMS2 was abrogated (*e.g.,* due to conformational changes). Another possibility is that, in the case of an unclassified *MLH1* variant, an unidentified pathogenic mutation in *PMS2* is responsible. We also do not know what exactly happened with the second *MLH1* allele in these tumors, which potentially might influence the staining results. Finally, technical problems with the MLH1 staining in individual cases and perhaps also the type of the MLH1 antibody used might play a role (31, 32). An illustration of the arguments above follows: seven cases in our database with an identical *MLH1* mutation (K618del) were tested (18). Only one of these (case 27, Table 5) was concluded to have retained nuclear MLH1 staining in tumor cells, but with loss of PMS2 staining. We identified three tumors (Table 5), all from *hMLH1* carriers, that stained +/++ for MLH1. In the literature, it is known that in individual cases, abnormally high sensitivity of the IHC can account for false positive interpretation (31). Whatever the explanation, the latter three cases illustrate the additional value of adding the +/++ score to the traditional scoring scheme.

Overall, we found that immunohistochemical staining using four antibodies confirmed the results of MSI analysis in 93% of the cases. This is nearly identical to that reported in the recent literature in studies using only three antibodies [hMLH1, hMSH2, and hMSH6 (19)] or even two antibodies [hMLH1 and hMSH2 (30)]. This discordance might be explained by the consecutive case series used in the study of Rigau *et al.* (19). The majority of their MSI-H tumors (very few HNPCC cases) are most likely due to methylation of hMLH1. The concordance between MSI-H and loss of MLH1 expression in the sporadic cases will be 100%, as expected. In the study of Lindor *et al.* (30), the concordance in the consecutive case series was indeed 100%. In the other three series, included in the same study, all from centers from a Cooperative Family Registry for Colon Cancer Studies, the concordance varied widely, from 84% to 95%. The exact reason for the discordance is unknown (30).

We classified the MSI-L tumors in our series into two groups: tumors with instability of only a mononucleotide marker (MSI-Lm); or tumors with instability of only a dinucleotide marker (MSI-Ld). The MSI-Lm tumors seem more informative for a true MMR deficiency than the MSI-Ld tumors, which seems to be in line with the views of Perucho (21): "The alterations in di-, tri- or tetranucleotide repeats can be also due to spontaneous errors of replication of these highly unstable sequences."

Ninety-five percent of all microsatellite stable tumors showed positive staining for the four MMR proteins, which implies that additional IHC in microsatellite stable tumors is often redundant. However, the value of MSH6 staining in microsatellite stable tumors might although not neglectable (33, 34). In our study, four of six microsatellite stable tumors with abnormal IHC showed an absence of MSH6 (Table 5, cases 2–5), although no mutation has been detected in this set of patients. On the basis of these results and the results of Wahlberg *et al.* (35), we recommend a possible decision scheme for (suspected) HNPCC as suggested previously (18). Rigau *et al.* (19) suggested that MSH6 (and possibly PMS2) can be considered as useful only in second line, when MLH1 and MSH2 show no abnormalities in MSI-H tumors or in suspected HNPCC. At our department, however, the costs for performing two or four stainings at the same time are almost equal, whereas performing them in two sessions is more labor intensive.

We recommend testing a second tumor from another relative in our decision scheme when MSI analysis of a tumor (from a family suspected of HNPCC) shows no evidence of instability because it is possible that we are dealing with a phenocopy within a HNPCC family. In the present series, we analyzed a second colon tumor in 69 families in which the first tumor showed MSS. MSI in the second tumor was found in 36% of the families. Furthermore, on basis of our results and those of others (36), we recommend, if possible, not to test a rectal tumor as first choice.

We noticed several cases in the whole database with discordant results (Table 5). The number of patients (8 patients) with a microsatellite stable tumor with a MMR mutation [five of eight were unclassified variants (in total, 11% of all subjects with a MMR mutation in our database)] falls within the range of about 10% published in the literature (12). In total, there were 70 cases (MSS, MSI-L, or MSI-H) in the whole database in which the search for a mutation in *hMLH1*, *hMSH2,* or *hMSH6* was negative. Five of 70 cases had a MSI-H tumor with absence of one or more proteins. This number (7%) is comparable with that seen in the literature (25).

In sum, on the basis of the present study, we recommend the inclusion of PMS2 staining in the panel of antibodies to identify families eligible for mutation analysis. The addition of PMS2 staining will lead to a marked increase of detection of *hMLH1* mutation carriers. Moreover, we suggest the following revisions to the Bethesda criteria: include late-onset families (three or more cases of CRC diagnosed at age > 50 years) and raise the age at diagnosis of CRC from 45 to 50 years in the original criteria.

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CHAPTER 3

Genome-wide copy neutral LOH is infrequent in familial and sporadic microsatellite unstable carcinomas

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Genome-wide copy neutral LOH is infrequent in familial and sporadic microsatellite unstable carcinomas

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Abstract Mismatch repair deficiency in tumors can result from germ line mutations in one of the mismatch repair (MMR) genes (*MLH1, MSH2, MSH6* and *PMS2*), or from sporadic promoter hypermethylation of *MLH1*. The role of unclassified variants (UVs) in MMR genes is subject to debate. To establish the extend of chromosomal instability and copy neutral loss of heterozygosity (cnLOH), we analyzed 41 archival microsatellite unstable carcinomas, mainly colon cancer, from 23 patients with pathogenic MMR mutations, from eight patients with UVs in one of the MMR genes and 10 cases with *MLH1* promoter hypermethylation. We assessed genome wide copy number abnormalities and cnLOH using SNP arrays. SNP arrays overcome the problems of detecting LOH due to instability of polymorphic microsatellite markers. All carcinomas showed relatively few chromosomal aberrations. Also cnLOH was infrequent and in Lynch syndrome carcinomas usually confined to the locus harbouring pathogenic mutations in *MLH1, MSH2* or *PMS2* In the carcinomas from the MMR-UV carriers such cnLOH was less common and in the carcinomas with *MLH1* promoter hypermethylation no cnLOH at *MLH1* occurred. MSI-H carcinomas of most MMR-UV carriers present on average with more aberrations compared to the carcinomas from pathogenic MMR mutation carriers, suggesting that another possible pathogenic MMR mutation had not been missed. The approach we describe here shows to be an excellent way to study genome-wide cnLOH in archival mismatch repair deficient tumors.

Keywords Lynch syndrome · HNPCC · MSI-H · Chromosomal instability · Copy neutral loss of heterozygosity · Mismatch repair (MMR) genes · Unclassified variants · *MLH1* hypermethylation · SNP array

Abbreviations

CGH	Comparative genomic hybridization
CIN	Chromosomal instability
CNA	Copy number aberrations
cnLOH	Copy neutral loss of heterozygosity
CRC	Colorectal cancer
FFPE	Formalin-fixed paraffin-embedded
GCS	Gene call score
GTS	Gene train score
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
LP	Linkage panels
MMR	Mismatch repair
MSI	Microsatellite instability
MSI-H	Microsatellite instability
MSS	Microsatellite stable
rGCS	Relative gene call score
SRO	Smallest region of overlap
UVs	Unclassified variants

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Introduction

In colorectal cancer (CRC) there are two classical pathways that direct tumorigenesis: microsatellite instability (MSI or MIN) and chromosomal instability (CIN). MSI results from a defective DNA mismatch repair (MMR) system and therefore characterises tumors from patients with Lynch syndrome (previously HNPCC, hereditary nonpolyposis colorectal cancer). In addition 15% of sporadic CRC displays MSI due to *MLH1* promoter hypermethylation [1–3]. Tumor cells with abrogated MMR function accumulate small deletions and insertions in stretches of short repetitive DNA sequences distributed throughout the genome. These mutations lead to frameshifts within coding sequences and thus inactivation of genes, thereby contributing to tumor development and progression [4–6]. MSI carcinomas most often show a diploid or near-diploid genome [7], while up to 73% of sporadic CRC tumors show aneuploidy, the equivalent of a gross amount of CIN [8]. In sporadic microsatellite unstable (MSI-H) carcinomas the most frequent aberrations are gains of chromosome 8, 12 and 13 while chromosomal losses occurred predominantly at 15q14 [9]. In sporadic microsatellite stable (MSS) CRC, CIN is characterized by losses and amplifications of arms of, or complete, chromosomes [10–12]. In general, physical loss of chromosomes 17p and 18q, and gain at 8q, 13q, and 20 occur at early stages during the transition from adenoma to carcinoma, whereas loss of 4p is associated with transition from Dukes' A to B–D. Chromosomal loss of 8p and gain of 7p and 17q is reported to be associated with the transition from primary carcinoma to local and distal metastases. Loss of 14q and gains of 1q, 11, 12p, and 19 are considered late events [13, 14]. Both chromosomes 5 and 17p are more often targeted by copy number neutral LOH than by copy number variations [15, 16].

Clinically, the uncertainty about the contribution of an MMR unclassified variant (MMR-UV) to the risk of developing cancer is a major problem. While carriers of a pathogenic MMR mutation are at increased risk, those with an MMR-UV could also represent rare variants without increased risk of cancer. For pathogenic MMR carriers, clinical geneticists offer pre-symptomatic testing for the detection of neoplasia at an early stage. For patients carrying an MMR-UV with unproven pathogenicity, offering pre-symptomatic testing is difficult.

Since 2001 evidence for differences between sporadic and familial MSI-H carcinomas with respect to both genotype and phenotype is accumulating. [17, 18] To expand this knowledge we determined the possible difference in genomic tumor profiles of patients with pathogenic MMR mutations, MMR-unclassified variants and of sporadic carcinomas with *MLH1* promoter hypermethylation.

Material and methods

Thirty-seven formalin-fixed paraffin-embedded (FFPE) microsatellite unstable (MSI-H) colorectal tumors from 37 patients selected from the pathology archives were included in our study. Corresponding histological normal tissue from 30 of these patients and leukocyte DNA for seven patients was available. In addition, four FFPE endometrial carcinomas with corresponding normal DNA were analyzed. Thirty-one of these samples originated from patients with familial MMR deficiency; the following mutation carriers were included: 11 *MLH1* (6 pathogenic, 5 UVs), 10 *MSH2* (7 pathogenic, 3 UVs), 5 *MSH6* (all pathogenic), and 5 *PMS2* (all pathogenic) mutation carriers. One *MLH1*–UV carrier also showed a mono-allelic G382D mutation in *MUTYH* and one *PMS2* carrier showed a V878A UV in *MSH6* as well. A subset of these cases has been reported previously [19, 20]. The mean age at diagnosis of cancer was 49 years for the pathogenic MMR mutation carriers, and 43 years for the MMR-UV carriers. Clinical and mutation data are given in Table 1. The additional 10 samples originate from 10 patients that present with sporadic MMR deficient right sided (RST) colon carcinomas based on *MLH1* promoter hypermethylation with a mean age of 76 years. The study was approved by the Medical Ethical Committee of the LUMC (protocol P01-019) and the tumors were analyzed following the guidelines described in the code for proper secondary use of human tissue established by the Dutch federation of medical sciences (http://www.federa.org/).

MSI analysis and immunohistochemistry (IHC) of the MMR genes

MSI analysis and immunohistochemical staining of the MMR proteins was performed as described by de Jong et al. [19].

DNA isolation

Normal and tumor tissue was selected by a pathologist (HM), guided with microscopy of a hematoxylin eosin-stained slide. DNA of the selected tissue was extracted from FFPE material as described [19]. The DNA was subsequently cleaned up using protein precipitation solution (Promega, Leiden, The Netherlands) and 2-propanol precipitation. Leukocyte DNA was obtained by salting out precipitation. DNA concentrations were measured using picogreen (Invitrogen-Molecular Probes, Carlsbad, CA, USA).

Hypermethylation analysis of the *MLH1* promoter

The *MLH1* promoter hypermethylation status of the five *MLH1*-UVs and the 10 sporadic MSI-H right-sided tumors

Table 1 Characteristics of pathogenic MMR mutation, MMR-UV carriers and patients with sporadic MSI-H carcinomas

Sample ID	Tumor location	Stage[a]	Age of onset	MSI	IHC-MLH1	IHC-MSH2	IHC-MSH6	IHC-PMS2	Mutated gene	Exon
1	Colon right	3	47	H	0	+	0	0	*MLH1*[b]	3
3	Colon right	3	30	H	0	+	+	0	*MLH1*	12
4	Colon right	2	47	H	0	+	0	0	*MLH1*	16
5	Colon right	2	68	H	0	+	+	0	*MLH1*	16
6	Colon right	3	45	H	0	+	0	na	*MLH1*	10
7	Colon right	3	38	H	0	+	+	0	*MLH1*	1
8	Colon uns	na	54	H	+	0	0	na	*MSH2*	7
9	Colon left	2	67	H	+	0	0	na	*MSH2*	9
10	Colon right	3	58	H	+	0	0	+	*MSH2*	3
11	Colon right	2	38	H	+	0	0	+	*MSH2*	3
12	SmB	2	39	H	+	0	0	+	*MSH2*	13
13	Colon left	1	26	H	+	0	0	+	*MSH2*	7
14	Colon uns	2	49	H	+	0	0	+	*MSH2*	12
15	Colon uns	1	47	H	+	+	0	+	*MSH6*	9
16	Colon left	1	44	H	+	+	0	na	*MSH6*[c]	3
17	Colon right	3	54	H	+	+	0	na	*MSH6*[d]	4
18	Colon right	2	59	H	+	0	0	+	*MSH6*	4
19	Colon left	3	49	H	+	+	0	+	*MSH6*	4
20	Colon right	2	42	H	+	+	0	0	*PMS2*	11
21	Colon right	2	46	H	+	+	+	0	*PMS2*	–
22	Colon left	1	52	H	0	+	0	0	*PMS2*	3_7
23	EC	Na	81	H	+	+	0	na	*PMS2*	10
24	EC	Na	47	H	+	+	0	0	*PMS2*[e]	8
25a	Colon right	2	54	H	0	+	0	na	*MLH1* UV[f]	3
27	Colon right	2	38	H	0	+	+	na	*MLH1* UV	16
28a	Colon left	2	36	H	+	+	+	+	*MLH1* UV	1
29	EC	Na	34	H	+	+	+	+	*MLH1* UV	1
31	EC	Na	45	H	+	+	+	+	*MLH1* UV	1
32	Colon left	2	48	H	+	+	0	+	*MSH2* UV	15
33	Colon left	3	36	H	+	+	+	na	*MSH2* UV	11
34	Colon left	3	53	H	+	+	+	+	*MSH2* UV	14
S10	Colon right	2	80	H	0	+	+	0	–	
S16	Colon right	3	80	H	0	+	+	0	–	
S19	Colon right	2	75	H	0	+	+	0	–	

Table 1 continued

Sample ID	Tumor location	Stage[a]	Age of onset	MSI	IHC-MLH1	IHC-MSH2	IHC-MSH6	IHC-PMS2	Mutated gene	Exon
S20	Colon right	3	75	H	0	+	+	0	–	
S32	Colon right	3	76	H	0	+	+	0	–	
S39	Colon right	2	90	H	0	+	+	0	–	
S43	Colon right	2	61	H	0	+	+	0	–	
S51	Colon right	2	69	H	0	+	+	0	–	
S69	Colon right	2	76	H	0	+	+	0	–	
S78	Colon right	3	82	H	na	+	+	0	–	

Sample ID	DNA nucleotide change	Predicted protein change	Predicted biological type	Clinical status	Missing LP (1,2,3,4)	CNA		Segment size[g]		
						Gain/*loss*	cnLOH	CNA	cnLOH	Total
1	c.298 C > T	p.Arg100X	Nonsense	Path	2			0	0	0
3	c.1046dupT	p.Pro350fsX12	Frameshift	Path	2		3p26.3–14.1 19p13.3	0	22	22
4	c.1852_1854del 3	p.Lys618del	1 amino acid deletion	Path	–		3p25.1–21.1; 3q27.1	0	14	14
5	c.1852_1854del 3	p.Lys618del	1 amino acid deletion	Path	4		3p26.3–21.31	0	16	16
6	c.806C > G	p.Ser269X	Nonsense	Path	–		3p26.3–21.31	0	16	16
7	c.18_34del17	p.Val7fsX18	Frameshift	Path	4	**3q26.6 9p24.3–22.3**	3p26.3–21.31	6	16	22
8	c.379del T	p.?	Frameshift	Path	–		2p25.3–13.3 6q24.3–25.3	0	23	23
9	c.1408_1410delGTAinsCT	p.Val471fsX11	Frameshift	Path	3			0	0	0
10	c.367–?_645+?del	p.?	Exon(s) deletion	Path	–	*2p21–16.2*	2p22.1	3	1	4
11	c.367–?_645+?del	p.?	Exon(s) deletion	Path	3		2p25.3–14 6p25.3–22.3	0	26	26
12	c.2006–?_2210+?del	p.?	Exon(s) deletion	Path	–		17q23.2–25.3	0	8	8
13	c.1221_1222delCT	p.Tyr408fsX8	Frameshift	Path	1,2			0	0	0
14	c.1861C > T	p.Arg621X	Nonsense	Path	–	*20p13–20q13.32*	6q24.2–25.2	19	4	23
15	c.4001G > A	p.Arg1334Gln	Splice site	Path	1			0	0	0
16	c.467C > G	p.Ser156X	Nonsense	Path	–		22q arm	0	12	12
17	c.1444C > T	p.Arg482X	Nonsense	Path	–	*9p24.3–24.1*	12p arm 19q13.11–13.43	3	22	25
18	c.1784delT	p.Leu595fsX15	Frameshift	Path	–			0	0	0
19	c.2191C > T	p.Gln731X	Nonsense	Path	–		15q arm	0	28	28
20	c.1882C > T	p.Arg628X	Nonsense	Path	1	**19q13.31–13.41**	15q21.3–22.2	4	7	11

Table 1 continued

Sample ID	DNA nucleotide change	Predicted protein change	Predicted biological type	Clinical status	Missing LP (1,2,3,4)	CNA		Segment size[g]		
						Gain/*loss*	cnLOH	CNA	cnLOH	Total
21	2 kb insertion intron 7	p.?	Intron variant	Path	–		7p arm	0	18	18
22	deletion exon 3_7	p.?	Exon(s) deletion	Path	–		2p25.3–15	0	17	17
23	deletion exon 10	p.?	Exon(s) deletion	Path	–		7p22.3–p13	0	13	13
24	c.856delG	p286, fsX	Frameshift	Path	–		10q26.13–26.3 22q12.1–13.33	0	11	11
25a	c.277A > G	p.Ser93Gly	Missense	UV[h,i]	–	*6p25.3–12.3*	1p36.33–31 3p26.3–21.31 18q21.31–23	16	26	42
27	c.1744C > T	p.Leu582Phe	Missense	UV[i]	–	**9p24.3**	Complete Chr. 12	1	41	42
28a	c.114C > G	p.Asn38Lys	Missense	UV[i]	3	*8q23.3–24.13* *9p24.3–24.1*	3p26.3–p22.3	7	11	18
29	c.112A > C	p.Asn38His	Missense	UV[i]	–	**17p13.3–q21.31** *Complete Chr. 8*	10p15.3–q21.1; 10q22.3–26.3	52	38	90
31	c.109G > A	p.Glu37Lys	Missense	UV[i]	–		3p26.3–21.31	0	16	16
32	c.2579C > T	p.Ser860Leu	Missense	UV	2		10q arm 21q arm 22q arm	0	50	50
33	c.1387–8G > T + c.1737A > G	p.? + p.Lys579Lys	Intron variant + silent	UV	–			0	0	0
34	c.2276G > A	p.Gly759Glu	Missense	UV	–	**Complete Chr. 7**	17p arm 21q21.1–22.3	44	14	58
S10				Meth	3			0	0	0
S16				Meth	–	*Complete Chr. 4*		47	0	47
S19				Meth	–	*8p23.3–p21.3*, **Complete Chr. 12**		46	0	46
S20				Meth	–	**Complete Chr. 9**	12q arm	43	29	72
S32				Meth	1			0	0	0
S39				Meth	–	*6q21–q22.31*, *Complete Chr. 10*		42	0	42
S43				Meth	–	**1q42.2–q44,** **6q25.3–q27**	6p25.3–q25.2, 19p13.3–p13.11	8	13	21
S51				Meth	–	**Complete Chr. 12**		41	0	41

Table 1 continued

Sample ID	DNA nucleotide change	Predicted protein change	Predicted biological type	Clinical status	Missing LP (1,2,3,4)	CNA		Segment size[g]		
						Gain/*loss*	cnLOH	CNA	cnLOH	Total
S69				Meth	–			0	0	0
S78				Meth	1		6p25.3–p22.1	0	10	10

[a] TNM classification of the colon tumors, http://tnm.uicc.org
[b] Mutation in a sister not in this patient
[c] Mutation in family, this person was not tested
[d] Obligate carrier
[e] Patient carried additional, *MSH6* UV [V878A]
[f] Patient carried additional *MUTYH* G382D mutation
[g] Size of segment is measured in chromosomal sub-bands
[h] Most likely a polymorphism
[i] *MLH1* promoter not hypermethylated

Abbreviations: MSI, microsatellite instability; IHC, immunohistochemistery; LP, linkage panel; CNA, copy number aberrations; cnLOH, copy neutral loss of heterozygosity; path, pathogenic; na, information not available; uns, unspecified; p.?, exact effect on the protein sequence unknown; SmB, small bowel; EC, endometrial carcinoma; UV, unclassified variant; Chr., chromosome; meth, *MLH1* promoter hypermethylation

In bold, physical loss; in italics, gain; 0, protein abrogated in tumor nuclei; +, protein expression in tumor nuclei

were determined by hypermethylation analysis of the *MLH1* promoter using a methylation-specific MLPA assay as previously described [21].

Single nucleotide polymorphism array analysis

DNA was tested using Illumina BeadArrays and the GoldenGate assay (Illumina, San Diego, CA, USA). The GoldenGate assay was carried out according to the manufacturer's protocol with minor differences: 1 µg DNA was used as input in a multi-use activation step and subsequently dissolved in 60 µl resuspension buffer. For each sample, four SNP panels (linkage panel, LP), LP1-4, were tested together covering the genome: LP1 covers chromosomes 1–3 and 22, LP2 for chromosomes 5–9, LP3 for chromosomes 10–15 and 21, and LP4 for chromosomes 4, 16–20, X and Y. Each panel was analyzed separately on a beadarray. Due to the limited availability of archival tumor tissue some of the LPs could not be analyzed. In 13 cases one LP, and in one case two LPs could not be analyzed. Two carcinomas (cases 13 and 15,) could therefore not be analyzed for loss at *MSH2* or *MSH6*, respectively, and two for the hypermethylated *MLH1* locus (case S32 and S78, Table 1). Overall, we were able to analyze 91% of the genome in the three groups we corrected for the missing information in subsequent calculations.

We used linkage mapping panel version IV_B containing 6008 SNP markers distributed evenly over the genome with an average physical distance of 482 kb. Gene calls were extracted using GeneCall (version 6.0.7) and GTS Reports (4.0.10.0) (Illumina, San Diego, CA, USA). The software provides two quality scores: an experiment-wide gene train score (GTS) and a sample-specific gene call score (GCS).

Copy number and loss of heterozygosity (LOH)

Copy numbers were determined from the signal intensity of the individual SNPs. LOH was analyzed by comparing the genotypes from paired normal and tumor DNA. Both genomic profiles were generated with the R-package BeadArray SNP [22]. In addition, chromosome visualization of LOH was performed in Spotfire DecisionSite (Spotfire, Somerville, MA, USA) [15]. Furthermore, LOH was computed from the GCS and the GTS. LOH was called for high quality heterozygous SNPs in the normal tissue (relative gene call score (rGCS) > 0.8) that were, in the paired tumor, either homozygous or showed an rGCS/GTS ratio <0.8. In practice, regions of LOH always presented with stretches of markers showing LOH. LOH at one or two SNPs was ignored [15, 23]. Our interpretation of LOH has been verified in separate experiments with tumors using microsatellite and FISH probes (results not shown).

When both physical loss and LOH were detected at a specific region, we considered the detected LOH as an additional indication of physical loss. If no copy number change was detected, LOH was interpreted as copy neutral LOH (cnLOH).

Statistics

With a one-way ANOVA *F* test the amount of chromosomal aberrations in the three MSI-H groups was compared. A Scheffe-post hoc test was performed between the contrasts when the 0-hypothesis was rejected.

Results

We studied genome wide copy number changes and copy neutral LOH (cnLOH) in formalin-fixed paraffin-embedded (FFPE) tumor tissue using 6 K SNP arrays. The cohort consisted of 23 MSI-H tumors of 23 Lynch syndrome patients with pathogenic mutations eight tumors of patients with unclassified variants in *MLH1*, or *MSH2* genes. In addition, 10 sporadic MSI-H carcinomas with *MLH1* promoter hypermethylation from 10 patients were analyzed (Table 1).

Lynch syndrome cases with pathogenic MMR mutations

Immunohistochemical analysis revealed that in all carcinomas of pathogenic MMR mutation carriers the protein of the mutated gene was abrogated. In 14 of these cases both proteins of the heterodimer (MLH1/PMS2 or MSH2/MSH6) were abrogated. In six of these carcinomas (from three *MLH1* and three *PMS2* mutation carriers) also MSH6 was not expressed, this might be due to a frameshift in the C8 repeat which is located in the coding region of *MSH6* [24] (Table 1).

As expected from the literature [7, 25, 26], very few copy number aberrations were observed in the carcinomas from the carriers of pathogenic MMR mutations (Table 1). Only five of 23 (22%) MSI-H tumors presented with copy number abnormalities. Four of these cases, showed a single loss or gain of a chromosomal region. The fifth tumor presented with gain in two chromosomal regions. The chromosomes 2p, 3q, 9p, 19q and 20p were targeted in these tumors and the size of the affected segments ranged from 1 to 19 chromosomal sub-bands. Chromosome band 9p24.3 was targeted twice, in cases 7 and 17 by physical loss and gain, respectively. Physical loss of the MMR gene involved was only detected in case 10. Interestingly, in this case two different types of alterations were detected around chromosomal sub-band 2p21 harbouring the (mutated) *MSH2* gene; physical loss adjacent to cnLOH. We designated this alteration as physical loss.

Table 2 Copy neutral LOH at MMR loci and mean percentage of aberrant sub-bands in cases from pathogenic MMR mutation versus MMR-UV carriers

Gene	cnLOH at locus	SRO at locus
MLH1	5/6	3p25.1–22.2
MSH2	2/6	2p25.3–14
MSH6	0/5	–
PMS2	2/5	7p22.3–13
MLH1 UV	2/5	3p26.3–21.31
MSH2 UV	0/3	–
MLH1 hypermethylation	0/8	–

Abbreviations: LOH, loss of heterozygosity; UV, unclassified variant; SRO, smallest region of overlap

Also genome wide cnLOH was infrequent in these tumors (Table 1). However cnLOH around the locus of the mutated MMR gene was frequently observed in the 23 carcinomas from patients with pathogenic mutations. Five of six tumors from *MLH1* mutation carriers showed cnLOH at the *MLH1* locus (3p22.2) (Table 2). The extend of the LOH ranged from chromosome 3p26.3 to 3p14.1, and the smallest region of overlap (SRO) spanned 3p25.5–21.31, which encompasses *MLH1*. Two of the six *MSH2* tumors showed cnLOH of the *MSH2* locus at chromosome 2p21 (the interval of LOH ranged from 2p25.3 to 2p13.3; SRO 2p25.3–14) (Table 2). For *PMS2* mutation carriers, cnLOH was seen in two of five tumors (interval of LOH, 7p22.3–11.1; SRO, 7p22.3–13) (Fig. 1, Table 2). None of the five tumors from *MSH6* mutation carriers showed cnLOH at the *MSH6* locus (2p16, Table 2).

In addition, two of seven *MSH2* carcinomas presented with cnLOH at 6q with SRO; 6q24.3–25.2 (cases 8 and 14, Table 1). One patient with a pathogenic *PMS2* germline mutation (case 22, Table 1) presented with additional LOH of the chromosomal region 2p25.3–15 that harbours *MSH2* and *MSH6.* In this left-sided colon carcinoma, the protein expression of MLH1, MSH6 and PMS2 was abrogated and MSH2 expression was retained. *MLH1* and *MSH6* germline mutation analysis were negative.

MSI-H carcinomas with unclassified variants in MMR genes

Eight carcinomas from MMR-UV carriers were tested. Five of the eight cases showed normal positive staining of MMR proteins tested. Two *MLH1*-UV cases showed absent staining of at least MLH1, whereas one *MSH2*-UV case showed only absence of MSH6 protein. The five *MLH1*-UVs did not show promoter hypermethylation of *MLH1*.

Copy number abnormalities were detected in five of eight (62%) carcinomas from MMR-UV carriers. These carcinomas were from four *MLH1*-UV and one *MSH2*-UV carriers. Four tumors displayed a single copy number abnormality and the fifth tumor displayed two copy number abnormalities. The affected segments ranged in size from 1 to 52 chromosomal sub-bands. The copy number abnormalities affected chromosome 6p, 7, 8, 9p and 17. Chromosome 9p24.3 was affected in two of these five tumors (a gain in tumor 27 and physical loss in tumor 28a, Table 1). None of the analyzed tumors from MMR-UV carriers showed physical loss at the specific MMR gene locus involved.

CnLOH at the locus of the mutated MMR gene was found to a lesser extent than in tumors from pathogenic MMR mutation carriers (Table 2). Two of the five *MLH1*-UV carcinomas showed cnLOH at the *MLH1* locus on chromosome 3p22.2 while none of *MSH2*-UV carriers showed cnLOH at chromosome 2p21 (Table 2). Also genome wide cnLOH was limited. Five of the eight carcinomas showed cnLOH, ranging from one to three genomic regions at eight different chromosomes (Table 1).

Sporadic MSI-H carcinomas with *MLH1* promoter hypermethylation

Genome-wide profiles of copy number abnormalities and cnLOH were determined from 10 MSI-H carcinomas with *MLH1* promoter hypermethylation. Protein expression of MLH1 and PMS2 was abrogated in all 10 carcinomas as determined by immunohistochemistry. In six of the 10 (60%) sporadic MSI-H carcinomas limited copy number changes were detected. Three of these tumors exhibited one copy number abnormality and the other three displayed two changes. The affected segments ranged in size from 8 to 47 sub-bands of the genome of these carcinomas, affecting chromosome 1q, 4, 6, 8p, 9, 10 and 12. Amplification of complete chromosome 12 occurred in two cases (case S19 and S51) all additional copy number changes were unique. The locus of *MLH1* showed neither physical loss nor cnLOH in eight tumors that could be tested (Table 2). CnLOH was observed in 3 of the 10 carcinomas (30%). Two tumors showed one segment of cn LOH and the other tumor displayed two segments of cnLOH, affecting chromosomes 6, 12q and 19p (Table 1).

Comparison of three groups

We compared the average number of segments with cnLOH or copy number abnormalities detected in the carcinomas of the different groups (Table 3). The fraction of aberrant segments in each group and the distribution over the chromosomes is shown in Fig. 2. This comparison

Fig. 1 LOH view of tumors from a pathogenic *PMS2* mutation carrier generated with Spotfire DecisionSite (Spotfire, Somerville, MA). Heterozygous SNPs (upper diamonds in the figure) are dispersed over the chromosomes. These were analyzed in both tumor and corresponding normal DNA. For LOH, ≥3 SNPs in a specific region that are altered from heterozygote in normal to homozygote in tumor (lower diamonds) are scored as LOH. In practice, regions of LOH always presented with stretches of markers showing LOH. LOH at one or two SNPs was ignored. In this case LOH of *PMS2* is seen on chromosome 7 and none of the pseudogenes on chromosome 7q are affected

shows that the carcinomas of patients carrying an UV in one of the MMR genes display more aberrations (on average 2.79, range 0–4), than the carcinomas of patients with a pathogenic MMR mutation (on average 1.44, range 0–3) and the carcinomas with *MLH1* promoter hypermethylation (on average 1.32, range 0–4). The average number of aberrant segments of the three groups were compared with a one-way ANOVA test. A significant difference was found ($P = 0.045$) comparing the total number of segments per group, in a post hoc test (Scheffe test) no significant difference was revealed between the individual groups. The average size (chromosomal sub-bands) of the aberrant segments is larger in the tumors of patients carrying an UV in *MLH1* or *MSH2* (13 sub-bands) and in tumors with *MLH1* promoter hypermethylation (20 sub-bands), compared to the tumors of patients with a pathogenic MMR mutation (8 sub-bands).

Although subtle, the distribution of the types of chromosomal events—copy number aberrations versus cnLOH—is different in the carcinomas with *MLH1* promoter hypermethylation compared to the carriers of a pathogenic mutation or an UV in one of the MMR genes. Whereas in these last two groups the majority of events comprise cnLOH, copy number aberrations are more prevalent in carcinomas with *MLH1* promoter hypermethylation (Table 3). The one-way ANOVA test identified a significant difference ($P = 0.027$) between the number of cnLOH events in the three carcinoma groups; the Scheffe test assigned this result to the difference between the sporadic carcinomas with *MLH1* promoter hypermethylation and carcinomas from MMR-UV carriers ($P = 0.027$). A comparison of the percentage of chromosomal gain, loss and/or cnLOH was made for the three groups. The increase of chromosomal aberrations in carcinomas from MMR-UV carriers compared to the other two groups is again evident. The chromosomes involved and the distribution of the events over the chromosomes is different between the groups. Chromosomes 6p, 9p, 10q and 12p are affected by events in all three groups although to a different level. The suggested increase in events on chromosome 3p in the tumors from MMR-UV (Fig. 2) carriers can be explained by an unequal distribution of *MLH1* carriers (pathogenic 6/26 vs UVs 5/8) in the groups. Furthermore, one *MLH1*-UV case with cnLOH on 3p does not comprise the *MLH1* locus.

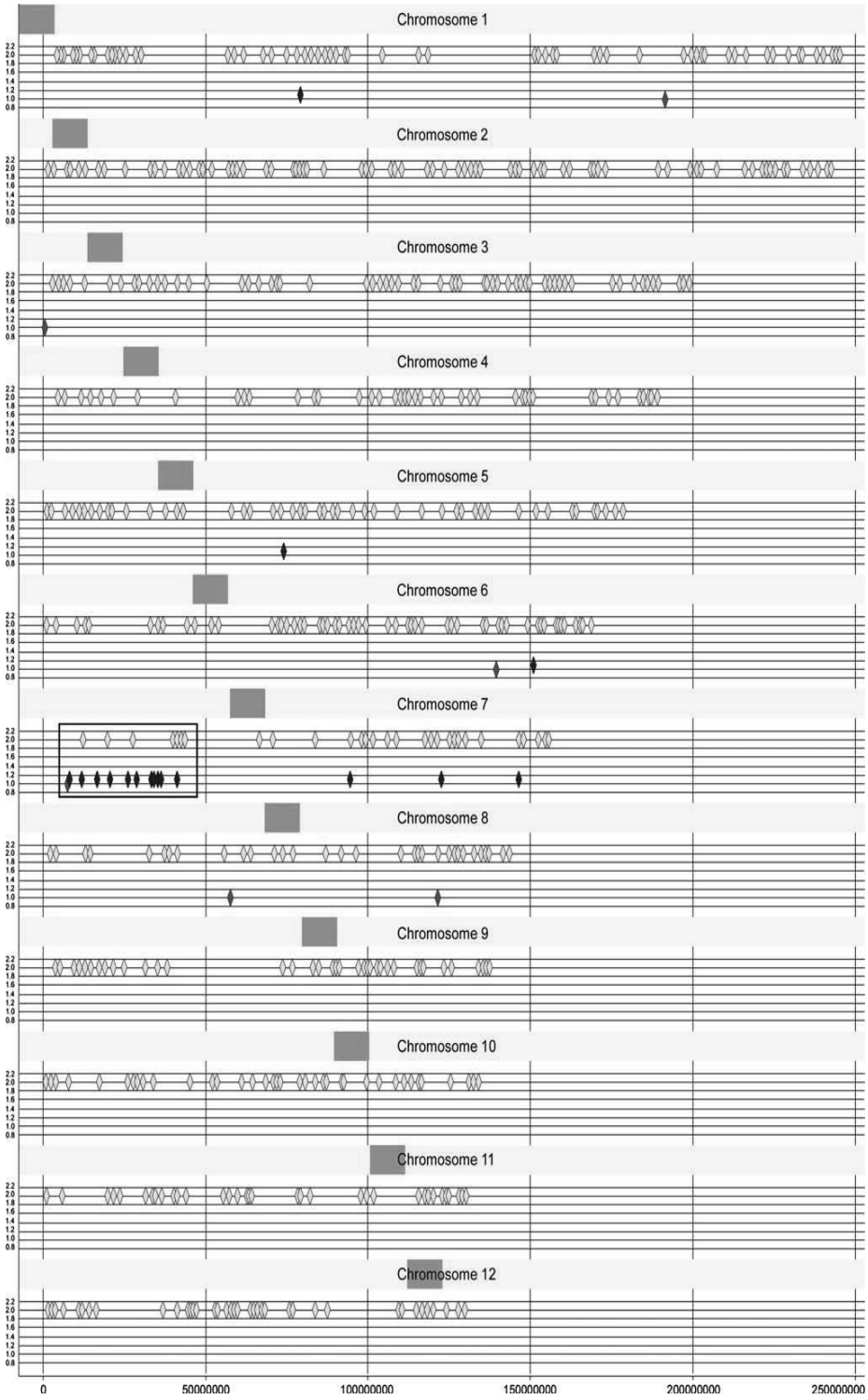
Chromosome 1
Chromosome 2
Chromosome 3
Chromosome 4
Chromosome 5
Chromosome 6
Chromosome 7
Chromosome 8
Chromosome 9
Chromosome 10
Chromosome 11
Chromosome 12
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
50000000
100000000
150000000
200000000
250000000

Table 3 Average number of chromosomal segments with cnLOH and genomic aberrations

	Pathogenic MMR mutation carriers	MMR-UV carriers	*MLH1* promoter hypermethylation
Gain	0.22	0.38	0.4
Loss	0.09	0.62	0.4
cnLOH	1.14	1.79	0.52
Total	1.44	2.79	1.32
Size	11.96	35.81	25.73
	$N = 23$	$N = 8$	$N = 10$

Abbreviations: MMR, mismatch; repair; UV, unclassified variant; cnLOH, copy neutral LOH; Size, average number of aberrant chromosomal sub-bands per carcinoma; N, number of carcinomas

Discussion

This is the first study that compares genome wide SNP array profiles of MSI-H carcinomas from MMR pathogenic mutation carriers, MMR-UV carriers and carcinomas with promoter hypermethylation of *MLH1*. With both comparative genomic hybridization (CGH) and SNP arrays, copy number information can be obtained however with SNP arrays also genome wide copy neutral LOH (cnLOH) can be studied which provide us with additional information. We used Illumina 6K SNP arrays on FFPE material and analyzed the data with the BeadArray SNP package [22].

Overall we did not detect extensive cnLOH in MSI-H carcinomas. Most of the cnLOH we found in carcinomas from pathogenic MMR mutation carriers, involved the MMR gene locus. Especially, for *MLH1* such cnLOH was seen in tumors from pathogenic mutation carriers (in five of six tumors). In the MMR-UV cases, cnLOH at the MMR locus was less frequent. In literature a varying frequency of LOH has been described on the *MLH1* and *MSH2* locus in series of pathogenic MMR mutation carriers and MMR-UV carriers. LOH at chromosome 3p has been reported in 35–85% of all tumors with a germline mutation (pathogenic as well as UVs) in *MLH1* [3, 27–34]. LOH at chromosome 2p has been described in 14–50% of all tumors with a germline *MSH2* mutation (pathogenic as well as UVs) [3, 28, 31, 35]. We detected cnLOH of *PMS2* in 40% of tumors, which to our knowledge has not been published previously. Using SNP arrays we have delineated the intervals of LOH around the affected genes. The LOH at the *PMS2* locus on chromosome 7p (SRO 7p22.3–13) points at the sensitivity of the technique in view of the existence of about 14 pseudogenes of *PMS2* [36–39] that were not targeted by the specific cnLOH of the *PMS2* locus.

Of interest is the increased number of aberrant segments in carcinomas from MMR-UV carriers compared to pathogenic MMR mutation carriers and carcinomas with *MLH1* promoter hypermethylation. Apparently, CIN is added to microsatellite instability in these MMR-UV cases during tumorigenesis. This could suggest that such additional CIN is necessary for tumorigenesis in cases with a priori weak mutator effects. Furthermore, this finding supports the observations that CIN and MIN are not mutually exclusive [9, 40–42]. With the detection of an unclassified variant in one of the MMR genes in patients that are highly suspected to be affected with Lynch syndrome, the uncertainty that a pathogenic mutation has been missed remains. We now suggest that finding a relatively increased CIN might make this less likely, as was seen in five of eight MMR-UV cases. However, the finding of MSI-H with absence of nuclear staining in cases from MMR-UV carriers does not definitively prove the pathogenicity of such UV. The five tumors from MMR-UV carriers, in which all MMR proteins tested are expressed, suggest the presence of a stable protein that is defective in MMR. It should be remembered that the staining and MSI results also depend on the nature

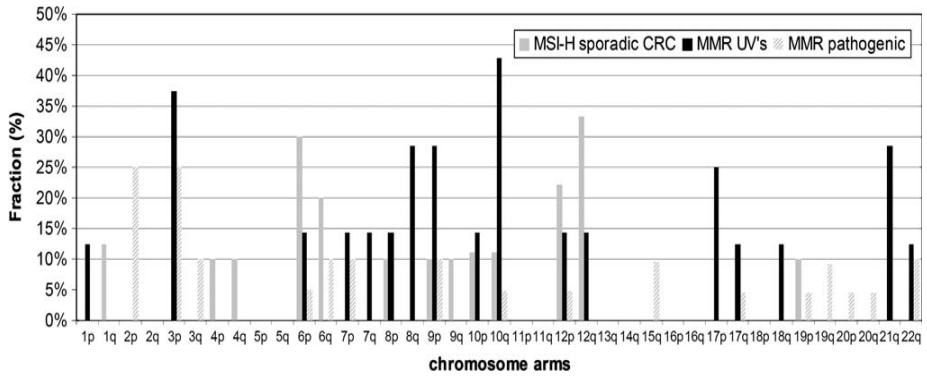

Fig. 2 Fraction of chromosomal events, per chromosome arm, in MSI-H carcinomas. The shaded bars indicate the percentage of 23 carcinomas from pathogenic mutation carriers and the black bars represent the eight carcinomas from MMR-UV carriers. The grey bars indicate the MSI-H carcinomas with hypermethylation of the *MLH1* promoter that exhibit events of chromosomal aberration of a chromosome. This percentage has been calculated for the respective chromosome arms

of the second somatic hit that occurred in the tumor. Furthermore, in series of cases with specific MMR-UVs not always the same results are obtained [19].

We see that the chromosomal segment that is targeted is larger in the tumors of patients carrying an UV in *MLH1* or *MSH2* and in tumors with *MLH1* promoter hypermethylation, compared to the tumors of patients with a pathogenic MMR mutation. Aberrations of whole chromosomes are found in, respectively, five of the eight MMR-UV carcinomas, in five of the 10 *MLH1* methylated carcinomas and only in two of the 23 MMR pathogenic carcinomas. In addition, the distribution of the types of chromosomal events—copy number aberrations versus cnLOH—is slightly different in the carcinomas with *MLH1* promoter hypermethylation compared to the carriers of a pathogenic mutation or an UV in one of the MMR genes. Whereas in these last two groups the majority of abnormalities concerns cnLOH (79% and 64%, respectively), copy number aberrations are the more prevalent abnormality seen in carcinomas with *MLH1* promoter hypermethylation (60%). In contrast to other publications we detected equal amounts of gain and physical loss of parts or whole chromosomes in the sporadic MSI-H carcinomas [9, 42]. Trautmann et al. studied 23 sporadic MSI-H carcinomas with array CGH and identified gains on chromosomes 8, 12 and 13. We also identified gain of chromosome 12 in two out of 10 carcinomas.

Moreover, we could identify several small regions with copy number changes and cnLOH that were present in more than one MSI-H tumor with a pathogenic MMR defect on chromosomes 9p24.3 and 6q24.2–25.2 respectively. These regions might harbour genes that are important for tumorigenesis. Recent association studies identified polymorphic sequences at 8q24 as associated with an increased risk for CRC. Interestingly, chromosome 9p24 was also implicated in two of these studies pointing at a role for 9p24 in carcinogenesis [43–46].

The approach we describe here appears to be an elegant way to detect (genome wide) cnLOH in MSI-H formalin fixed paraffin embedded carcinomas. Studying LOH in these type of carcinomas was often hampered due to instability of polymorphic microsatellite markers. We also suggest that the SNP array platform, as described here and applicable to FFPE tissue, may be a crucial tool in finding the genetic cause of unexplained familial colorectal cancer, since we were able to identify distinct small regions of LOH and/or copy number alterations.

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CHAPTER 4

Identification of (atypical) MAP patients by *KRAS2* c.34 G>T prescreening followed by *MUTYH* hotspot analysis in formalin-fixed paraffin-embedded tissue

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Identification of Patients with (Atypical) *MUTYH*-Associated Polyposis by *KRAS2* c.34G > T Prescreening Followed by *MUTYH* Hotspot Analysis in Formalin-Fixed Paraffin-Embedded Tissue

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Abstract **Purpose:** To assess the feasibility of identifying patients with (atypical) *MUTYH*-associated polyposis (MAP) by *KRAS2* c.34G > T prescreening followed by *MUTYH* hotspot mutation analysis in formalin-fixed paraffin-embedded tissue (FFPE).

Methods: We collected 210 colorectal FFPE tumors from 192 individuals who presented with <10 adenomas or familial mismatch repair proficient colorectal carcinomas with <10 concomitant adenomas. The tissues were tested for somatic *KRAS2* mutations and for three Dutch hotspot *MUTYH* germ line mutations (p.Tyr165Cys, p.Gly382Asp, and p.Pro391Leu) by sequencing analysis.

Results: The c.34G > T, *KRAS2* transversion was detected in 10 of 210 tumors. In one of these 10 cases, a monoallelic p.Gly382Asp *MUTYH* mutation was found and a full *MUTYH* analysis in leukocyte DNA revealed an unclassified variant p.Met269Val. This was in a 61-year-old patient with a cecum carcinoma and three adenomas. After further requests, her family case history revealed that her brother had had between 10 and 15 adenomas and turned out to carry both *MUTYH* germ line mutations. *MUTYH* hotspot mutation screening in 182 patients without the somatic c.34G > T *KRAS2* mutation led to the detection of three monoallelic germ line *MUTYH* mutation carriers.

Conclusion: *KRAS2* c.34G > T somatic prescreening, followed by *MUTYH* hotspot mutation analysis when positive, can identify patients with (atypical) MAP. If heterozygous hotspot *MUTYH* mutations are identified, a complete germ line *MUTYH* mutation screening should be carried out if possible. Immediate *MUTYH* hotspot mutation analysis is a practical alternative in patients with >10 adenomas or in cases of multiple colorectal carcinomas in one generation for which only FFPE tissue is available.

The aim of this study was to explore the feasibility of identifying patients with (atypical) MAP using *KRAS2* c.34G > T somatic prescreening followed by *MUTYH* hotspot analysis in patients that presented with <10 adenomas or familial mismatch repair proficient colorectal carcinomas (CRC) with <10 concomitant adenomas.

In 2002, the first autosomal recessive colorectal cancer and polyposis syndrome, *MUTYH*-associated polyposis (MAP), was described (1). Biallelic germ line *MUTYH* mutations predispose carriers to somatic G > T transversions in genes involved in the tumorigenesis of CRCs, such as *APC* and *KRAS2*, due to failure of base excision repair to remove the purine adenine aberrantly coupled to 8-oxo-guanine by DNA polymerase (1–4).

In most cases, patients with MAP develop between 10 and 500 polyps at a mean age of ~50 years (5–7). Previously, in large cohorts of patients with CRC (with or without polyps), ~1% of patients with biallelic MAP were detected, some of whom were without polyps (8, 9). Although in other cohorts of patients with <10 polyps, no *MUTYH* mutation carriers were detected (10), the question remains of how prevalent the (biallelic) *MUTYH* mutations are in familial CRC cases with <10 polyps, with or without concomitant CRC.

In the Netherlands, clinical geneticists advise diagnostic testing for *MUTYH* germ line mutations based on the number of adenomas, age at diagnosis, and the family history. *MUTYH* will be analyzed in patients with 10 to 100 adenomas at ages under 70 years, whereas in CRC patients with a history of <10 adenomas, Lynch syndrome could also be considered. In patients with classic polyposis (>100 adenomas), germ line *APC* mutations can be excluded prior to *MUTYH* testing (11).

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Table 1. Basic clinical characteristics of the familial microsatellite stable cases

	No. of patients	Carcinomas			Adenomas		
		Right	Left	Unspecified	<5	5-10	>10
Adenoma <40 y	7	—	—	—	6	1	—
Adenoma 40-50 y	14	—	—	—	13	1	—
Adenoma >50 y	18	—	—	—	17	1	—
Carcinoma <50 y	74	18	46	10	8	1	—
Carcinoma >50 y	79	18	48	13	18	1	1*

*Patient, at 71 years old; left-sided colon carcinoma, no polyps identified and at 77 years old; right-sided colon carcinoma and 10 to 20 polyps (therefore not immediately eligible for germ line *MUTYH* testing).

Two missense mutations (p.Tyr165Cys and p.Gly328Asp) account for 73% of the *MUTYH* mutations that have been reported thus far (12). In addition, there seems to be population-specific *MUTYH* mutations, such as the Italian 1395delGGA, the Portuguese 1186-1187insGG, and the Indian p.Glu466OCHer (5, 10, 13). In the Netherlands, we identified p.Pro391Leu as a possible founder mutation. Three hotspot mutations (p.Tyr165Cys, p.Gly328Asp, and p.Pro391Leu) represent 89% of the *MUTYH* mutations that are found in Dutch patients with MAP, and at least one of these mutations is present in all biallelic germ line *MUTYH* mutation carriers of Dutch origin identified thus far, and 79% of these carriers have two hotspot mutations (7). Up to 64% of MAP carcinomas showed a specific G > T transversion in *KRAS2* c.34G > T, p.Gly12Cys (3, 4). The latter somatic mutation is infrequent in consecutive series of sporadic CRC (14).

Materials and Methods

Patient cohort. We analyzed 210 tumors from 192 patients who were referred to the Department of Pathology, as part of the familial cancer clinics, and who presented with <10 adenomas or familial mismatch repair proficient CRCs with <10 concomitant adenomas. Microsatellite instability analysis and additional immunohistochemistry was done in order to exclude a mismatch repair gene defect.

Basic clinical characteristics of these familial cases are summarized in Table 1. Complete pedigree information was available in only 62 cases (data not shown). Informed consent was obtained for DNA testing according to protocols approved by the local ethics review boards, and the cases were analyzed following the medical ethnical guidelines described in the Code for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences.[4]

DNA isolation. Genomic DNA of normal colon and colorectal tumor tissue was extracted from formalin-fixed paraffin-embedded (FFPE) material as described by De Jong et al. (15). Microsatellite analysis was done as described (15).

Somatic KRAS2 mutation analysis. Nested *KRAS2* mutation analysis (16), and an improved *KRAS2* mutation analysis was used (preventing the amplification of chromosome 6 *KRAS2* pseudogene sequences; detailed information will be given on request).

Somatic APC mutation analysis. Samples were screened for the presence of mutations in the mutation cluster region codons 1286-1513 of *APC* by sequence analysis as previously described (16).

Dutch MUTYH mutation hotspot (p.Tyr165Cys, p.Gly382Asp, and p.Pro391Leu) analysis in FFPE material. Mutation analysis was done by direct sequencing of a PCR product which was obtained under standard PCR conditions. The following primer sets were developed: forward 5′-CCC ACA GGA GGT GAA TCA ACT-3′, and reverse 5′-GTT CCT ACC CTC CTG CCA TC-3′ for *MUTYH* (p.Tyr165Cys), and forward 5′-GGC AGT GGC ATG AGT AAC AAG-3′ and reverse 5′-CTT GCG CTG AAG CTG CTC T-3′ for *MUTYH* (p.Gly328Asp) and (p.Pro391Leu).

Germ line MUTYH mutation analysis. When a *KRAS2* c.34G > T mutation was found, or when *MUTYH* hotspot analysis showed a monoallelic *MUTYH* mutation, mutation analysis of the whole *MUTYH* gene was done in leukocyte DNA (when available) as described by Nielsen et al. (7). For further details, see the LUMC web site.[5]

Results

Frequency of somatic KRAS2 mutations. We identified 34% (54 of 159) and 27% (14 of 51) *KRAS2* mutations in mismatch repair proficient carcinomas and adenomas, respectively (Table 2). The majority of carcinomas showed G > A transitions (36 of 54, 67%), of which 75% (27 of 36) were c.35G > A transitions. G > T transversions were detected in 26% (14 of 54), whereas G > C transitions were detected in only 6% (3 of 54) of the carcinomas. Preferential occurrence of G > A transitions over G > T transversions was not seen in adenomas (6 of 10 versus 7 of 10, respectively), although we only had a low number of cases.

Cases with somatic KRAS2 c.34G > T transversions. The c.34G > T, p.Gly12Cys *KRAS2* mutation was detected in 10 cases (six carcinomas, four adenomas; Table 3). Six of the 10 showed inactivating *APC* somatic mutations other than G > T transversions (Table 3). One patient with a somatic c.34G > T *KRAS2* mutation in her carcinoma carried a monoallelic p.Gly382Asp germ line *MUTYH* mutation, and subsequent complete germ line *MUTYH* analysis in leukocyte-derived DNA revealed an unclassified variant c.805A > G, p.Met269Val. No somatic *APC* mutation was found. This female patient (III.1) presented with a right-sided cecum carcinoma and three adenomas at 61 years old. Her pedigree is shown in Fig. 1. Only after further requests did her family case history reveal that her brother (living abroad) had had between 10 and 15 adenomas and turned out to carry both *MUTYH* germ line mutations (III.2). The nine remaining cases with c.34G > T *KRAS2* mutations showed no hotspot *MUTYH* mutations in FFPE material. Leukocyte DNA was available in three of nine

[4] http://www.federa.org/?s=1&m=78&p=&v=4

[5] http://www.lumc.nl/4080/DNA/MUTYH.html

Table 2. Somatic mutation analysis of codons 12 and 13 of *KRAS2*

	Patients	Carcinomas (159)		Adenomas (51)	
		% *KRAS2* mutations	No. of *KRAS2* mutations	% *KRAS2* mutations	No. of *KRAS2* mutations
Familial MRR proficient	192	(54) 34%	1 (c.34G > A) + (=) 6 (c.34G > T) + (=) 2 (c.34G > C) + (=) 27 (c.35G > A) + (=) 1 (c.35G > C) + (=) 8 (c.35G > T) + (=) 9 (c.38G > A) + (=)	(14) 27%	4 (c.34G > T) + (=) 4 (c.35G > A) + (=) 1 (c.35G > C) + (=) 3 (c.35G > T) + (=) 2 (c.38G > A) + (=)

Abbreviations: ca, carcinoma; ad, adenoma; (=), wild-type.

cases to complete *MUTYH* germ line mutation analysis but showed no *MUTYH* mutations.

MUTYH *germ line hotspot mutation carriers without a somatic* KRAS2 *c.34G > T transversion.* In 182 patients without the c.34G > T *KRAS2* mutation, *MUTYH* hotspot analysis revealed three heterozygotes: two with the p.Gly382Asp mutation and one with the p.Tyr165Cys mutation. The complete *MUTYH* gene could be analyzed in two of the three patients, but no additional mutation was detected. One of the two heterozygous p.Gly382Asp patients (not fully tested for *MUTYH*) carried a somatic c.35G > A mutation in *KRAS2* in his tumor. He presented with a well-differentiated right-sided adenocarcinoma when he was 74 years old. The second patient (fully tested for *MUTYH*) with the monoallelic *MUTYH* p.Gly382Asp mutation had no mutation in *KRAS2* in his tumor and presented with a rectal carcinoma at age 41 years. The third patient (fully tested for *MUTYH*), with a monoallelic p.Tyr165Cys *MUTYH* mutation, presented with five adenomas at age 43 years, three of which were tested and showed no somatic *KRAS2* mutations.

Discussion

Because MAP carcinomas show a specific c.34G > T *KRAS2* mutation (2–4), we investigated whether somatic *KRAS2* prescreening could be used to detect patients with atypical MAP among individuals who presented with <10 adenomas or with familial mismatch repair proficient CRCs with <10 or no concomitant adenomas. For the same purpose, we did *MUTYH* hotspot analysis in FFPE material. In the Netherlands, it is logical to search for hotspot *MUTYH* mutations because MAP patients of Dutch origin always have at least one of the hotspot mutations (data not shown). If a *MUTYH* hotspot mutation

Table 3. Patients with c.34G > T, p.Gly12Cys mutations

Patient ID	Age of onset (y)	Tumor	MSI	Germ line *MUTYH* mutation	Somatic *KRAS2* mutation	Somatic *APC* mutation	
						Nucleotide change	Amino acid change
1	35	Sigmoid carcinoma	S	wt*	(c.34G > T) + (=) †	(c.4468delC) + (=) ‡	(p.His1490fs) + (=)
2 T1	35	Cecum adenoma	S	wt	(c.34G > T) + (=) †	(c.4497delA) + (=) ‡	(pSer1501fs) + (=)
2 T2	35	Cecum carcinoma	S	wt	wt	wt ‡	
3	49	Cecum adenoma	S	wt*	(c.34G > T) + (=) †	(c.4285C > T) +(=) §	(p.Gln1429X) + (=)
4	40	Sigmoid adenoma	S	wt	(c.34G > T) + (=) †	(c.4285C > T) + (=)	(p.Gln1429X) + (=)
5	71	Sigmoid carcinoma	S	wt*	(c.34G > T) + (=) †	wt ‡	
6	47	Cecum adenoma	S	wt*	(c.34G > T) + (=) †	wt §	
7	45	Sigmoid carcinoma	S	wt	(c.34G > T) + (=) †	(c.3922_3929del AAAGAAAA) + (=)	(p.Lys1308fs) + (=)
8	45	Sigmoid carcinoma	S	wt*	(c.34G > T) + (=) †	wt §	
9	51	Cecum carcinoma	S	wt*	(c.34G > T) + (=) †	(c.3949G > C) + (=) §	(p.Glu1317Gln) + (=)
10	61	Cecum carcinoma ‖	S	(c.805A > G) + (c.1145G > A) ¶	(c.34G > T) + (=) †	wt §	

Abbreviations: MSI, microsatellite instability; S, stable; wt, wild-type; T1, tumor 1; T2, tumor 2; (=), wild-type.
*Patients were only tested for three *MUTYH* hotspots (p.Tyr165Cys, p.Gly382Asp, and p.Pro391Leu).
†(c.34 G > T, p.Gly12Cys) + (=).
‡SNP rs 41115 (c.4479G>A) + (=) confirmed in normal DNA.
§SNP rs 41115 (c.4479G>A) + (c.4479G > A) confirmed in normal DNA.
‖This patient also presented with three adenomas.
¶(c.805A > G,p.Met269Val) + (c.1145G > A, p.Gly382Asp).

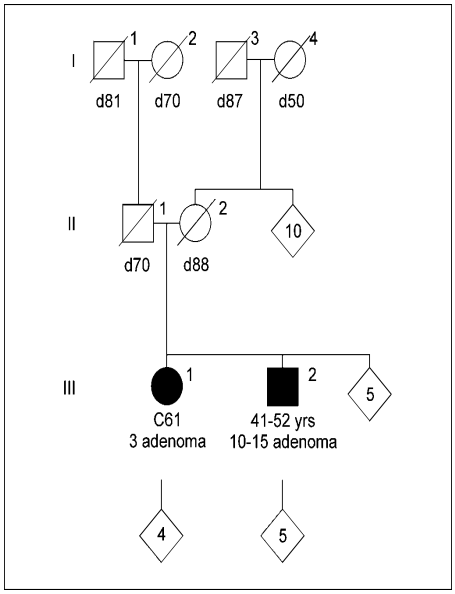

Fig. 1. Pedigree of a Dutch family in which two members were found to carry a heterozygous pGly382Asp germ line *MUTYH* mutation and an unclassified variant of *MUTYH*, c805A › G, pMet269Val. C, colorectal cancer; d, age at death.

is present, the gene should be screened for additional rare mutations in *MUTYH*.

This study identified one compound heterozygote *MUTYH* mutation carrier (p.Gly382Asp, p.Met269Val) with *KRAS2* mutation screening for the specific c.34G > T somatic mutation and three other monoallelic *MUTYH* germ line mutation carriers with the *MUTYH* hotspot analysis.

In our total cohort of 192 cases, 10 tumors had a somatic c.34G > T *KRAS2* mutation (six carcinomas and four adenomas). Of these, one turned out to carry a germ line *MUTYH* mutation, although this patient would a priori not have been tested for *MUTYH* mutations. This patient (and later her brother, who turned out to have >10 adenomas) carried both a proven pathogenic *MUTYH* mutation p.Tyr165Cys and an unclassified variant, c.805A > G, p.Met269Val. The c.805A > G, p.Met269Val unclassified variant in *MUTYH* was identified only after a full *MUTYH* gene mutation screening as a next step. This *MUTYH* unclassified variant described by Lejeune et al. is evolutionarily strongly conserved and locates within the adenine recognition motif (17). Although it was not predicted to be damaging by Polyphen software, the above family characteristics might suggest otherwise.

In the remaining nine patients with c.34G > T *KRAS2* somatic mutations, six also had inactivating *APC* somatic mutations. However, none of these mutations were G > T transversions and no germ line hotspot *MUTYH* mutations were identified.

In conclusion, we have shown that *KRAS2* c.34G > T, p.Gly12Cys somatic prescreening followed by *MUTYH* (hotspot) mutation analysis of cases (presenting with <10 adenomas or familial mismatch repair proficient CRCs with <10 or no concomitant adenomas) could be used successfully to identify patients with (atypical) MAP. If monoallelic (hotspot) *MUTYH* mutations are identified subsequently, full germ line *MUTYH* mutation analysis should also be carried out to exclude additional rare mutations. *KRAS2* c.34G > T prescreening only followed by *MUTYH* hotspot analysis when positive, is cost-effective especially when transformed into an allele-specific PCR. We estimate that the cost would be at least five times higher if immediate *MUTYH* hotspot mutation analysis would be done in all cases. The latter, however, is a practical alternative in patients with >10 adenomas or in family cases of multiple CRCs in one generation, for which only FFPE tissue is available.

Since finishing our study, we implemented *KRAS2* c.34G > T prescreening in our diagnostic setting. We recently identified a second atypical MAP family. The female index patient was diagnosed with metastasized colon cancer at age 41. No polyps were described. After identification of the c.34G > T transversion in *KRAS2* in her tumor, subsequent *MUTYH* hotspot analysis identified a monoallelic p.Gly382Asp *MUTYH* mutation. Full germ line *MUTYH* mutation analysis showed a 956-13 G > T splice variant.

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CHAPTER 5

High frequency of copy neutral LOH in *MUTYH*-associated polyposis carcinomas

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High frequency of copy-neutral LOH in *MUTYH*-associated polyposis carcinomas

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Abstract

Genetic instability is known to drive colorectal carcinogenesis. Generally, a distinction is made between two types of genetic instability: chromosomal instability (CIN) and microsatellite instability (MIN or MSI). Most CIN tumours are aneuploid, whereas MSI tumours are considered near-diploid. However, for *MUTYH*-associated polyposis (MAP) the genetic instability involved in the carcinogenesis remains unclear, as near-diploid adenomas, aneuploid adenomas and near-diploid carcinomas have been reported. Remarkably, our analysis of 26 MAP carcinomas, using SNP arrays and flow sorting, showed that these tumours are often near-diploid (52%) and mainly contain chromosomal regions of copy-neutral loss of heterozygosity (LOH) (71%). This is in contrast to sporadic colon cancer, where physical loss is the main characteristic. The percentage of chromosomal gains (24%) is comparable to sporadic colorectal cancers with CIN. Furthermore, we verified our scoring of copy-neutral LOH versus physical loss in MAP carcinomas by two methods: fluorescence *in situ* hybridization, and LOH analysis using polymorphic markers on carcinoma fractions purified by flow sorting. The results presented in this study suggest that copy-neutral LOH is an important mechanism in the tumorigenesis of MAP.

Keywords: ***MUTYH*; tumour profiling; copy-neutral LOH; SNP arrays; MAP carcinomas; colorectal adenomatous polyposis; colorectal cancer**

Introduction

MUTYH-associated polyposis (MAP) is the first colorectal cancer syndrome shown to be inherited in an autosomal recessive fashion. Biallelic mutations in the base excision repair (BER) gene *MUTYH* have been shown to cause colorectal adenomatous polyposis, and correlate with a high risk of developing carcinomas [1]. BER is a DNA repair mechanism that guards oxidative DNA damage and other metabolic DNA damage. Upon oxidative DNA damage, MUTYH removes incorrectly incorporated adenines opposite to an 8-oxo-guanine. Consequently, MAP patients show somatic G : C → T : A mutations in crucial genes such as *APC* and *KRAS*. In *APC*, these G : C → T : A transversions seem to occur primarily in GAA sequences [1,2]. In *KRAS*, a specific GGT → TGT mutation (c.34 G → T, p.Gly12Cys) is found in up to 64% of MAP carcinomas [3]. Interestingly few *p53* and *SMAD4* mutations are found in MAP carcinomas, whereas these genes are frequently affected in sporadic colorectal cancer [3]. Although MUTYH deficiency triggers carcinogenesis by G : C → T : A transversions, the exact role of MUTYH deficiency in the tumour progression in MAP patients is still unknown.

For colorectal cancers, different types of genetic instability are known to drive carcinogenesis. The two main types of genetic instability are microsatellite instability (MIN or MSI) and chromosomal instability (CIN). CIN is defined as an accelerated rate of chromosomal missegregation resulting in an aberrant chromosomal content, and is found in the vast majority of sporadic colorectal cancers [4]. On the other hand, ∼15% of the sporadic colorectal cancers show MSI, due to *MLH1* promoter hyper-methylation [5]. Moreover, MSI is typically seen in the carcinomas of Lynch syndrome patients. Colon carcinomas that display neither CIN nor MSI have also been described [6]. More recently, abnormal epigenetic modification has been described in colorectal cancer, exhibiting the CpG island methylator phenotype (CIMP) [7,8].

The genomic profile of MAP tumours has been described in three studies to date. Using flow

cytometry, Lipton *et al* found MAP carcinomas to be predominantly near-diploid. Comparative genomic hybridization (aCGH) of two near-diploid MAP carcinomas showed no detectable chromosomal gains or losses. Furthermore, they analysed chromosomes 1p, 2p, 5q, 10p, 15q, 18q and 20q for LOH, using microsatellite markers, and reported a high frequency of LOH for chromosome 18q but low levels of LOH for the other regions [3]. Recently, the same research group identified only a small number of copy number changes in MAP adenomas [9]. These changes were mainly restricted to chromosomes 1p, 13, 17p, 19 and 22. Additionally, in a single MAP adenoma, copy-neutral LOH (cnLOH) of whole chromosome 7 and 12 was reported. On the other hand, Cardoso *et al* identified chromosomal copy number aberrations in MAP adenomas using aCGH analysis. The most prevalent aberrations identified were gains at chromosomes 7 and 13, as well as physical losses on chromosomes 17p, 19p and 22q [10]. However, the ploidy status of these adenomas was not determined.

Although these studies seem to be contradictory, Lipton *et al* studied carcinomas, whereas the other studies analysed adenomas. In addition, different technical platforms were used, i.e. flow cytometry vs. aCGH after amplification of laser capture microdissected DNA.

In order to gain further insight into the genetic instability involved in MAP carcinogenesis, we analysed formalin-fixed paraffin-embedded tumour tissue from 26 carcinomas for patterns of chromosomal losses and gains and copy-neutral LOH using SNP arrays [11,12].

Materials and methods

Samples

From 19 MAP patients, 26 formalin-fixed paraffin-embedded (FFPE) carcinomas and corresponding normal tissue were selected (Table 1). This series of carcinomas included metastases of primary colon carcinomas (t10 and t11). Corresponding normal tissue was either histological normal colon tissue or tissue from unaffected lymph nodes. The carcinomas originated from 11 biallelic Y165C mutation carriers, two biallelic P391L mutation carriers, three Y165C/G382D compound heterozygotes, one 1105delC/G382D, one P391L/G382D and one P391L/R233X compound heterozygote. Clinical details of patients 2, 3, 8, 9, 10, 12, 13, 14, 15, 16 and 17 were previously described

Table 1. Characteristics of the MAP carcinomas

Tumour	Patient	*MUTYH* mutation	Site CRC*	Age at diagnosis	Tumour stage	DNA index
t1	1	Y165C/Y165C	Distal	52	I	0.9 + 1.7†
t2	2	Y165C/Y165C	Distal	49	II	1.1 + 1.4†
t3	3	Y165C/Y165C	Proximal	39	II	1.0
t4	4	Y165C/Y165C	Proximal	49	III	1.0 + 1.5†
t5	5	Y165C/Y165C	Distal	56	I	1.6
t6	6	Y165C/Y165C	Proximal	53	II	1.0
t7.1	7	Y165C/Y165C	Proximal	43	II	1.0 + 1.5†
t7.2	7	Y165C/Y165C	Distal	43	II	1.0 + 1.5†
t8.1	8	Y165C/Y165C	Proximal	41	III	na
t8.2	8	Y165C/Y165C	Proximal	41	III	na
t8.3	8	Y165C/Y165C	Proximal	41	III	1.0
t8.4	8	Y165C/Y165C	Distal	41	III	1.0
t9	9	Y165C/Y165C	Ileum	77	II	1.0
t10	10	Y165C/Y165C	Metastases‡	45	IV	1.5 + 2.7§
t11	11	Y165C/Y165C	Metastasis‡	64	IV	1.5
t12	12	Y165C/G382D	Proximal	67	III	1.0
t13.1	13	Y165C/G382D	Proximal	43	II	1.0 + 1.1†
t13.2	13	Y165C/G382D	Proximal	46	II	1.0
t14	14	Y165C/G382D	Proximal	59	II	1.0
t15.1	15	P391L/P391L	Proximal	37	III	1.1 + 1.4†
t15.2	15	P391L/P391L	Proximal	37	III	Na
t16	16	P391L/P391L	Distal	58	II	1.0
t17.1	17	1105delC/G382D	Distal	42	I	1.1
t17.2	17	1105delC/G382D	Distal	42	I	1.0
t18	18	R233X/P391L	Proximal	48	II	1.4
t19	19	G382D/P391L	Proximal	51	III	1.1

The tumours were located before (proximal) or after (distal) to the splenic flexura of the colon. T11 is a metastasis of an earlier colon carcinoma. T10 consists of two metastases of a colorectal carcinoma from patient 10.The DNA index was measured by multiparameter DNA flow cytometry. When two populations were identified in the keratin-positive fraction, the DNA index of both tumour fractions is shown in the table. Tumour staging was performed according to the TNM classification (http://tnm.uicc.org).

na, could not be analysed for technical reasons.

* All tumours were colorectal with the exception of t9 (ileum).

† Multiple clones.

‡ Exact location of the primary tumour in the colon not known.

§ Two metastases of a primary colorectal carcinoma with DNA index 1.5 and 2.7, respectively.

by Nielsen *et al* [13] (as the respective numbers 13, 4, 11, 12, 14, 20, 18, 16, 35, 34 and 30). Twenty-two previously published sporadic CRCs [14] were included as reference controls.

The study was approved by the local Medical Ethical Committee (protocol P01.019); samples were handled according to the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (www.federa.org). Tumour samples were enriched for tumour tissue by taking 0.6 mm tissue punches, using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA) guided by a haematoxylin and eosin (H&E)-stained slide. DNA was isolated by the previously described method, and subsequently cleaned using the Genomic Wizard kit (Promega, Leiden, The Netherlands) [15]. DNA concentrations were measured with the picogreen method (Invitrogen–Molecular Probes, Breda, The Netherlands).

Flow cytometry and cell sorting

For 23 carcinomas, the DNA index was determined by flow cytometry, as described previously with minor modifications [16]. In short, cell suspensions were prepared from FFPE samples and stained for keratin (APC), vimentin (RPE) and DNA (DAPI). Samples were analysed on a LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). From five MAP carcinomas (t2, t4, t10, t12 and t18) and one sporadic carcinoma (sp1), those cell fractions that were vimentin-positive, keratin-negative (V^+K^-) and vimentin-negative, keratin-positive (V^-K^+) were flow-sorted using a FACSAria cell sorter (BD Biosciences).

Single nucleotide polymorphism arrays

Illumina BeadArrays were used in combination with the linkage mapping panel IV_B4b (Illumina, San Diego, CA, USA) [11], which consists of four panels. Panel I covers chromosomes 1, 2, 3 and 22; panel II covers chromosomes 5, 6, 7, 8 and 9; panel III covers chromosomes 10, 11, 12, 13, 14, 15 and 21; and panel IV covers chromosomes 4, 16, 17, 18, 19, 20, X and Y. The GoldenGate assay was performed according to the manufacturer's protocol, with minor adjustments: 1 µg input DNA was used for multi-use activation and resuspended in 60 µl RS1 [17]. Genotypes were extracted using GenCall (version 6.0.7) and GTS Reports (version 4.0.10.0; Illumina). Tumours t3, t9, t10 and t14 could only be analysed for copy number abnormalities, since corresponding normal tissue was unavailable to determine cnLOH. For t1, t17.2 and t18 only three panels could be analysed, due to limited availability of the FFPE tumour DNA. We corrected for this missing information in our calculations.

Analysis of copy numbers and loss of heterozygosity

Copy number and cnLOH profiles were generated by analysing the carcinomas and corresponding normal tissue in 'Beadarray SNP' [12]. Criteria for the scoring of copy number aberrations were based on previous experiments [12]. LOH was determined as follows. The ratio between the GenCall Score (GCS) and the GenTrain Score (GTS) was computed as a relative measure for the quality of the clustering of the SNP. All high-quality heterozygous SNPs (GCS/GTS >0.8) in the normal sample were included in the analysis. For homozygous SNPs and those with a GCS/GTS <0.8 in the tumour, LOH was assigned. LOH at one or two SNPs was ignored. In practice, regions of LOH always presented as stretches of markers showing LOH. When both a copy number change and LOH were detected at a specific region, the detected LOH was considered to be a consequence of the copy number alteration. If no copy number change was detected, LOH was interpreted as cnLOH.

For verification, conventional LOH analysis was performed for chromosomes 17p and 18q, using microsatellite markers (D17S938, D17S921, D18S877, D18S65, D18S460 and D18S1137) in pure tumour DNA of five MAP carcinomas obtained after flow sorting. Normal DNA was used as a reference. As a positive control, one sporadic carcinoma with known physical loss of chromosomes 17p and 18q was included. A standard PCR protocol was used for amplification. Mixtures of 9.5 µl HiDi formamide, 0.5 µl ROX 500 size standard and 2.0 µl PCR product were run on an ABI 3130 Genetic Analyser (Applied Biosystems) and analysed using GeneMapper version 4.0 (Applied Biosystems).

Interphase fluorescence *in situ* hybridization (FISH)

FISH was performed on flow-sorted nuclei that were spotted onto glass slides, as described previously [18]. The nuclei of five carcinomas were hybridized with a BAC on 17p13.1 (RP11-199F11, spanning the *p53* locus), a BAC on 18q21.1 (RP11-748M14, spanning the *SMAD2* locus) and centromere probes for chromosome 17 and 18. For all carcinomas 50 nuclei were scored. For heterogeneous tumours, each cell population that represented at least one-third of the scored nuclei was considered as a separate fraction.

Statistics

The amount of chromosomal aberrations identified in the MAP carcinomas and the sporadic carcinomas was compared using a Mann–Whitney U-test for independent samples. The analyses were performed using SPSS 12.0.1.

Results

We studied a series of 26 Dutch carcinomas from 19 biallelic *MUTYH* mutation carriers. All patients

were diagnosed with >10 colon polyps (median age at diagnosis 49 years, range 37–77 years), ranging from 10–50 polyps to polyposis with >50–100 polyps. The carcinomas were predominantly located proximal to the splenic flexura (15/24 reported) (Table 1). Most carcinomas were stage II (11/26 or 42%) or stage III (9/26 or 35%); 68% of the MAP carcinomas contained a somatic mutation in *KRAS* (16/17 mutations: c.34 G > T, p.Gly12Cys). A low level of mutations (12%) in the mutation cluster region of *APC* was identified and all carcinomas were microsatellite-stable (data not shown).

Using SNP arrays suitable for analysis of FFPE tissue, we were able to study the 26 carcinomas for genome-wide copy number abnormalities and genome-wide copy-neutral loss of heterozygosity (cnLOH) (see Supplementary Table 1, available at: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2375.html). Remarkably, this analysis revealed that 71% of all changes in the MAP carcinomas concerned cnLOH, whereas only 29% comprised copy number abnormalities (mainly chromosomal gains). On average, 5.1 (range 1–14) cnLOH events were identified per carcinoma. The cnLOH involved chromosome arms or complete chromosomes, but cnLOH of smaller chromosomal regions was also frequently identified. The regions most commonly affected by cnLOH in these tumours were chromosome 17p (57%), 18q (52%) and 15q (52%). Copy-neutral LOH was also frequently present at chromosome 6p (36% of the carcinomas). Lower frequencies of cnLOH were found for chromosomes 4p (24%), 4q (29%), 6q (23%), 8p (23%), 10q (24%), 18p (24%), 21q (24%) and 22q (29%) (Figure 1, Supplementary Table 1).

The MAP carcinomas we studied displayed only a few copy number abnormalities (on average 2.5, range 0–9). This is in contrast to sporadic colorectal cancer, where many chromosomal gains and losses are generally seen [4]. In all patients, the tumours showed five or fewer changes, except for patients 10 and 11, who showed eight, nine and six aberrations, respectively. Gain of chromosome 13q was the most prevalent aberration, seen in 9/26 (35%) carcinomas. Chromosome 11q was amplified in 6/26 (23%) carcinomas. Very limited physical chromosomal loss occurred in the MAP carcinomas (Figure 1, Supplementary Table 1).

The absence of gross chromosomal copy number alterations in our series of MAP carcinomas may reflect a near-diploid genome. For 23 carcinomas, we were able to measure ploidy status using flow cytometry. This analysis concluded that 12/23 (52%) MAP carcinomas were, indeed, near-diploid (DNA index, 1.0 ± 0.1). We found three cases with a near-triploid DNA index (1.5 ± 0.1). In addition, flow cytometry revealed that seven carcinomas contained two fractions, each with a different DNA index. In all seven of these carcinomas, one of the fractions was near-diploid, while the other fraction had a DNA index of 1.5 in five of the seven cases. Tumour 10 consisted of two metastases from the same primary tumour, each with a different DNA ploidy (Table 1).

We further compared the 19 MAP carcinomas to the CIN profile of sporadic carcinomas (Figure 1). For accurate comparison, we used a series of 22 microsatellite-stable sporadic carcinomas with CIN that were analysed previously using the same SNP methodology [14] and displayed the typical CIN profile of sporadic carcinomas [4]. Our comparison showed that the amount of cnLOH in MAP carcinomas

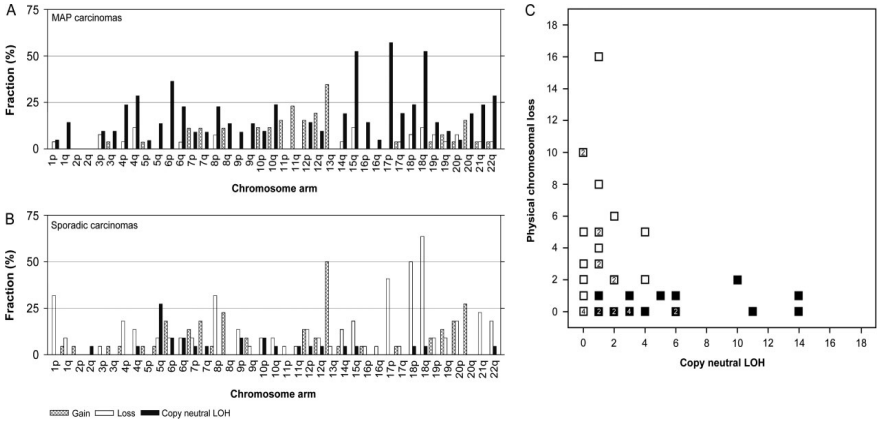

Figure 1. Chromosomal aberrations in MAP carcinomas versus sporadic CRCs. (A, B) The bars indicate the percentage of the 26 MAP carcinomas and 22 sporadic carcinomas, respectively, that exhibit an event of gain, loss or cnLOH of a chromosome. This percentage has been calculated for the respective chromosome arms. White bars, chromosomal gains; checked bars, physical losses of chromosomes; black bars, cnLOH. (C) In this graph the number of cnLOH events versus the number of physical losses is depicted for the 19 MAP carcinomas for which all genomic information was collected (see Materials and methods) versus 22 sporadic carcinomas. White squares, sporadic carcinoma; black squares, MAP carcinoma. The numbered squares represent multiple carcinomas that share the same amount of copy-neutral LOH and physical chromosomal loss

is significantly increased compared to sporadic carcinomas ($p < 0.001$). Moreover, the amount of physical chromosomal losses is significantly ($p < 0.001$) decreased compared to sporadic carcinomas (Figure 1). No differences were seen in the number of chromosomal gains between MAP carcinomas and the sporadic carcinomas. The majority of chromosomal events that are targeted by cnLOH in MAP comprise physical loss instead of cnLOH in sporadic CRC.

The observed pattern of cnLOH versus physical loss was confirmed for five representative MAP carcinomas (t2, t4, t10, t12 and t18) after flow sorting, by FISH for chromosome 17p and 18q on tumour nuclei, in combination with LOH analysis using microsatellite markers. One sporadic carcinoma was included as a control (Table 2). The SNP arrays revealed that four of these five MAP carcinomas exhibited cnLOH on chromosome 17p (t2, t4, t12 and t18) and three exhibited cnLOH on chromosome 18q (t2, t12 and t18). Two MAP cases and the sporadic CRC displayed physical loss of chromosomes 17p and/or 18q. All FISH results that could be obtained were in agreement with our estimation based on the DNA index in combination with the SNP array results. For example, in the tumours with a near-diploid genome content, two copies of chromosome 17p and 18q were identified by FISH in case of cnLOH and in tumours with a near-triploid genome three copies were identified in case of cnLOH (Figure 2). However, within MAP carcinoma t18 (DI = 1.4) only half of the tumour nuclei showed three chromosomal arms of 18q, indicating intratumour heterogeneity. The sporadic carcinoma also harboured two cell populations, with different copy numbers on chromosomal arms 17p and 18q. LOH was unambiguously identified for all informative microsatellite markers in all these cases, also in the cases with cnLOH in the context of a triploid genome content (implying the presence of three copies of a single allele), except for D17S921 in the diploid fraction of MAP carcinoma t4, which showed retention. These results are concordant with the results obtained with the SNP array analysis.

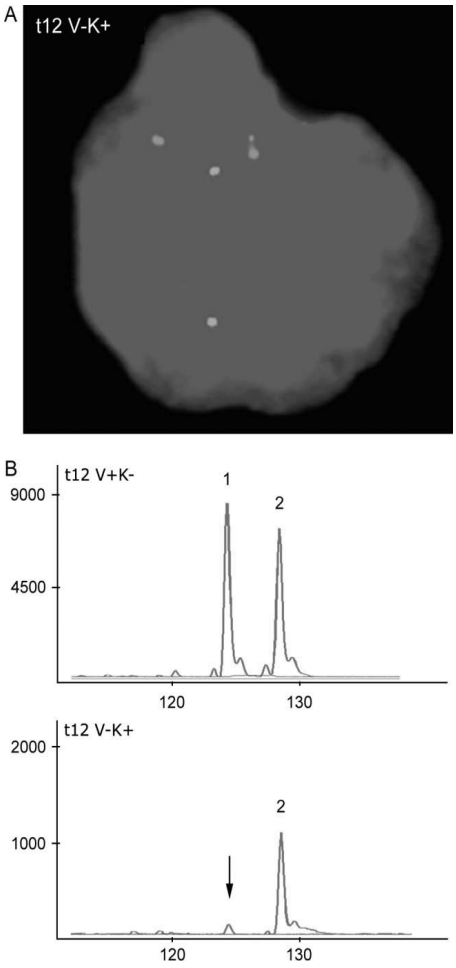

Figure 2. Microsatellite LOH analysis and fluorescent *in situ* hybridization on chromosome 18q21.1 after flow sorting of MAP carcinoma t12 (see also Table 2). (A) FISH showed two centromeric chromosome 18 signals (red) and two signals on 18q21.1 (green) for MAP carcinoma t12 (DNA index = 1.0). (B) Microsatellite LOH analysis (D18S877) on the flow-sorted MAP carcinoma t12 is shown: (upper panel) vimentin-positive, keratin-negative (normal) fraction; (lower panel) the vimentin-negative, keratin-positive (tumour) fraction. Unambiguous LOH is seen of allele 1 in the tumour. In combination with the FISH result shown in (A), copy-neutral LOH for chromosome 18q can be concluded

Discussion

Three studies have reported on the genetic profiles of MAP tumours [3,9,10]. Unfortunately, the results of these studies are seemingly contradictory. Copy number changes in adenomas have been reported, as well as near-diploidy in adenomas and carcinomas. In order to gain more insight into the genetic instability in MAP tumours we studied a series of 26 MAP carcinomas using SNP array analysis in FFPE tissue. In contrast to sporadic colorectal cancer, copy-neutral LOH (cnLOH) appears to be a prevalent characteristic of MAP carcinomas, while only a few copy number abnormalities were identified (4). However, the percentage of chromosomal gains (24%) is comparable to sporadic colorectal cancers with CIN. Such a genomic tumour profile of colon cancer has, to our knowledge, not been described before. With the recent availability of SNP arrays, more detailed information can be obtained on genome-wide cnLOH and several studies now report on cnLOH in cancers [19,20]. However, no study has described cnLOH to the extent seen in our series of MAP carcinomas.

The relative absence of chromosomal loss in our series of MAP carcinomas indeed reflects a

Table 2. Confirmation of copy-neutral LOH by FISH and microsatellite analysis

		p53 locus (17p)				SMAD4 and SMAD2 locus (18q)					
Tumour	DNA index	SNP array Chr. 17	D17S938	D17S921	FISH 17p	SNP array Chr. 18	D18S877	D18S65	D18S460	D18S1137	FISH 18q
t2 K^+ Dip	1.1	17p cnLOH*	LOH	LOH	na	18pq cnLOH*	LOH	na	na	LOH	na
t2 K^+ An	1.4	17p cnLOH*	LOH	LOH	3/3	18pq cnLOH*	LOH	na	LOH	na	na
t4 K^+ Dip	1	17pq cnLOH*‡	U	R	2/2	18pq phLoss*	na	LOH	LOH	U	2/2†
t4 K^+ An	1.5	17pq cnLOH*‡	U	LOH	3/3	18pq phLoss*	LOH	LOH	LOH	U	
t10 K^+	1.5	17p phLoss	U	U	na	18q phLoss	LOH	LOH	U	na	na
t12 K^+	1	17p cnLOH	LOH	LOH	2/2	18pq cnLOH	LOH	na	LOH	na	2/2
t18 K^+	1.4	17p cnLOH	U	LOH	3/3	18pq cnLOH	LOH	LOH	U	LOH	2/2, 3/3
sp1 K^+	1	17p phLoss	U	LOH	2/1, 1/1	18pq phLoss	LOH	na	U	na	2/2, 1/1

Microsatellite LOH analysis and FISH after flow sorting of five MAP carcinomas and one sporadic carcinoma was concordant with our estimation based on the DNA index and SNP array results. Chr., chromosome, K^+, keratin-positive, vimentin-negative (tumour) fraction after flow sorting; Dip, diploid fraction; An, aneuploid fraction; PhLoss, physical loss; cnLOH, copy-neutral LOH. For the LOH analysis: LOH, loss of heterozygosity; R, retention of both alleles; U, uninformative; na, could not be analysed for technical reasons. For the FISH results, the first number indicates the amount of centromeres and the second number indicates the amount of chromosomal arms 17p and 18q, respectively.

* Assay performed on unsorted tumour material.

† The FISH for t4 on chromosome 18q was, due to technical limitations, not performed on flow-sorted tumour nuclei, but on a tissue slide.

‡ cnLOH of complete chromosome 17.

near-diploid genome. Ploidy analysis using flow cytometry concluded that 12/23 (52%) MAP carcinomas analysed were near-diploid (DNA index, 1.0 ± 0.1). Lipton *et al* [3] found a near-diploid genome in 12/13 MAP carcinomas tested, with one carcinoma showing a polyploid status. We found three cases with a near-triploid DNA index (1.5 ± 0.1). In addition, flow cytometry revealed that seven carcinomas contained two fractions, each with a different DNA index. In all seven of these carcinomas, one of the fractions was near-diploid, while the other fraction had a DNA index of 1.5 in five of the seven cases. Interestingly, the distribution of the DNA ploidy of the MAP carcinomas is very different from sporadic colorectal cancers, which are primarily highly aneuploid. A DNA index of $\sim$1.5 is uncommon in sporadic CRC, although near-triploidy has been described for sporadic CRC [21,22]. We confirmed the scoring of our SNP results in a purified set of tumours by a combination of FISH and LOH analysis, using polymorphic chromosomal microsatellite markers on chromosomes 17p and 18q. In the tumours with a near-diploid genome content, two copies of chromosome 17p and 18q were identified by FISH in case of cnLOH, and in tumours with a near-triploid genome three copies of a single allele were identified in case of cnLOH. Possible limited sensitivity in detecting copy number aberrations, especially in heterogeneous tumours, is unlikely in view of the FACS sorting in combination with FISH and conventional LOH analysis. Moreover, the results we obtained on the sporadic CRC are reassuring in this respect, since these are analysed and scored in exactly the same way as the MAP tumours.

Recently, we studied by SNP analysis of FFPE tissue a series of microsatellite-unstable sporadic and Lynch syndrome colon carcinomas, often with a near-diploid DNA content. All MSI-H carcinomas showed few chromosomal aberrations. CnLOH was infrequent in these tumours and usually confined to the locus harbouring a pathogenic mutation in *MLH1, MSH2* or *PMS2* [23]. These results further underline the uniqueness of the phenotype of the MAP carcinomas.

Interestingly, the cnLOH events identified in the MAP carcinomas frequently involve the same chromosomes affected by physical loss in sporadic colorectal cancer, indicating that the same tumorigenic pathway may be involved in tumour initiation and progression. For example, chromosomes 17p and 18q are commonly affected by physical loss in sporadic colorectal cancer, whereas cnLOH is identified primarily on these chromosome arms in MAP carcinomas. How frequent the genes that are targeted in sporadic colorectal cancer on these respective chromosomes, e.g. *p53* and the *SMAD* genes, are targeted in MAP carcinomas remains elusive. Lipton *et al* found only three *p53* (located on chromosome 17) somatic mutations in 14 MAP carcinomas analysed, although immunohistochemistry for p53 over-expression (indicative for mutation) was positive in four tumours that were negative for mutation testing. *SMAD4* mutations on 18q were not found in the MAP carcinomas analysed by Lipton *et al* [3], although analysis of two chromosome 18q microsatellite markers showed a high frequency of 18q LOH in 7/14 cases analysed.

Our studies also indicate that chromosome 15q is often targeted by cnLOH in the MAP carcinomas. Physical loss of this chromosome has been associated with distant metastasis of sporadic colorectal cancer [4].

Copy-neutral LOH can arise via mitotic recombination, non-disjunction, or deletion and reduplication events. In our series, we identified cnLOH on whole chromosomes and on parts of chromosomes. The high prevalence of cnLOH in MAP carcinomas suggests a relationship between mitotic recombination and the MUTYH deficiency. However, it is difficult to explain why MAP cancers show few copy number aberrations. First, the occurrence of copy-neutral LOH might be

directly linked to BER malfunctioning. Secondly, in parallel to a mismatch repair deficiency, the mutational burden might be relatively high due to the BER defect, favouring mitotic recombination but not physical loss. Therefore, further research into this possible relation is important.

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Supplementary material

Supplementary material may be found at the web address: http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2375.html

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CHAPTER 6

The natural history of a combined defect in *MSH6* and *MUTYH* in a HNPCC family

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The natural history of a combined defect in *MSH6* and *MUTYH* in a HNPCC family

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Abstract In the inherited syndromes, *MUTYH*-associated polyposis (MAP) and hereditary nonpolyposis colorectal cancer (HNPCC), somatic mutations occur due to loss of the caretaker function that base-repair (BER) and mismatch repair (MMR) genes have, respectively. Recently, we identified a large branch from a *MSH6* HNPCC family in which 19 family members are heterozygous or compound heterozygous for *MUTYH* germ line mutations. *MSH6/MUTYH* heterozygote mutation carriers display a predominant HNPCC molecular tumour phenotype, with microsatellite instability and underrepresentation of G>T transversions. A single unique patient is carrier of the *MSH6* germline mutation and is compound heterozygote for *MUTYH*. Unexpectedly, this patient has an extremely mild clinical phenotype with sofar only few adenomas at age 56. Four out of five adenomas show characteristic G>T transversions in *APC* and/or *KRAS2*, as seen in *MUTYH* associated polyposis. No second hit of *MSH6* is apparent in any of the adenomas, due to retained MSH6 nuclear expression and a lack of microsatellite instability. Although this concerns only one case, we argue that the chance to find an additional one is extremely small and currently a mouse model with this genotype combination is not available. Moreover, the patients brother who is also compound heterozygous for *MUTYH* but lacks the *MSH6* germline mutation presented with a full blown polyposis coli. In conclusion, these data would support the notion that abrogation of both MSH6 DNA mismatch repair and base repair might be mutually exclusive in humans.

Keywords Base excision repair · Colorectal cancer · HNPCC · Mismatch repair · *MUTYH* · Urinary tract

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Abbreviations

BER	Base excision repair
MMR	Mismatch repair
MAP	*MUTYH*-associated polyposis
HNPCC	Hereditary nonpolyposis colorectal cancer
8-oxoG	8-oxo-guanine
CRC	Colorectal cancer
MCR	Mutation cluster region
MSI	Microsatellite instability
LOH	Loss of heterozygosity
IHC	Immunohistochemistry
MSS	Microsatellite stable

Introduction

Somatic genetic alterations direct the development of colorectal malignancies. In the majority of cases, such mutations occur in an apparently sporadic context.

In a group of distinct inherited syndromes however, many somatic mutations occur as a consequence of the loss of caretaker function of the base-repair (BER) or mismatch repair (MMR) systems in, *MUTYH*-associated polyposis (MAP) and hereditary nonpolyposis colorectal cancer (HNPCC), respectively [1, 2]. Loss of MMR function is also seen in 15% of sporadic colorectal cancer (CRC) due to promoter methylation [3].

BER is a multi-step process that repairs frequently occurring 8-oxo-guanine (8-oxoG) DNA lesions [4]. Until recently inherited deficiencies in the BER pathway had not been causally linked with any human genetic disorder. However, in 2002 it was discovered that biallelic mutations in *MUTYH* (formerly *MYH*) lead to the autosomal recessive syndrome exerting adenomatous colorectal polyposis and CRC [1]. The MMR pathway consists of a highly conserved set of proteins in humans, which are primarily responsible for the post-replicative correction of nucleotide mispairs and extra-helical loops. The MMR system includes *hMLH1* and *hPMS2*, which form a heterodimer (hMutLα) and *hMSH2* and *hMSH6*, forming the hMutSα-heterodimer. hMutsSα has been shown to bind specifically to G*T DNA mismatches, other base–base DNA mismatches and to 1-, 2- or 3 nucleotide insertion–deletion loops [5]. Germline mutations in one of the MMR genes underlie the autosomal dominant HNPCC syndrome.

Due to the reduced ability of mutant *MUTYH* to recognize and repair A/8-oxoG mismatches, in tumours of MAP patients specific G:C>T:A somatic transversions can be found in genes such as *APC* and *KRAS2* with an incidence of up to 40 and 60%, respectively [6]. In *APC* the G>T transversions appear to have a preference for G bases in GAA sequences whereas in *KRAS2* a preferential GGT>TGT [c.34G>T, p.Gly12Cys] transition of codon 12 can be found [1, 7].

In MMR deficiency apart from the frameshift mutations in repetitive DNA stretches, under representation of G>T transversions and possibly preferential G>A somatic alterations in *APC* and *KRAS2* are found, this in contrast to the G>T transversions in BER deficiency [8, 9].

Although *MUTYH* is the most important cellular player in the removal of adenine in an A/8-oxoG mismatch, also MMR has been shown to play a role since *MSH2* and *MSH6* are activated upon recognition of 8-oxoG [10, 11]. Moreover, it was recently demonstrated that amino acid residues 232–254 of *MUTYH* interact with MutsSα via *MSH6* and this interaction stimulates the glycosylase activities of *MUTYH* [12].

In order to determine the effect of different combinations of BER and MMR defects we studied the branch of a HNPCC family in which *MSH6* and *MUTYH* germline mutations co-segregate [13]. Nineteen family members are heterozygous or compound heterozygous for [c.494A>G, p.Tyr165Cys] and/or [c.1145G>A, p.Gly382Asp] in *MUTYH*, 11 also carry a pathogenic *MSH6* [c.1784del T, p.Leu595fs] germline mutation. We analysed the somatic mutation spectrum of *APC* and *KRAS2*, microsatellite instability including *MUTYH/OGG1* repeats, MSH2/MSH6 protein expression and studied the clinical phenotype.

Materials and methods

Patients

We studied a branch of a Dutch HNPCC family in which *MSH6* and *MUTYH* germline mutations co-segregate (Fig. 1, Table 1) [12]. Cases were analysed following the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences; http://www.fmwv.nl/gedragscode/goedgebruik/code.

Germline mutation analysis

Mutation analysis was performed as described for *MSH6* and *MUTYH* [13, 14]. For further details see http://www.lumc.nl/4080/DNA/MSH6.html and http://www.lumc.nl/4080/DNA/MUTYH.html.

DNA isolation

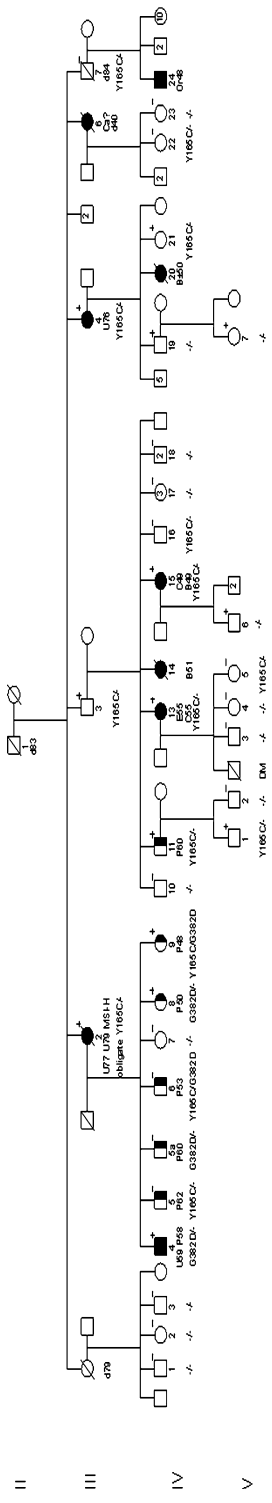
From nine patients 18 tumours were collected. Genomic DNA of normal colon and colorectal tumour tissue was extracted from paraffin embedded material as described [15].

Microsatellite instability (MSI) analysis

Microsatellite analysis was performed as described [15].

APC and *KRAS2* somatic mutation analysis

Samples were screened for the presence of mutations in the Mutation Cluster Region (MCR) codons 1286–1513 of *APC* and for mutations in codon 12 and 13



◀ **Fig. 1** Pedigree of a HNPCC family in which *MSH6* and *MUTYH* germline mutations co-segregate. *Abbreviations*: C, colorectal cancer; E, endometrial cancer; U, urinary tract cancer; P, polyp; B, breast cancer; Or, Oral squamous cell carcinoma; DM, diabetes mellitus; +, carrier of *MSH6* [c.1784delT, p.Leu595fs] mutation, –, wt *MSH6*, –/–, *MUTYH* mutation negative. *Note*: The pedigree is slightly different depicted than the one previously published because of some minor intentional changes in the latter (i.e. the number of unaffected siblings and one patient with C32 belonging to the other branch) for privacy reasons. For further questions the corresponding author can be contacted [12]

of *KRAS2*, by sequencing analysis as described [16]. For detection of known HNPCC associated somatic mutations outside the MCR of *APC*, eight different primersets for eleven target sequences were used (Table 2) [9]. PCR is performed under standard conditions (33 cycles with an annealing temperature of 60°C) PCR products were sequenced at the Leiden Genome Technology Center (LGTC; http://www.lgtc.nl) and analysed with the Mutation Surveyor software package (Softgenetics, State College, PA).

Loss of heterozygosity (LOH)

Analysis was done by direct sequencing as described [17]. PCR was performed on DNA from paired tumour and normal tissue under standard conditions with primer sets for [Tyr165Cys] and [Gly382Asp] as described in Table 2.

Microsatellite analysis of *MUTYH/OGG1*

Analysis of repeats in *MUTYH* and *OGG1* was done by direct sequencing. PCR was performed under standard conditions with primer sets for 2 (A)5 repeats in the coding region of *MUTYH* of which one is known to be located in the binding site of *PCNA* [18]. In the coding region of *OGG1*, two repeats were tested; a (C)5 and a (T)5 repeat, primers described in Table 2.

Immunohistochemistry (IHC) of MSH6 and MSH2

Staining of the MMR proteins was done as described [15].

Results

The clinical phenotype of the HNPCC family (Fig. 1) in which *MSH6* and *MUTYH* germline mutations co-segregate is described in Table 1 [12]. The molecular characteristics are summarized in Table 3.

Table 1 (Pre) malignant tumours in the extended HNPCC family in which *MSH6* and *MUTYH* germline mutations co-segregate

Patient	Tumour	Age at diagnosis	Age 12-2005	*MSH6* mutation	*MUTYH* mutation
III.2	Transitional cell carcinoma right renal pelvis and transitional cell carcinoma left ureter	77	d89	+[a]	[Tyr165Cys]+[=][a]
III.3	None	79	FU ends at 86	+	[Tyr165Cys]+[=]
III.4	Transitional cell carcinoma renal pelvis	76	93	+	[Tyr165Cys]+[=]
III.6	Anamnestic carcinoma	40	d40	na	na
III.7	Unknown		d84	wt	[Tyr165Cys]+[=]
IV.4	Transitional cell carcinoma ureter and anamnestic 1 polyp of the colon (adenomatous)	59	66	+	[–]+[Gly382Asp]
IV.5	4 Polyps left-sided (adenomatous and hyperplastic)	62	69	wt	[Tyr165Cys]+[=]
IV.5a	1 Hyperplastic polyp	60	68	wt	[=]+[Gly382Asp]
IV.6	Polyposis coli; > 100 adenomatous polyps	53	61	wt	[Tyr165Cys] + [Gly382Asp]
IV.8	2 Polyps (adenomatous and hyperplastic polyp)	50	58	+	[–]+[Gly382Asp]
IV.9	5 Adenomas	48	56	+	[Tyr165Cys]+[Gly382Asp]
IV.11	Tubulovillous adenoma	60	66	+	[Tyr165Cys]+[=]
IV.13	Endometrial carcinoma and rectal carcinoma	55	65	+	[Tyr165Cys]+[=]
IV.14	Breast carcinoma (ductal, invasive)	51	d52 (±)	na	na
IV.15	Breast carcinoma and colon carcinoma	49	55	+	[Tyr165Cys]+[=]
IV.16	None		61	wt	[Tyr165Cys]+[=]
IV.19	None		59	+	wt
IV.20	Breast carcinoma	±50	d50 (±)	na	na
IV.21	None		58	+	[Tyr165Cys]+[=]
IV.22	None		48	wt	[Tyr165Cys]+[=]
IV.24	Oral squamous cell carcinoma	48	FU ends at 48	na	na
V.1	None		34	+	[Tyr165Cys]+[=]
V.5	None		32	wt	[Tyr165Cys]+[=]
V.6	None		30	+	wt
V.7	None		30	+	wt

Abbreviations: d, death; +, carrier of *MSH6* [c.1784delT, p.Leu595fs] mutation; FU, follow up; na, not analysed; wt, wild type

[a] Obligate carrier

Table 2 Primers used for HNPCC related *APC* mutation screening, *MUTYH* LOH analysis and MSI analysis in *MUTYH* and *OGG1*

Primer	*APC* nucleotide	5′–3′ forward	5′–3′ reverse	Annealing temperature
Ca6 and Ca18	731–786	gcaaataggcctgcgaagta	gatgagatgccttgggactt	58
Co8/K39 and Cx7	780–860	cccaaggcatctcatcgtag	tagaccaattccgcgttctc	58
K10	877–930	tttgcagatctccaccactg	tatgggcagcagagcttctt	58
Co86 and Co39	923–986	aagaagctctgctgcccata	ggattcaatcgagggtttca	58
Cx10	1901–1966	acctccaaccaacaatcagc	tgagaaaagcaaaccggagt	58
22–18	1525–1585	atgcctccagttcaggaaaa	tgttggcatggcagaaataa	58
Co88	1768–1828	gaaaaagaaaccaacttcacca	tgggagcttatcattgaagacc	58
Co10	1093–1160	tggacagcaggaatgtgttt	ttggtctctcttcttcttcatgc	58
MUTYH [Tyr165Cys]		cccacaggaggtgaatcaact	gttcctaccctctgccatc	60
MUTYH [Gly328Asp]		ggcagtggcatgagtaacaag	cttgcgctgaagctgctct	60
MUTYH (A)5 repeat (*PCNA* binding site)		ctacaaggcctccctccttc	ctgcactgttgaggctgtgt	60
MUTYH (A)5 repeat		aagtatatgggctggccttg	caacaaagacaacaaaggtagtgc	60
OGG1 (C)5 repeat		aaaggtggctgactgcatct	tttcctcacccagttccttg	60
OGG1 (T)5 repeat		gggtcagataacttagtctcatcactt	aggaaacctagggaggacacc	60

Heterozygous *MUTYH* [Tyr165Cys] mutation carriers with a wild type *MSH6* germline status

Patient IV.5 developed four colon polyps, whereas three other family members; IV.16, IV.22 and V.5 show no abnormalities. From patient III.7 the tumour status is unknown. Two polyps (one hyperplastic and one adenoma) from patient (IV.5), displayed a microsatellite stable (MSS) phenotype and expressed MSH6 and MSH2. The adenoma showed a [c.35G>A,

Table 3 Clinical information and molecular characteristics

Category	Patient number	Age of diagnosis	Age 12-2005	Gender	*MSH6* germline mutation[a]	*MUTYH* germline amino acid change	LOH MUTYH	MSI	MSI repeat *MUTYH/OGG1*	*APC* somatic mutation	*APC* amino acid change	*KRAS2* somatic mutation	*KRAS2* amino acid change	MSH2 staining	MSH6 staining	Tumour
A	IV.5	62	69	M	wt	[p.Tyr165Cys] +[=]	no	S	no	wt	wt	wt	wt	+	+	Sigmoid HP
A	IV.5	62				[p.Tyr165Cys] +[=]	no	S	no	wt	wt	[c.35G>A] +[=]	[p.Gly12Asp] +[=]	+	+	Rectal, tub. vill. ad. LG
B	IV.13	56	65	F	+	[p.Tyr165Cys] +[=]	no	H	no	wt[b]	wt	wt	wt	0	na	Rectal ca.
B	IV.13	56					no	H	no	wt[b]	wt	wt	wt	+	na	Endometrial ca.
B	IV.15	49	55	F	+	[p.Tyr165Cys] +[=]	no	L	no	[c.4487_4499del CTCCAGA-TGGATT]+[=][c]	[p.Thr1496fs] +[=]	[c.34G>T] +[=]	[p.Gly12Cys] +[=]	+	0	Colon ca. left
B	IV.15	49				[p.Tyr165Cys] +[=]	no	S	no	[c.4487_4499del CTCCAGA-TGGATT]+[=][c]	[p.Thr1496fs] +[=]	[c.34G>T] +[=]	[p.Gly12Cys] +[=]	+	0	Colon ad. left[d]
B	IV.15	49					no	H	no	wt[c]	wt	wt	wt	+	0	Breast ca. left
B	III.4	76	93	F	+	[p.Tyr165Cys] +[=]	no	L	no	na	na	[c.34G>T] +[=]	[p.Gly12Cys] +[=]	+	0	Renal pelvis, pap. transitional cell ca., Gr III
B	III.2	77	d89	F	+[e]	[p.Tyr165Cys] +[=][e]	nma	H	nma	nma	nma	wt	wt	+	0	Ureter left, pap. transitional cell ca., GrII
B	III.2	79				[p.Tyr165Cys] +[=][e]	nma	H	nma	wt	wt	wt	wt	na	na	Renal pelvis right, transitional cell ca. GrII

Table 3 continued

Category	Patient number	Age of diagnosis	Age 12-2005	Gender	*MSH6* germline mutation[a]	*MUTYH* germline amino acid change	LOH MUTYH	MSI	MSI repeat *MUTYH/OGG1*	*APC* somatic mutation	*APC* amino acid change	*KRAS2* somatic mutation	*KRAS2* amino acid change	MSH2 staining	MSH6 staining	Tumour
C	IV.4	59	66	M	+	[=]+[p.Gly382Asp]	no	H	no	wt	wt	wt	wt	+	0	Distal ureter right, transitional cell ca. GRII
C	IV.8	50	58	F	+	[=]+[p.Gly382Asp]	no	S	no	[c.4475_4476-delCC]+[=]	[p.Ala1492fs] +[=]	wt	wt	+	+	Colon tub. ad. LG
D	IV.6	53	61	M	wt	[p.Tyr165Cys] +[p.Gly382Asp]	no	S	no	wt	wt	[c.34G>T] +[=]	[p.Gly12Cys] +[=]	+	+	Polyposis coli with HG
E	IV.9	48	56	F	+	[p.Tyr165Cys] +[p.Gly382Asp]	no	S	no	wt	wt	[c.34G>T] +[=]	[p.Gly12Cys] +[=]	+	+	Sigmoid ad. LG
E	IV.9	54					no	S	no	[c.4612G>T] +[=]	[p.Glu1538X] +[=]	wt	wt	+	+	Rectal villous ad. HG
E	IV.9	54					no	S	no	[c.4618G>T] +[=]	[p.Glu1540X] +[=]	[c.34G>T] +[=]	[p.Gly12Cys] +[=]	+	+	Caecum villous ad. LG
E	IV.9	54					no	S	no	[c.4612G>T] +[=]	[p.Glu1538X] +[=]	wt	wt	+	+	Rectal villous ad. LG
E	IV.9	54					no	S	no	wt	wt	[c.38G>A] +[=]	[p.Gly13Asp] +[=]	+	+	Caecum villous ad. LG

Abbreviations: M, male; F, female; na, not analysed; nma, no material available; wt, wild type; ad, adenoma; ca, carcinoma; HP, hyperplastic; HG, high grade dysplastic; LG, low grade dysplastic

Note: Tumours were categorized based different on germline mutation combinations. Category A; heterozygous *MUTYH* [Tyr165Cys] mutation carrier with wild type *MSH6* germline status. Category B; heterozygous *MUTYH* [Tyr165Cys] mutation carriers with *MSH6* [c.1784delT, p.Leu596fs] germline mutation. Category C; heterozygous *MUTYH* [Gly382Asp] mutation carriers with *MSH6* [c.1784delT, p.Leu596fs] germline mutation. Category D; compound heterozygous *MUTYH* [Tyr165Cys, Gly382Asp] mutation carrier with wild type *MSH6* germline status. Category E; compound heterozygous *MUTYH* [Tyr165Cys, Gly382Asp] mutation carrier with *MSH6* [c.1784delT, p.Leu596fs] germline mutation

[a] *MSH6* [c.1784delT, p.Leu595fs] mutation

[b] SNP rs 41115 heterozygote [c.4479G>A]
+[=]

[c] SNP rs 41115 homozygote [c.4479G>A]+[c.4479G>A]

[d] Precursor adenoma next to carcinoma

[e] Obligate carrier

p.Gly12Asp] *KRAS2* mutation. No *APC* somatic mutations were detected (Table 3, category A).

Heterozygous *MUTYH* [Tyr165Cys] mutation carriers with a *MSH6* [c.1784del T, p.Leu595fs] germline status

Five of eight mutation carriers, showed a diverse spectrum of tumour types (Table 3) including colon adenomas (IV.15, IV.11), a colon and a breast carcinoma (IV.15), a rectum and a endometrium carcinoma (IV.13), two papillary transitional cell carcinomas of the renal pelvis (III.4, III.2) and one of the ureter (III.2). Three family members V.1, IV.21, and III.3 did so far not present with any HNPCC or MAP associated lesion. Five tumours (a rectum, endometrium, breast renal pelvis papillary transitional cell and ureter papillary transitional cell carcinoma) of three patients (IV.13, IV.15, III.2) are MSI-High with diminished or abrogated MSH2 staining or abrogation of MSH6 staining if tested. No *KRAS2* and *APC* somatic mutation was identified in three of the five tumours. Two tumours however, of patients IV.15 and III.4; a colon carcinoma including its precursor adenoma and a papillary transitional cell carcinoma, showed limited or no instability, with minor shifts of *BAT25* and *BAT40*. Nonetheless MSH6 staining was abrogated. Surprisingly only in these latter tumours the typical, MAP associated [c.34G>T, p.Gly12Cys] *KRAS2* mutation was found. In both the colon carcinoma and its precursor adenoma, a somatic deletion of 13 nucleotides in *APC* was identified (Table 3, category B).

Heterozygous *MUTYH* [Gly382Asp] mutation carrier with a wild type *MSH6* germline status

One patient (IV.5a) presented with one hyperplastic polyp, not further molecular characterized.

Heterozygous *MUTYH* [Gly382Asp] mutation carriers with a *MSH6* [c.1784del T, p.Leu595fs] germline status

Patient IV.4 showed a transitional cell carcinoma, patient IV.8 showed one low-grade dysplastic adenoma. The papillary transitional cell carcinoma of IV.4 tested MSI-High with abrogation of MSH6 expression. No mutations in *KRAS2* or *APC* were identified. A low-grade dysplastic adenoma from IV.8 showed a MSS phenotype with retained MSH6 staining. No somatic mutation in *KRAS2* was identified. In *APC* a [c.4475_4476delCC, p.Ala1492fs] mutation was found (Table 3, category C).

Compound heterozygous *MUTYH* [Tyr165Cys] + [Gly382Asp] mutation carrier with a wild type *MSH6* germline status

Patient IV.6 showed a full-blown polyposis phenotype of colorectal adenomas. In one adenoma the MAP characteristic *KRAS2* mutation; [c.34G>T, p.Gly12Cys] was identified. No somatic mutations were identified in the tested areas of *APC*. As expected, the specimen had a MSS phenotype and showed normal protein expression of MSH2 and MSH6 (Table 3, category D).

Compound heterozygous *MUTYH* [Tyr165Cys,Gly382Asp] mutation carrier with a *MSH6* [c.1784del T, p.Leu595fs] germline status

The phenotype of patient IV.9 with the triple mutations is remarkably mild. The patient to date developed five pathologically verified colon adenomas (Table 3) only one with high-grade dysplasia, the other four are low-grade dysplastic (minimal mucosal changes have been coagulated during endoscopy). All five tumours from patient (IV.9) showed a MSS phenotype and retained nuclear expression of MSH6, suggesting the absence of a second hit in *MSH6*. Two rectum adenomas lack *KRAS2* mutations but carry an *APC* [c.4612G>T, p.Glu1538X] somatic mutation (Table 3, category E). One caecum adenoma carried the *MUTYH* associated somatic *KRAS2* [c.34G>T, p.Gly12Cys] mutation. This specimen also showed a [c.4618G>T, p.Glu1540X] mutation in *APC*. A second caecum adenoma showed a *KRAS2* [c.38G>A, p.Gly13Asp] mutation and no *APC* somatic mutations (Table 3, category E). Although the [Gly13Asp] alteration is found in a low frequency in our *MUTYH* family cohort (data not shown), this mutation represents the most frequent somatic mutation found in *KRAS2* in HNPCC patients with a MMR mutation [8].

In all tested specimens neither LOH of *MUTYH* nor microsatellite instability, in the tested repeats in *MUTYH* and *OGG1*, was detected (Table 3).

Discussion

We identified a branch from a previously described Dutch HNPCC family where *MSH6* and *MUTYH* germline mutations co-segregate. In order to determine the effect of different combinations of BER and MMR defects we analysed somatic mutation spectra of *APC* and *KRAS2*, microsatellite instability including *MUTYH/OGG1* repeats, MSH2/MSH6 protein expression and studied the clinical phenotype.

In this family of the 34 *MSH6* [c.1784del T, p.Leu595fs] mutation carriers 11 also carry a *MUTYH* mutation, of which one bi-allelic [11]. The remaining 23 individuals lack *MUTYH* mutations, either tested or obligatory negative (not taking in account the possibility of a "new" *MUTYH* mutation in this branch, as *MUTYH* mutations are found in 1–2% of the general population) [1, 19].

In individuals with a combined defect in *MSH6* and *MUTYH* (heterozygous) a higher incidence of urothelial cancers was found compared to a *MSH6* defect alone (three out of 10 versus none out of 23, $P = 0.022$ Fisher exact), suggesting that a single *MUTYH* mutation modifies the risk for developing for urothelial cancers in *MSH6* mutation carriers.

A predominant HNPCC molecular phenotype was observed in tumours from patients heterozygous for *MUTYH* and *MSH6* defects, which suggest that a second inactivating somatic hit on *MSH6* took place and MMR deficiency is the leading cause of tumourigenesis in these patients, although in two out of nine tumours the *MUTYH* characteristic [c.34G>T] somatic transversion in *KRAS2* was observed. Microsatellite instability seemed less extensive in the latter cases, with MSH6 expression abrogated. Remarkable is that in one of these two (including the precursor adenoma) a genomic 13 bp *APC* deletion was found not typical for HNPCC. In cases where no *APC* alteration was identified it should be noted that only the major cluster region for somatic mutations in *APC* was screened including published hot spots for specific somatic HNPCC mutations.

Out of eight *MSH6* and *MUTYH* (heterozygous [Tyr165Cys]) mutation carriers two present with late onset tumours (III.2, III.4). The age of onset in three other cases (IV.15, IV.13, IV.11) is lower with five different tumours (three colon tumours) at an age range of 49–60, the remaining three cases did so far not present with tumours (III.3, IV.21, V.1). Croitoru et al. [19] concluded that heterozygote mutation carriers for [Tyr165Cys] have an increased risk (although not significant) for colorectal cancer (CRC) with an odds ratio of 2.1.

The relative mild clinical phenotype of patient IV.9, who is compound heterozygous for *MUTYH* [Tyr165Cys] and [Gly382Asp] and also carrying the *MSH6* germline mutation might be explained, at least in part, by a selection against MSH6 mismatch repair deficient cells. Such is in line with Kambara et al. [20] who suggested that BER and DNA MMR pathways are mutually exclusive implying that cells with abrogation of both pathways are not viable and undergo apoptosis.

The molecular phenotype of the tumours of this patient occur most likely as a result of *MUTYH* dysfunction, while no mismatch repair deficiency seems evident despite the presence of a germline *MSH6* defect. These results are remarkable in view with the natural mutation rate in cells, estimated at 1×10^{-6} cells per gene, per cell division. There are 1×10^{10} epithelial cells in the colon of which potentially one percent is dividing. That would imply that every cell division 10^2 intestinal cells are at risk for a second hit in *MSH6.* In *MUTYH* compound heterozygotes the mutation rate is increased by a factor 100 (10^4 cells are then at risk for a second mutational hit in *MSH6*). So far this does not appear to be the case in the triple mutation case (IV.9). Unfortunately a mouse model with this genotype combination is not available.

Although the number of cases is low, a striking potentiating effect of a combined heterozygote *MSH6* and *MUTYH* mutation status is not evident except perhaps for urothelial tumours. However, recently, a *MUTYH* mutation combined with non-pathogenic (or low penetrant) *MSH6* missense mutation is reported to be associated with an increased cancer risk for colorectal cancer [21]. Other combined defects of *APC* and *MLH1 or MSH2* have been reported to accelerate tumourigenesis (summarized in [22]). The finding of an unexpectedly mild clinical phenotype in an individual with combined *MUTYH* deficiency and a heterozygote pathogenic *MSH6* germline mutation should be seen with caution considering the variable expression of MAP and HNPCC in general. The molecular characteristics of the tumours of this patient studied, however, point to selection against MSH6 abrogation.

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CHAPTER 7

Mass spectrometry-based loss of heterozygosity analysis of single-nucleotide polymorphism loci in paraffin embedded tumors using the MassEXTEND assay

single-nucleotide polymorphism loss of heterozygosity analysis of the protein tyrosine phosphatase receptor type J in familial colorectal cancer

J Mol Diagn. (2005) 7:623-630.

Mass Spectrometry-Based Loss of Heterozygosity Analysis of Single-Nucleotide Polymorphism Loci in Paraffin Embedded Tumors Using the MassEXTEND Assay

Single-Nucleotide Polymorphism Loss of Heterozygosity Analysis of the Protein Tyrosine Phosphatase Receptor Type J in Familial Colorectal Cancer

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As the number of identified single-nucleotide polymorphisms (SNPs) increases, high-throughput methods are required to characterize the informative loci in large patient series. We investigated the feasibility of MassEXTEND LOH analysis using Sequenom's MassArray RT software, a mass spectrometry method, as an alternative to determine loss of heterozygosity (LOH). For this purpose, we studied the c.827A>C SNP (1176A>C p.Gln276Pro) in protein tyrosine phosphatase receptor type-J (*PTPRJ*), which is frequently deleted in human cancers. In sporadic colorectal cancer (CRC), c.827A>C showed allele-specific LOH of the c.827A allele, which is important because LOH of *PTPRJ* may be an early event during sporadic CRC. To elucidate the impact of this low-penetrance gene on familial CRC, we studied c.827A>C in 222 familial CRC cases and 156 controls. In 6.2% of the A/C genotyped CRC samples, LOH of c.827A was observed with MassEXTEND LOH analysis and confirmed by conventional sequencing. Furthermore, a case with LOH of c.827A showed no LOH in 22 synchronously detected adenomas, including one with malignant transformation. The importance of the *PTPRJ*- c.827A>C SNP appears to be limited in familial CRC. We conclude that MassEXTEND LOH analysis (using Sequenom's MassARRAY RT software) is a sensitive, high-throughput, and cost-effective method to screen SNP loci for LOH in formalin-fixed paraffin-embedded tissue. *(J Mol Diagn 2005, 7:623–630)*

Loss of heterozygosity (LOH) analysis has been commonly used to provide (indirect) evidence for the presence of a tumor suppressor gene within a genomic region.[1] Standard LOH studies with polymorphic microsatellite markers compare individual allele intensities of normal and tumor DNA. LOH analysis of specific single-nucleotide polymorphism (SNP), however, requires a different approach such as allele-specific amplification or direct sequencing. The former requires thorough optimization of PCR protocols (especially in cases of A/T polymorphisms), whereas the latter is not quantitative. Furthermore, direct sequencing is labor intensive and expensive, with relatively low throughput. For the characterization of the increasing number of informative SNPs in large patient series, high-throughput methods are required. Moreover, for many such series only formalin-fixed paraffin-embedded (FFPE) tissue is available for retrospective testing.

In this study, we used a novel form of LOH analysis, MassEXTEND LOH analysis based on matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).[2,3] This method is less labor intensive and expensive than sequencing with potential for enormous throughput. MALDI-TOF MS has been used to solve a variety of biochemical and molecular genetic questions.[4] The inherent high-molecular weight resolution of MALDI-TOF MS gives high specificity and good signal-to-noise ratio to perform accurate quantification.

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The MassEXTEND LOH analysis introduced here is based on such quality.[5]

In FFPE tissue from familial colorectal cancer (CRC) cases, we have studied LOH of the c.827A >C SNP in protein tyrosine phosphatase receptor type-J (*PTPRJ*). Recently, MALDI TOF MS genotyping of *PTPRJ* was published including limited LOH analysis. No validation for LOH was done, and the spectra were not automatically analyzed.[6]

PTPRJ is a member of the receptor protein tyrosine phosphatases, which play specific and active roles in setting the levels of tyrosine phosphorylation in cells, and as such, they are important in the regulation of many physiological processes.[7] Furthermore, recent mutation analysis in human colorectal cancer suggests that tyrosine phosphatases may function as "true" tumor suppressor genes regulating a wide variety of pathways, which may be susceptible for therapeutic intervention.[8]. In the mouse, *Ptprj* has been identified as a colon cancer susceptibility gene.[9]. Frequent LOH of the *PTPRJ* locus was shown in human sporadic colorectal, breast, and lung tumors[9] and in human thyroid carcinomas.[10] Additionally, Ruivenkamp et al[11] concluded that LOH of *PTPRJ* frequently occurs in the adenoma stage of sporadic human CRC.

The c.827A>C (also known as 1176A>C) SNP in exon 5 of PTPRJ encodes the p.Gln276Pro amino acid change. Preferential loss of the c.827A versus c.827C allele was described, which suggests that the putative "cancer resistance" A allele is lost whereas the (potential less active) C allele is retained in sporadic colorectal cancer.[9]. In this study, we focused on the feasibility of using MassEXTEND LOH analysis to determine LOH of the c.827A>C SNP in FFPE tumor tissue.

We show that the results obtained with the MassEXTEND LOH analysis (using Sequenom's MassARRAY RT software) are as reliable as conventional sequence methods and document the utility of this new technique to detect LOH of a specific SNP in a sensitive, cost-effective manner in FFPE tissue from archival samples. Furthermore, our results suggest limited importance of the c.827A>C polymorphism in familial CRC, including (suspect) Hereditary Non–Polyposis Colorectal Cancer (HNPCC) cases.

Materials and Methods

Cases

At the Unit Molecular Diagnostics, Department of Pathology, Leiden University Medical Center, The Netherlands, 222 cases recorded as familial-CRC (fulfilling either Amsterdam II criteria for HNPCC, Bethesda criteria, or being registered as late onset familial [three or more cases of CRC all diagnosed at age >50 years]) were registered between November 1999 and December 2002. These cases were analyzed following the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (*www.fmwv.nl/gedragcodes/goedgebruik/CodeProperSecondaryUseOfHumanTissue.pdf*).

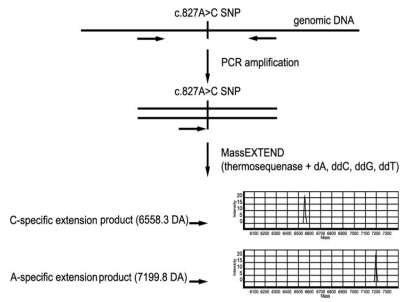

Figure 1. Design of the MassEXTEND genotyping of c.827A>C SNP assay. 1) PCR amplification generated a product including the c.827A>C SNP. 2) MassEXTEND reaction that results in two products with different mass: c.827C allele, 6558.3 d, and c.827A allele, 7199.8 d.

The mean age of diagnosis of the 222 patients was 54 years. Appearance of tumor sites was distributed as follows: coecum, 27; left colon, 15; colon transversum, 3; right colon, 38; sigmoid, 30; recto-sigmoid, 19; and rectum, 35. In 55 cases, the location was unspecified. One hundred and thirty-one cases showed a microsatellite stable phenotype, 88 cases had a microsatellite (MSI) instable (MSI-high, 71; MSI-low, 17) phenotype, and in three cases, the phenotype was unknown. As a control group, lymphocyte DNA of 156 healthy Dutch blood donors was used. Before analysis, MassEXTEND analysis of c.827A>C was validated with a standard control panel of 96 human genomic DNAs (BD Biosciences Clontech).

DNA Isolation

Normal colon, carcinoma tissue was collected as 0.6-mm-diameter punches with a tissue microarrayer (Beecher Instruments, Inc., Sun Prairie, WI) based on evaluation of hematoxylin- and eosin-stained slides. Conventional microdissection (dissection with a needle of selected areas from a 10 μm hematoxylin-stained paraffin slide under microscopic examination with an inverted microscope.) was performed on the 22 adenomas of case 02031. Furthermore, flow sorting was carried out in three carcinomas containing <60% tumor cells (case 02031, 02395, and 01362) and one metastasis (02031).[12] Genomic DNA was isolated from FFPE using a chelex extraction method as described by De Jong et al.[13]

MassEXTEND Genotyping of c.827A>C

DNA samples isolated from normal tissue of the patient and control group were genotyped with the MassEXTEND assay (Sequenom, Inc., San Diego, CA). This SNP scoring is based on the mass difference of allele-specific primer extension products. Design of the assay Figure 1. First, a 110-bp amplicon was generated using a standard PCR protocol with a forward primer, 5′-ACGTTGGATGGTTCAATACAACATCAACCCG-3′, and a reverse primer, 5′-ACGTTGGATGTTGTAACTCACCCAAGCCAC-3′. Note that

the PCR primers incorporate a 10-nucleotide-long generic tag at their 5′ end. Second, the PCR was treated with shrimp alkaline phosphatase (SAP) to remove the dNTPs subsequently. SAP was, in turn, heat inactivated at 80ºC for 5 minutes. The primer extension reaction was initiated by the addition of a primer, 5′-ACATCAACCCGTATCTTCTAC-3′, that matches with the target sequence adjacent to the interrogated SNP. Thermosequenase and a substrate mix consisting of dATP and the dideoxynucleotides G, C, and T substrate mix was chosen to maximize the mass difference between all possible extension products, thus facilitating automated calling of the genotypes. Forty rounds of primer extension were performed by temperature cycling. The resulting reactions were treated with a cation-exchange resin (SpectroCLEAN; Sequenom, Inc., San Diego, CA) to remove extraneous salts that interfere with the mass spectrometrical analysis. The amplification, SAP treatment, primer extension reaction, and cleaning step were all performed in a single well of a 384 microtiter plate. Finally, ~15 nl of each reaction was spotted onto the pads of a 384-format SpectroCHIP and subjected to MALDI-TOF MS (Bruker-Sequenom Biflex III array mass spectrometer). In addition to the unextended primer (6285.2 d), a C-specific extension product (5′-ACATCAACCCGTATCTTCTAC-ddC-3′; 6558.3 d), an A-specific extension product (5′-ACATCAACCCGTATCTTCTAC-AAddT-3′; 7199.8 d), as well as two possible polymerase pausing products (5′-ACATCAACCCGTATCTTCTAC-A-3′, 6598.4 d; and 5′-ACATCAACCCGTATCTTCTAC-AA-3′, 6911.6 d) are discernable in the mass spectra. The genotypes were called in real-time using Sequenom's MassARRAY RT software. The assay protocol was validated by means of a commercially available human genomic DNA preparation as well as four representative FFPE samples.

MassEXTEND LOH Analysis of the c.827A>C SNP in PTPRJ

The MassEXTEND assay described above was also used to determine loss of heterozygosity for 64 heterozygous cases. The quantification of the allele-specific mass signals generated in a MassEXTEND assay has previously been exploited to assess SNP allele frequencies in DNA pools.[14] The use of the MassEXTEND assay to measure LOH at the c.827A>C SNP was validated by means of a control experiment among 48 independent measurements of the c.827A>C SNP allele frequencies in a pool of samples (unrelated to the samples of the present study). In 64 cases, paired normal/tumor DNA samples were assayed in triplicate. The analysis of the spectra and the automated quantification of the alleles by comparison of the peak areas were performed with Sequenom's MassARRAY RT software. The C/A frequency ratios for tumor samples were divided by the C/A frequency ratio of the corresponding "normal" tissue. To obtain an allelic imbalance factor, the threshold for LOH was defined as 40% reduction of one allele, equating to a allelic imbalance factor of $\geq$1.7 or $\leq$0.59; the threshold for retention ranged from 0.76 to 1.3; for so-called gray areas with ratios of 0.58 to 0.75 and 1.31 to 1.69, no definitive decision was made.[15,16]

Sorting/Fluorescence Activated Cell Sorter (FACS) Flow Cytometry

On three tumors and one metastasis, with <60% tumor cells, flow sorting was performed following procedures as described previously.[12] For each measurement, data from 10,000 single-cell events were collected using a FACSCalibur (BD Biosciences, San Jose, CA). Propidium iodide fluorescence (DNA stain) was pulse-processed for FL3-area versus FL3-width that enabled us to discriminate single cells from debris (nuclear fragments) and cell aggregates. Simultaneous staining for keratin with anti-keratin antibody AE1/AE3 (Chemicon International, Inc., Temecula, CA), enabled discrimination between keratin-positive tumor cells and keratin-negative stromal and infiltrating inflammatory cells. Data were analyzed using WinList 5.0 and ModFit LT 3.0 software packages (Verity Software House, Inc., Topsham, ME). Cell fractions were sorted using a FACSVantage flow-sorter (BD Biosciences).

LOH Analysis at the PTPRJ Locus with Microsatellite Markers

Four tumors, one metastasis, and one adenoma with malignant transformation with LOH calling using MassEXTEND LOH analysis were tested for conventional LOH at the *PTPRJ* locus using five microsatellite markers: D11NKI01, D11S4117, D11S4183, D11S1350, and D11S1326.[9] The density of the tumor cells varied from 60 to 100% per case. PCR was performed under conditions recommended by the manufacturer (Applied Biosystems, Inc.) with 2 pmol of the primer pairs as mentioned above with exception of D11S1350 from which 10 pmol was used. The following PCR conditions were used in Gene Amp 9700 thermocycler (Applied Biosystems, Inc.): initial denaturation step, 5 minutes at 96°C, followed by 33 cycles of 45 seconds at 94°C, 1.5 minutes at 58°C, and 45 seconds at 72°C thereafter; and a final elongation step of 7 minutes at 72ºC was performed. Mixtures of 24 μl of deionized formamid, 1 μl of TAMRA 500 size standard (Applied Biosystems Inc.), and 1.0 μl of PCR product were run on an ABI 310 Genetic Analyzer (Applied Biosystems, Inc.) for 24 minutes with run profile GS STR POP 4 (1.0 ml) C and analyzed with Gene Scan. A threshold characterizes conventional LOH, comparing normal and tumor DNA; this threshold was defined as described under MassEXTEND LOH analysis of the c.827A>C SNP in *PTPRJ*.

PTPRJ Sequencing

Sequencing analysis of PCR products was done at the Leiden Genome Analysis Center. Sequencing reactions were run on an ABI3730 (Applied Biosystems, Inc.) and analyzed with chromas 1.5. (*www.technelysium.com.au/chromas.html*).

Table 1. Distribution of the Genotype c.827A>C SNP in Exon 5 of *PTPRJ*: A/C, A/A, and C/C Genotypes in 222 Familial CRC and Suspected HNPCC Cases Compared with 156 Healthy Blood Donors Showed Comparable Frequencies

Genotype	A/C	A/A	C/C
Control (n = 156)	47	103	6
Normal CRC (n = 222)	64*	149	9
Tumor CRC			
–/C*	4	–	–
A/C	60	–	–
A/–	0	–	–

*In 4 of 64 tested tumors from patients with an c.827A>C genotype, loss of the A allele was detected.

Results

Genotyping of the c.827A>C Polymorphism in PTPRJ Using the MassEXTEND Analysis

The c.827A>C SNP in PTPRJ was genotyped in 156 healthy blood donors and normal DNA from 222 patients with familial CRC (including cases with HNPCC) using the MassEXTEND analysis. The distribution of the three possible genotypes (A/A, A/C, and C/C) was the same in the two groups analyzed (Table 1). The A/A (Figure 2A) genotype was present in 66% of the control cases versus 67% in CRC cases; the A/C genotype (Figure 2C) was present in 30% of the control cases versus 29% in CRC cases; whereas the C/C genotype (Figure 2B) was found in 4% of the control and CRC cases. Among the cancer cases, no significant difference was found among the three genotypes with regard to distant metastases, tumor size, tumor site, age, or MSI status.

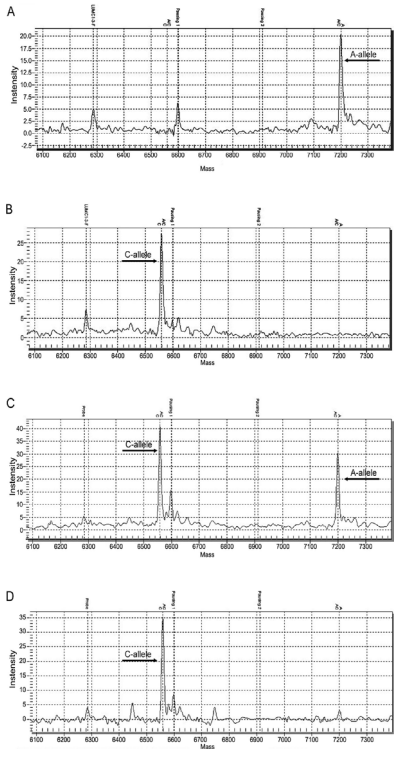

Figure 2. Mass spectra of the three c.827A>C SNP genotypes (**A,** A/A; **B,** C/C; **C,** A/C) and tumor 02031 with loss of the A allele (**D**). The alleles are indicated with thick horizontal **arrows**. Pausing and probe peaks are indicated above the graph.

LOH Analysis of the c.827A>C SNP in PTPRJ with a MassEXTEND LOH Assay and Its Validation

In a control experiment (see Materials and Methods), among 48 independent measurements, the c.827A allele was observed with a frequency of 0.766 ± 0.02 and 0.234 ± 0.02 for the c.827C allele (0.02 is the SD). In the 64 patients with an A/C genotype (Table 1), LOH using the MassEXTEND LOH assay was determined in triplicate (Table 2). The dropout rate was ~5%, and there were no discrepancies among the replicate measurements. In 4 of 64 (6.2%) cases, LOH with selective loss of the A allele was found with Sequenom's MassARRAY RT software; the mean allelic imbalance factors (AIFs) were 6.09, 13.3, 4.72, and 3.60 (A, B, C, and D) (Table 2).

In four carcinomas, LOH was validated using conventional LOH analysis at the *PTPRJ* locus with flanking polymorphic markers. These were two cases with an AIF of, respectively, 6.09 (A) and 13.3 (B) and two cases with ambiguous (gray value) AIFs of 0.65 (E) and 0.59 (F) (Table 2). In tumors (A and B), conventional LOH analysis showed high allelic imbalance in 22 of 23 informative markers with a mean value of 4.78. In those tumors (E and F) with ambiguous MassEXTEND LOH, limited allelic imbalances with conventional markers was seen in enriched tumor cell populations. In all six cases (A through F) (Table 2) and in an additional seven heterozygous tumors without apparent MassEXTEND LOH, the c.827A>C SNP was analyzed by sequencing. Cases A through D clearly show loss of the A allele in tumor cells. In the two tumors (E and F) with ambiguous MassEXTEND LOH values, an A/C heterozygote sequence is identified indicating retention of both the A and C alleles. Seven tumors, with a mean AIF of 1.03, all showed retention of the A and C alleles (G).

Interestingly, all four tumors showing loss of the A allele were microsatellite stable and located in the recto-sigmoid. Case 02031 (A) (Figure 2) demonstrating LOH of the c.827A allele (AIF of 6.09) concerns a 37-year-old female patient with a Dukes C rectal carcinoma and synchronously one separate adenoma with malignant transformation and at least 21 other adenomas (*APC* and *MYH* germline mutation analysis proved negative; C.M. Tops and M.M. Weiss, unpublished results). Flow cytometry analysis of this rectal carcinoma showed two aneuploid keratin-positive tumor cell fractions (one hypo- and one hypertetraploid fraction; Figure 3). Only the hypertetraploid tumor cell fraction was present in one of the lymph-node metastases analyzed (Figure 3). DNA sequencing of the sorted tumor cell fractions confirmed the

Table 2. Validation of the *MassEXTEND* LOH Analysis of the c.827A>C SNP of *PTPRJ* in Tumors with Conventional LOH of the PTPRJ Locus and Sequence Analysis

				MassEXTEND c.827A>C LOH	LOH *PTPRJ* locus					
ID	Sample ID	FACS sorting	Tumor percentage (%)	C/A ratio (AIF)	D11NKI01	D11S4117	D11S4183	D11S1350	D11S1326	PTPRJ sequencing
A*	02031 n.†	No			–§					A/C
	02031 ad. M. transform.‡	No		0.97 (0.89–1.03)	–	–	–	–	–	A/C
	02031 ca.¶	No	50	6.09 (5.39–6.81)	+‖	+	+	+	+	/C
	02031 ca. (fr1)(ker+)	Yes			+	+	+	±**	+	/C
	02031 ca. (fr2)(ker+)	Yes			+	+	+	+	+	/C
	02031 metastasis (ker+)	Yes			+	+	+	+	+	/C
B	02327 n.	No								A/C
	02327 ca.	No	60	13.3 (11.4–14.9)	+	NA	+	+	+	/C
C	02034 n.	No								A/C
	02034 ca.	No	60	4.72 (3.91–6.04)	NA	NA	NA	NA	NA	/C
D	00040 n.	No								A/C
	00040 ca.	No	60	3.60 (3.50–3.77)	NA	NA	NA	NA	NA	/C
E	02395 n.	No								A/C
	02395 ca.	No	40	0.65 (0.60–0.70)						A/C
	02395 ca.	Yes			–	NA	+	–	NA	A/C
F	01362 n.	No								A/C
	01362 ca.	No	50	0.59 (0.56–0.63)						A/C
	01362 ca.	Yes			+	±	+	±	+	A/C
G	7 ca.	No	>60	1.03 (0.82–1.27)	NA	NA	NA	NA	NA	A/C

*From carcinoma case 02031 with loss of the A allele, 21 additional adenomas were tested with sequence analysis; no loss of the A allele was found in any of these 21 samples.
†n., normal.
‡ad. M. transform., adenoma with malignant transformation.
§Retention AIF 0.76 to 1.3.
¶ca., carcinoma.
‖LOH AIF ≥1.7 or ≤0.59.
**Gray area AIF 0.58 to 0.75 and 1.31 to 1.69.

loss of the c.827A allele in both aneuploid tumor fractions, implying that loss of the A allele most likely was an early event during tumorigenesis. However, sequence analysis and conventional LOH analysis of the 22 adenomas (including MassEXTEND LOH of the adenoma with malignant transformation; Table 2) did not identify LOH of flanking microsatellite markers nor of the c.827 *PTPRJ* alleles (data of the 21 additional adenomas not shown).

Cost-Comparison MassEXTEND LOH Analysis versus Sequencing Analysis

A cost comparison between the on mass spectrometry bases MassEXTEND LOH analysis and sequencing analysis was made in Table 3 on the basis of our facilities. In our setting, the MassEXTEND LOH analysis is ninefold less expensive and the throughput is 10 times higher than conventional sequencing.

Discussion

We have shown that the MassEXTEND (LOH) assay is a reliable and cost-effective method for typing SNPs and detecting LOH of SNP loci using formalin-fixed paraffin-embedded (FFPE) tissue. The automated analysis of the spectra is made possible by Sequenom's MassARRAY RT software. Genotyping with MALDI TOF has already been described by Haff and Smirnov[2] as a high-volume application. Recently, MALDI TOF genotyping of *PTPRJ* is also published including limited LOH analysis, although no validation for LOH was done, and the spectra were not automatically analyzed.[6] For FFPE material, the MassEXTEND (LOH) assay is significantly less labor intensive than direct sequencing analysis (the main alternative for detecting LOH at specific SNP loci in tumors). Furthermore, in our setting, the MassEXTEND LOH assay is ninefold less expensive, and the throughput is 10 times higher than conventional sequencing. Lately, high-throughput SNP tools have become available for mass screening of leukocyte DNA and frozen tumor tissue. Such tools will lead to the identification of new markers for cancer susceptibility, tumor behavior, and prediction of treatment response. When selected markers need to be tested in FFPE, the MassEXTEND (LOH) assay may appear to be an excellent option.

For the *PTPRJ* c.827A>C SNP, we observed a similar distribution in familial CRC patients as in healthy blood donors, not supporting this polymorphism as an evident risk modifier in familial CRC. Recently, preferential loss of

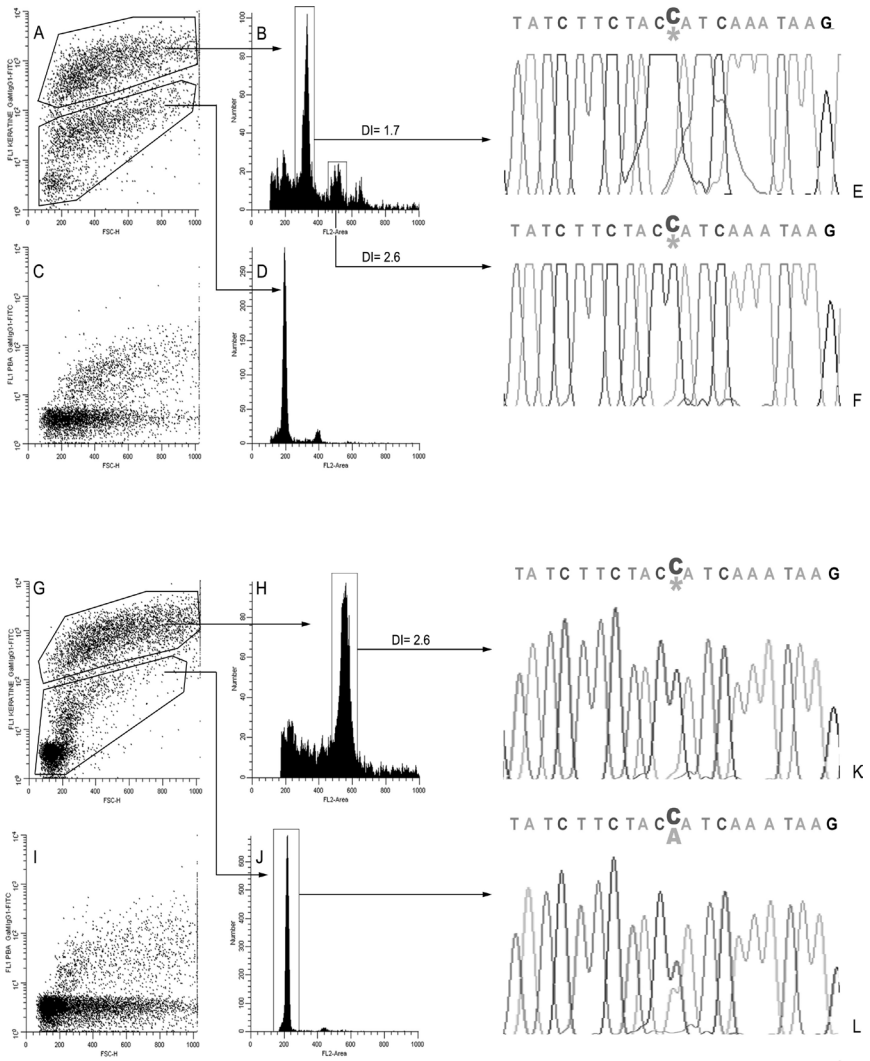

Figure 3. Sequence analysis of the c.827A>C SNP of *PTPRJ* of flow-sorted cell populations. Distinct cell populations were flow-sorted from the formalin-fixed paraffin-embedded primary tumor and lymph-node metastasis of case 02031. **A–F:** Primary tumor. **A:** Keratin positive (K pos.) cells can be clearly identified in the forward scatter versus keratin dot plot, compared with a negative control (**C**). **B:** After gating on the K pos. cells, a bimodal DNA histogram can be observed with two dominant cycling populations with a DNA index of 1.7 and 2.6, respectively. **D:** The Keratin negative (K neg.) cells, comprising inflammatory and stromal cells, revealed an unimodal DNA diploid histogram.[17] **E** and **F:** Sequence analysis of fraction ID 1.7 and fraction ID 2.6 showed loss of the A allele in both populations. **G–L:** Lymph-node metastasis. **G:** Forward scatter versus keratin dot plot. **I:** Negative control. **H:** Gating on the K pos. cells shows an unimodal DNA histogram with a DNA index of 2.6. These cells probably branched from the second DNA aneuploid population (DI = 2.6) of the primary tumor. **J:** Unimodal DNA diploid histogram of the K neg. cells. **K:** Sequence analysis of ID 2.6 fraction showed loss of the A allele. **L:** The K neg. cells are diploid and show the normal A/C genotype.

the putative cancer resistance allele c.827CA versus the potentially less active c.827C allele was shown in sporadic CRC of heterozygote c.827A>C patients.[9] Our study demonstrates that also in familial CRC, the A allele is preferentially lost, however, only 4 of 64 heterozygotes (6.25%) lost the A allele. The C allele was retained in all cases. Interestingly, loss of the A allele was only found in patients with microsatellite stable tumors that were located in the recto-sigmoid. The percentage of loss of c.827A>C in our study is much lower than the percentages published for CRC of 49 and 71%, respectively.[9,11] This discrepancy might partly be explained by technical reasons; we used a more stringent threshold for LOH, 40% instead of a 20 to 30% reduction of one allele when comparing normal and tumor DNA.[15,16] An additional explanation is that the tumors analyzed for LOH of c.827A>C by Ruivenkamp et al[9] had been preselected for LOH using flanking polymorphic markers. Further-

Table 3. Cost Comparison between the Mass Spectrometry Bases MassEXTEND LOH Analysis and Sequencing Analysis

	Mass spectrometry	Sequencing
Equipment	Brucker-Sequenom Biflex III array mass spectrometer	ABI prism 3730 genetic analyzer
Hands on time per sample	7 s (~6 hours for 3072 samples)	72 s (~6 hours for 288 samples)
Turn around time per sample	30 s (~24 hours for 3072 samples*)	300 s (~24 hours for 288 samples*)
Data analysis per sample	Negligible	30 s
Cost per reaction industrial laboratory	€ 2.00	€ 18.00
Cost per reaction academic laboratory	€ 0.30	€ 3.96
Instrument throughput (samples per day)	7680	960

*The number of samples that can be done in 1 day, assuming there are no limitations in equipment (PCR machines, etc.) and in people.
s, seconds.

more, we studied LOH of the c.827A allele in a cohort of familial CRC cases compared with sporadic colorectal cancer in other studies. Our results suggest that the c.827A>C plays a limited role in familial CRC and (suspect) HNPCC.

We did not detect any LOH of the *PTPRJ* locus using flanking markers or loss for the A1176 SNP allele in 21 early adenomas and 1 adenoma with malignant transformation, from one single case, having loss of the c.827A allele in a synchronous rectal carcinoma. This would appear to be in contrast with previous findings, suggesting loss of PTPRJ to be an early event in colon tumor development, ie, in the adenomatous stage.[11] Additionally, we conclude that in this case, the loss of the c.827A allele must be a relatively early event although only to have occurred in an early carcinoma phase. This conclusion is based on the observation that in all carcinoma cell fractions (a hypotetraploid and a hypertetraploid cell fraction, the latter of which was also found in a metastasis analyzed), loss of the c.827A allele was found. However, we cannot rule out that the clone with LOH could propagate so rapidly that it might have completely wiped out all non-LOH clones.

We show that the results obtained with the MassEXTEND LOH analysis are as reliable as conventional sequence methods, and we document the utility of this new technique to detect LOH of a specific SNP in a sensitive and automated manner in FFPE tissue from archival samples. Furthermore, our results suggest limited importance of the c.827A>C polymorphism in familial CRC, including (suspect) HNPCC cases.

The practical feasibility of the MassEXTEND LOH analysis in a basic molecular diagnostic laboratory on a routine day-to-day basis is limited and must be placed in verification of data in large series of cases. Examples might be the analysis of SNP profiles that, eg, determine chemosensitivity of all sorts of tumors that could be translated in use for daily practice.

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CHAPTER 8

Homozygosity for a *CHEK2**1100delC mutation identified in familial colorectal cancer does not lead to a severe clinical phenotype

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Homozygosity for a *CHEK2**1100delC mutation identified in familial colorectal cancer does not lead to a severe clinical phenotype

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Abstract

It has recently been suggested that the frequency of the germline *CHEK21100delC mutation is higher among breast cancer families with colorectal cancer, although the mutation does not seem to be significantly associated with familial colorectal cancer. Five hundred and sixty-four familial colorectal tumours were studied for expression of CHEK2 using tissue microarrays and an antibody against the NH_2-terminal SQ regulatory domain of the CHEK2 protein. Normal colonic tissue from patients whose tumours showed loss of CHEK2 expression was investigated further using fragment and sequence analysis for the presence of a *CHEK2**1100delC mutation and five other (R117G, R137Q, R145W, I157T, and R180H) known germline variants in *CHEK2*. Twenty-nine tumours demonstrated loss of expression for CHEK2. Analysis of matched normal colonic tissue from these patients revealed germline *CHEK2**1100delC mutation in three cases. In two of these, the mutation was heterozygous but, interestingly, the third patient proved to be homozygous for the deletion, using six different primer pair combinations. None of the other tested germline variants were identified. No *CHEK2**1100delC mutations were found in patients whose tumours stained positive. Homozygosity for the *CHEK2**1100delC mutation appears not to be lethal in humans. No severe clinical phenotype was apparent, although the patient died from colonic carcinoma at age 52 years. This observation is in line with recent knockout mouse models, although in the latter, cellular defects in apoptosis and increased resistance to irradiation seem to exist. It is also concluded that CHEK2 protein abrogation is not caused by the *CHEK2* germline variants R117G, R137Q, R145W, I157T, and R180H in familial colorectal cancer.**

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Introduction

CHEK2 on chromosome 22q is the human homologue of the yeast Cds1 and Rad53 G2 checkpoint kinases. The Chek2/Rad53/Cds1 family of proteins identifies DNA damage in eukaryotic cells [1]. Pseudo-genes of exons 10–14 of *CHEK2* are found on chromosomes 15 and 16 and, with lower homology, on chromosomes 2, 10, 13, X, and Y [2,3]. The protein truncating mutation *CHEK2**1100delC, present in exon 10 of the functional gene on chromosome 22q, abolishes the kinase function of CHEK2 [4,5]. The role of the *CHEK2**1100delC and other germline variants has been well studied in breast cancer. The 1100delC allele has been claimed to be a low penetrance susceptibility allele for breast cancer and carriers appear to have a two-fold increase in breast cancer risk [6]. CHEK2 protein is abrogated or reduced to a large extent in breast tumours of heterozygous *CHEK2**1100delC mutation carriers [7–9]. The incidence of the 1100delC mutation has been suggested to be higher among breast cancer families with colorectal cancer than in those without colorectal cancer, identifying a hereditary breast and colorectal cancer (HBCC) phenotype [10]. Recently, the incidence of the *CHEK2**1100delC mutation in familial and non-familial colorectal cancer (CRC) patients was determined to be 1.3% and 2.9%, respectively, which is not significantly higher than the 1.1–1.4% frequency with which this allele is found in the healthy European population studied so far. With an estimated range of 1.3–1.6%, this frequency seems similar in the Dutch population [11]. These results suggest that the *CHEK2**1100delC mutation may not be significantly associated with familial colorectal cancer or with colorectal cancer risk in the population, although a very

low penetrance effect on colorectal cancer could not be excluded [12,13].

In addition to the 1100delC mutation, other germline variants in *CHEK2* have been identified among families with cancer, only two of them with known reduced (R145W) or absent (1422delT) catalytic activity [5]. Mis-sense variants R117G, R137Q, and R180H have been detected with an increased incidence in affected individuals from breast cancer families. Tumours with these mutations have been demonstrated to show loss of the mutant allele, suggesting a mechanism for tumour genesis other than loss of the wild-type allele [14]. R145W was identified in a sporadic colon cancer cell line (HCT15), and I157T and 1422delT have been identified in Li–Fraumeni syndrome variants [15]. I157T has also been detected with an increased frequency in several tumour types including breast cancer [8,13], prostate cancer [13,16–18], and thyroid cancer cases [13], although the variant appears to exhibit wild-type activity [5].

To evaluate the frequency of the *CHEK2**1100delC mutation in a well-defined familial colorectal cancer cohort, and to study the possible role of five *CHEK2* germline variants (R117G, R137Q, R145W, I157T, and R180H) in abrogation of the CHEK2 protein, we used tissue microarrays (TMAs) and examined CHEK2 protein expression in tumours with immunohistochemistry. Patients with loss of the protein were investigated further at the molecular level with fragment and sequencing analysis.

Materials and methods

Patients

To protect the information on each patient analysed, protect patient privacy, and prevent misuse of data, we acted according to the national code for working with patient data. In The Netherlands, all patient-related data used for research are protected by the Code for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences: www.fmwv.nl/gedragscodes/goedgebruik/CodeProperSecondaryUseOfHumanTissue.pdf.

Five hundred and sixty-four Dutch cases recorded as familial CRC [397 microsatellite-stable (MSS) familial CRC, 140 microsatellite-instable (MSI-H) (suspect) hereditary non-polyposis colorectal cancer (HNPCC), and 27 familial adenomatous polyposis (FAP)] were used for the study. The 564 tumours were located respectively in the caecum (61), left colon (24), transverse colon (11), right colon (70), sigmoid (57), recto-sigmoid (29), and rectum (87): in 225 cases, the location was not specified. In addition, two cases, one with a rectal adenoma and one with two colon adenomas, were included.

Tissue microarray (TMA) construction

Triplicate tissue cores from tumour areas, selected by a pathologist (HM) on the basis of a haematoxylin and eosin (H&E)-stained slide, were taken from each specimen (Beecher Instruments, Silver Springs, MD, USA). The punches, which had a diameter of 0.6 mm, were arrayed on a recipient paraffin wax block, using standard procedures [19].

Immunohistochemistry and evaluation

Staining of CHEK2 was performed with anti-CHEK2 (clone DCS 270.1, 1 : 100; Novocastra Laboratories Ltd, UK). Clone DCS 270.1 localizes within the NH_2-terminal SQ regulatory domain of CHEK2 [20]. Sections from the constructed tissue arrays were transferred to glass slides using a paraffin sectioning aid system (Instrumedics Inc, Hackensack, NJ, USA). Next, tissue sections were dewaxed three times in xylene for a total of 15 min and subsequently rehydrated. Antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6.0) for 10 min using a microwave oven, after which the sections were cooled in this buffer for at least 2 h at room temperature. After rinsing in demineralized water and phosphate buffered saline (PBS), the tissue sections were incubated with the primary antibody diluted in 1% (w/v) PBS/bovine serum albumin overnight at room temperature. Sections were washed in PBS and endogenous peroxidase was blocked in 0.03% hydrogen peroxide PBS for 20 min, washed with PBS, and incubated with biotinylated rabbit anti-mouse (1 : 200; DAKO, Glostrup, Denmark) for 30 min, washed again with PBS, and incubated with streptavidin–biotin complex (1 : 100; DAKO) for 30 min. Sections were washed and developed in 3,3′-diaminobenzidine tetrahydrochloride substrate solution containing 0.002% hydrogen peroxide for 10 min. The sections were then counterstained with haematoxylin, dehydrated, cleared in xylene, and mounted with pertex. Microscopic analysis was done by a pathologist (HM). CHEK2 expression was scored positive or negative in tumour nuclei. In the majority of negative cases, no internal positive stromal and inflammatory control cells could be identified, including the three cases in which a *CHEK2**1100delC mutation was eventually identified. This might be explained by the fact that CHEK2 expression is lower in stromal and inflammatory cells than in epithelial cells and by the fact that in our hands using TMA, staining is often somewhat weaker in comparison with whole-slide analysis.

DNA isolation

Genomic DNA from normal colon (89 cases plus two affected family members described in the results) and colorectal tumour (6 cases) tissue was extracted from paraffin wax-embedded material as described previously by de Jong *et al* [21].

PCR and sequencing of the *CHEK2**1100delC mutation

PCR for the *CHEK2**1100delC mutation was performed as described previously by Cleton-Jansen *et al*

[22]. Mixtures of 24 µl of de-ionized formamide, 1 µl of TAMRA 500 size standard (Applied Biosystems Inc, Foster City, CA, USA), and 1 µl of PCR product were each run on an ABI 310 Genetic Analyzer (Applied Biosystems Inc) for 20 min with run profile GS STR POP 4 (1.0 ml) C and analysed with Gene Scan Analysis *3.1*. The *CHEK2**1100delC mutation is characterized by the generation of a PCR product that is one base shorter than the control sample. To confirm this mutation, sequence analysis was performed. Furthermore, alternative primers were designed to confirm the *CHEK2**1100delC mutation and to exclude technical problems caused by possible polymorphisms in the primer annealing site. Primers rv4 and rv5 were chosen on the basis of the mismatches that they have at the 3′ end with the pseudo-genes (Figure 1 and Table 1). Afterwards, sequencing of the PCR products was performed at Base Clear LABSERVICES and analysed with chromas 1.5.

PCR and sequencing of polymorphisms R117G, R137Q, R145W, I157T, and R180H

PCR was carried out in a total reaction volume of 12 µl, containing the same chemicals as used for the *CHEK2**1100delC mutation PCR and 10 pmol of the primer pairs as described in Table 1. The following PCR conditions were used in the Gene Amp 9700 thermocycler (Applied Biosystems Inc): initial denaturation step 5 min at 96 °C, followed by 33 cycles of 45 s at 94 °C, 1.5 min at 60 °C, and 45 s at 72 °C; thereafter, a final elongation step of 7 min at 72 °C was performed. Afterwards, sequencing analysis was performed.

Results

Twenty-three microsatellite-stable (MSS) familial CRC cases and six MSI-H (suspect) HNPCC cases showed loss of CHEK2 expression in their tumours by TMA immunohistochemistry (IHC) (Figure 2). In the majority of negative cases, no internal positive control cells could be identified.

The *CHEK2**1100delC mutation was present in normal tissue from three of these 29 cases (Figure 2). Sixty of the 475 cases with positive nuclear CHEK2 staining in tumour cells were used as controls and no *CHEK2**1100delC mutations were identified in these cases ($p = 0.011$). Two cases were heterozygous for the *CHEK2**1100delC germline mutation, while one case proved to be homozygous for the mutation with both fragment and sequence analysis. The homozygous status for the *CHEK2**1100delC mutation was also confirmed by five alternative primer pair combinations to exclude a possible polymorphism in the primer annealing site or amplification of pseudo-genes (Table 1 and Figure 1). Pedigree analysis for this homozygous case (case 01 272) is shown in Figure 3. The index case died at the age of 52 years with

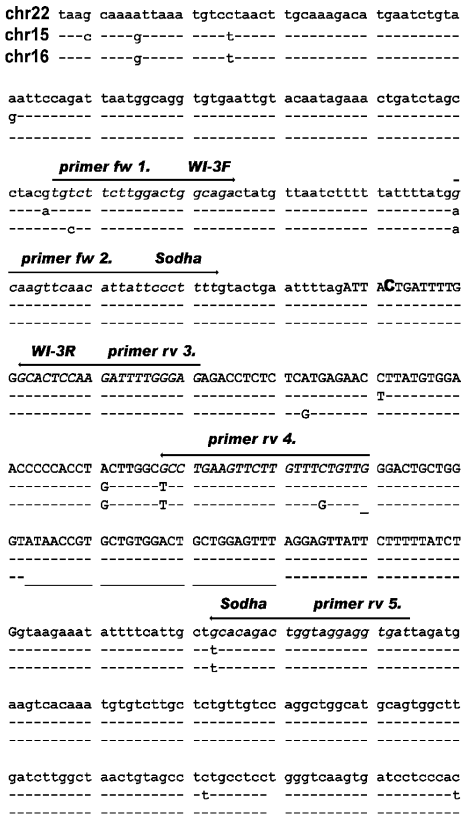


Figure 1. Detection of the *CHEK2**1100delC mutation in DNA extracted from archival paraffin wax-embedded tissue. Identification of the *CHEK2**1100delC mutation and other *CHEK2* variants in cases that stained negative for CHEK2 by IHC as well as analysis of positive staining control cases. *CHEK2* exon 10 (containing the *CHEK2**1100delC) on chromosome 22q is shown and compared with pseudo-genes containing the same region on chromosomes 15 and 16. The pseudo-genes on chromosomes 2, 7, 10, 13, X, and Y are not shown since the homology is limited. Sequence differences between *CHEK2* and the pseudo-genes are noted by the indicated nucleotide positions. The underscore in chromosome16 means that these specific nucleotides are not present on this chromosome. The different primers are indicated above the sequences, as well as in Table 1

metastatic disease from a sigmoid carcinoma. The mother (rectal adenoma at age 69) is heterozygous for the *CHEK2**1100delC mutation; the brother (two colon adenomas at age 45) has no *CHEK2**1100delC mutation. The father could not be tested. All the tumours tested in this pedigree were MSS with normal positive nuclear expression for the mismatch repair proteins MLH1, PMS2, MSH2, and MSH6, indicating mismatch repair proficiency. To exclude the involvement of a base excision repair defect, the mutational hotspots of *MYH* (Y165C, G382D, and P391L) [23] were shown to be absent in the mother and two affected sons tested (data not shown). Also,

Table 1. Different primers used for the identification of six *CHEK2* germline variants including 1100delC

CHEK2 mutation	Exon	Forward primer		Reverse primer	Sequencing Primer
1100delC nested	10	fw* 1	TGT CTT CTT GGA CTG GCA GA	rv‡ 5	ATC ACC TCC TAC CAG TCT GTG C	—
				rv 3	GTT TGT TCT CCC AAA ATC TTG GAG TGC	TGT CTT CTT GGA CTG GCA GA
ALT† 1 1100delC	10	fw 1	TGT CTT CTT GGA CTG GCA GA	rv 3	GTT TGT TCT CCC AAA ATC TTG GAG TGC	TGT CTT CTT GGA CTG GCA GA
ALT 2 1100delC	10	fw 1	TGT CTT CTT GGA CTG GCA GA	rv 4	CAA CAG AAA CAA GAA CTT CAG GC	TGT CTT CTT GGA CTG GCA GA
ALT 3 1100delC	10	fw 1	TGT CTT CTT GGA CTG GCA GA	rv 5	ATC ACC TCC TAC CAG TCT GTG C	TGT CTT CTT GGA CTG GCA GA
ALT 4 1100delC	10	fw 2	GCA AGT TCA ACA TTA TTC CCT	rv 4	CAA CAG AAA CAA GAA CTT CAG GC	CAA CAG AAA CAA GAA CTT CAG GC
ALT 5 1100delC	10	fw 2	GCA AGT TCA ACA TTA TTC CCT	rv 5	ATC ACC TCC TAC CAG TCT GTG C	ATC ACC TCC TAC CAG TCT GTG C
R117G	2	—	ATT CAA CAG CCC TCT GAT GC	—	GCA GTG GTT CAT CAA AGC AA	ATT CAA CAG CCC TCT GAT GC
R137Q	2	—	TTG CTT TGA TGA ACC ACT GC	—	TCC ATT GCC ACT GTG ATC TT	TTG CTT TGA TGA ACC ACT GC
R145W	2	—	TTG CTT TGA TGA ACC ACT GC	—	TCC ATT GCC ACT GTG ATC TT	TTG CTT TGA TGA ACC ACT GC
I157T	3	—	TTG CTT TGA TGA ACC ACT GC	—	TCC ATT GCC ACT GTG ATC TT	TTG CTT TGA TGA ACC ACT GC
R180H	3	—	ATC ACA GTG GCA ATG GAA CC	—	CTC CCA AAG TGC TGG GAT TA	ATC ACA GTG GCA ATG GAA CC

* Forward primer.
† Alternative primer combination.
‡ Reverse primer.

the typical somatic *K-RAS2* mutations described in *MYH*-defective tumours were not found [24] (data not shown). The two heterozygous *CHEK2**1100delC cases proved to be MSS tumours, one from a 63-year-old female (a left-sided colon carcinoma without lymph node metastasis; case 01 033) and the other from a 53-year-old male (rectal carcinoma without lymph node metastasis; case 00 207). LOH analysis was performed to analyse the wild-type allele in the latter two cases (Figure 2). A control case with positive immunohistochemical staining for CHEK2 in tumour nuclei (case 00 076) showed only wild-type 121 base-pair allele fragments in tumour as well as in normal colon DNA, as expected. The second heterozygous case showed loss of the wild-type allele of *CHEK2* in the tumour, while the first case did not show any LOH. Re-evaluation of the CHEK2 staining in the latter case showed that although strikingly diminished, there was a remnant of positive staining in the tumour nuclei, compared with control cases.

In addition, five other *CHEK2* germline variants (R117G, R137Q, R145W, I157T, and R180H) were examined in the 29 patients; none of them was identified.

Discussion

In this study, we analysed 564 tumours from patients with familial colorectal cancer for abrogation of the CHEK2 protein and examined the patients with abrogation for the *CHEK2**1100delC mutation and five other germline variants of *CHEK2*, R117G, R137Q, R145W, I157T, and R180H.

The homozygous *CHEK2**1100delC mutation that we identified has not been described previously in humans, but in view of the 1.1–1.4% allele frequency of *CHEK2** 1100delC in the general European population and the 1.3–1.6% frequency in the Dutch population [11], homozygous status should be encountered in about 1/10 000 individuals. Although the patient identified in this study died from colon cancer, no severe syndrome seemed clinically apparent. It is likely that another gene defect is responsible for this family's colorectal tumours (a brother does not carry the variant but has already had four polyps at an early age), although we have ruled out HNPCC and MYH-associated polyposis.

The fact that *Chek2* −/− knockout mice seem to appear normal is in line with our observation. However, the phenotype of *Chek2*-deficient mice is dominated by increased resistance to irradiation and by defects in apoptosis [25]. Hirao *et al* showed, on the basis of *Chek2* −/−mice, that *Chek2* is not essential for somatic growth, fertility, or immunological development [26]. Manipulated HCT-116 human colon carcinoma cells carrying a homozygous deletion for *CHEK2* yielded no defective phenotype with respect to p53, G1 or G2 cell-cycle arrest and apoptosis [27]. Whether these data suggest that *CHEK2* has a complementary or even redundant function in

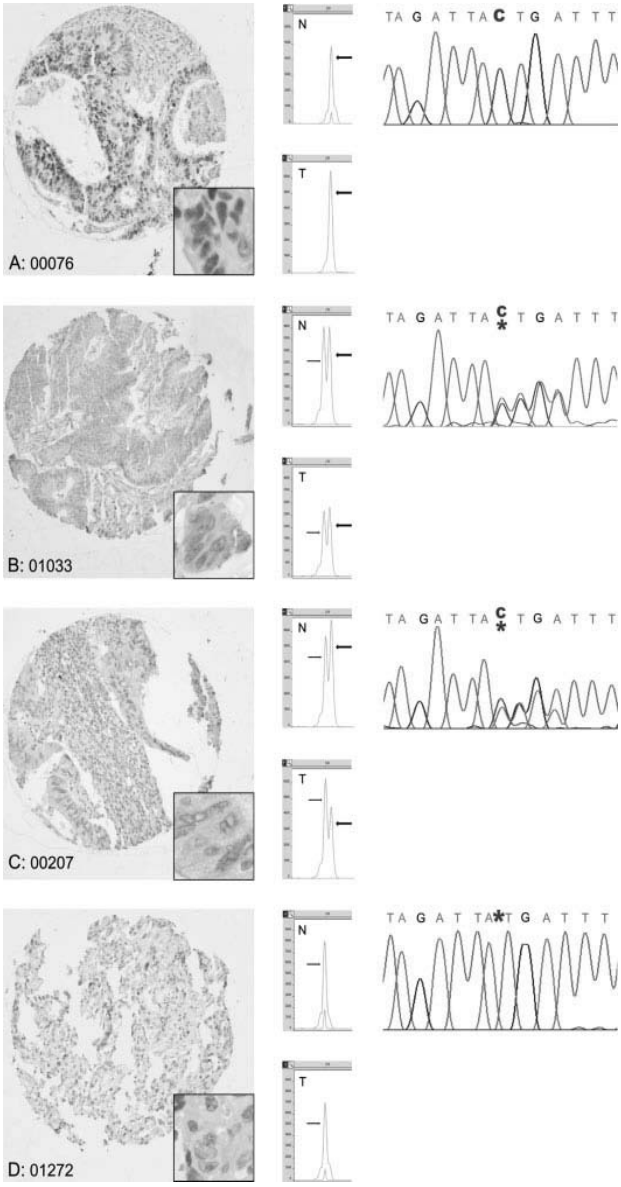
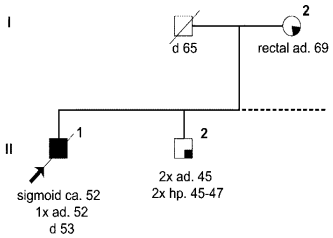


Figure 2. Three familial colorectal cancer cases with a *CHEK2**1100delC mutation and one control case. The *CHEK2**1100delC mutation (disease allele) was characterized by a PCR product that was one base shorter than the control sample. Mutation sequence analysis was performed to confirm this. (A) Patient (00 076) without mutation; positive immunohistochemical staining with CHEK2 antibody (magnification 50× and 200×). The wild-type alleles (thick arrows) are found in normal and tumour DNA and sequence analysis shows an 1100 C wild-type sequence in both alleles. (B) Patient (01 033) with a heterozygous 1100delC mutation; the tumour cells from this patient were initially scored as negative immunohistochemically. Re-evaluation showed some residual brown staining. In normal and tumour DNA, the wild-type (thick arrow) and mutant (thin arrow) allele appears, indicative of retention of the wild-type allele in the tumour. (C) Patient 00207 with heterozygous 1100delC mutation; staining for CHEK2 is negative. In normal DNA, the wild-type (thick arrow) and disease allele appear; LOH of the wild-type allele is present in tumour DNA. (D) Patient (01 272) homozygous for *CHEK2**1100delC; CHEK2 staining is negative. Only the mutant allele is present in normal and tumour DNA amplified with six different primer combinations (Table 1 and Figure 1); sequence analysis confirmed the homozygous *CHEK2**1100delC mutation



		MYH						IHC 4 MMR proteins			
Patient	*CHEK2* 1100delC	Y165C	G382D	P391L	Histology	*K-RAS2*	MSI-analysis	MLH1	PMS2	MSH2	MSH6
I-2	mut/wt	wt	wt	wt	ad.	wt	MSS	+	+	+	+
II-1	mut/mut	wt	wt	wt	ca.	wt	MSS	+	+	+	+
II-2	wt/wt	wt	wt	wt	ad.	wt	MSS	+	+	+	+

Figure 3. Pedigree of the family of the index patient (01 272). ad. = adenoma; hp. = hyperplastic polyp; ca. = carcinoma; d = age at time of death. Different analyses are shown schematically. mut = mutant; wt = wild type. MSI analyses were performed using markers recommended by Boland *et al* [31]. MMR = mismatch repair

human colon cells remains to be established. This finding is especially intriguing in view of the fact that *CHEK2**1100delC mutation is associated with familial breast cancer and is also strongly associated with bilateral breast cancer [6,7,28,29].

Overall, we identified only a low percentage of cases that exhibited abrogation of CHEK2 protein staining and actually carried the *CHEK2**1100delC mutation in our familial colorectal cancer cohort. The range of possible frequencies of this abnormality is 0.5% (3/564) to 3.4% (3/89). Sixty cases with positive staining were analysed genetically; if the number of positive staining cases were increased, the upper range would become much lower than 3.4%. This is in line with the observations of Kilpivaara *et al* [12], who identified *CHEK2**1100delC mutation in 1.3% of familial colorectal cancer cases. Furthermore, based on the results of our control group with positive staining (half of which showed weak positive intensity of staining), and the studies of Vahteristo *et al* [7] and Oldenburg *et al* [9], it is not likely that we missed many *CHEK2**1100delC mutation carriers by selecting cases on the basis of protein expression. The contribution of *CHEK2**1100delC mutation to the risk of multiple colorectal adenomas and carcinomas has been studied by Lipton *et al* [30]. Their data and a recent study by Cybulski *et al* [13] suggest that the 1100delC mutation is not associated with an increased risk for colorectal cancer.

None of the five other known germline variants in *CHEK2* (R117G, R137Q, R145W, I157T, and R180H) were identified and are thus not an explanation for the abrogation of CHEK2 staining. In breast cancer, it has already been shown that in cases with the I157T variant, the tumours stain positively for CHEK2 [8]. However, the protein stability of CHEK2 mutant R145W is questionable, considering its reduced kinase activity [5]. It is still possible that the unexplained negative staining for CHEK2 in some of the remaining cases is an artefact, although other causes such as promoter hypermethylation and the involvement of other components of the pathway(s) regulating the expression of CHEK2 protein have been suggested [9]. In two other studies, the percentage of cases with unexplained negative staining seems to be in the same range [7,9].

Taking our data together, we found that only a low percentage of patients whose tumours exhibited abrogation of CHEK2 protein staining actually carried the *CHEK2**1100delC mutation. Homozygosity for *CHEK2**1100delC appears not to be lethal in humans, although subtle molecular defects cannot be excluded. We conclude that CHEK2 protein abrogation is not caused by the germline variants R117G, R137Q, R145W, I157T, and R180H in familial colorectal cancer.

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CHAPTER 9

Concluding remarks and implications for the future

In this thesis, we evaluate the use of molecular pathology for identifying individuals with an increased risk for colorectal cancer (CRC) based on their genetic makeup, and for generating insight into the tumorigenesis of familial CRC.

The described work can be divided into:

1) The use of reliable methods that are applicable in formalin-fixed paraffin-embedded (FFPE) tissues, which is of utmost importance since the majority of tumor tissue from familial CRC is only available as FFPE tissue.

2) Tumor profiling to guide genetic testing strategies and clinical genetic decision making, to gain insight on tumorigenesis in familial CRC [including Lynch syndrome and *MUTYH*-associated polyposis (MAP)], and to study the role of *CHEK2* and *PTPRJ*.

1) The use of reliable methods to test FFPE tissues

We used high-throughput methods suitable for FFPE materials to study the characteristics of colorectal tumors. One of these techniques, the MassEXTEND loss of heterozygosity (LOH) analysis (using Sequenom's MassARRAY RT software) is a sensitive, high-throughput, and cost-effective method for genotyping large series of cases for a limited number of single nucleotide polymorphisms (SNP). Moreover, LOH at a particular SNP can be studied in FFPE tumor tissues (Chapter 7). Shortly after our study, Ollikainen *et al*. used the same method to detect LOH in tumors from patients with a mismatch repair (MMR) defect [1]. In the classical LOH analysis using microsatellite repeat markers, the applicability is impaired in mismatch repair deficient tumors due to the intrinsic instability of these markers. In a later phase, we showed that SNP arrays can also be an excellent way to genotype archival tissues and to identify copy neutral LOH (cnLOH) in mismatch repair deficient tumors (Chapter 3). The introduction of this whole genome SNP array analysis enabled the detection of distinct small regions of cnLOH as well as the identification of copy number alterations in FFPE tumor tissues [2,3]. We used this platform to investigate chromosomal instability (CIN) in microsatellite unstable (MSI-high) carcinomas and MAP carcinomas (Chapter 3 and 5). We also suggest that the SNP array platform may be an important tool for finding the genetic cause of unexplained familial CRC.

Another method that we applied was immunohistochemical (IHC) pre-screening of several hundred familial CRC cases that were compacted into tissue micro arrays. This approach was used to screen for loss of CHEK2 expression in familial CRC and also to identify several unexplained MSI-high cases with loss of PMS2 expression in which later germline mutations were identified (Chapter 8 and Hendriks *et al*. (2006) Gastroenterology 130:312-322. list of additional publications).

2) Tumor profiling

Microsatellite instability (MSI) analysis

MSI analysis and IHC of MMR proteins (including PMS2) in CRC from index patients fulfilling the Amsterdam II or (modified) Bethesda criteria have now become a cost effective approach to identify Lynch syndrome patients and to direct germline MMR testing. The presence of a MSI-high phenotype (sporadic and hereditary) has also been associated with an improved prognosis and altered responses to various chemotherapies when compared to microsatellite stable (MSS) tumors [4-6]. There is now debate as to whether to refrain from 5-FU compounds in cases of CRC with MSI-high phenotypes [7].

MMR unclassified variant (MMR-UV)

The identification of pathogenic MMR mutations in Lynch syndrome can be used to offer pre-symptomatic testing in currently unaffected family members. However, in the case of finding only an MMR-UV, the uncertainty about the contribution of such UV to the risk of developing cancer is a major problem, as these UVs could also represent rare variants without increased risk of cancer. Furthermore, the possibility remains that a true pathogenic mutation has been missed. Besides the existing test modalities (segregation assays, MSI status, IHC of MMR proteins, functional testing, etc.), additional proof is still needed [8]. Therefore, it is of great importance to search for additional tools that can provide insight on pathogenicity. We performed whole genome SNP arrays in MSI-high tumor materials from MMR-UV carriers. In five out of eight MMR-UV cases, additional chromosomal instability (although subtle) was found in comparison with tumors from true pathogenic mutation carriers (Chapter 3). This suggests that such additional CIN was necessary for tumorigenesis in cases with *a priori* weak mutator effects and that another mutation has not been missed. The validity of this observation should now be confirmed in a large series of MMR-UV cases. We recommend the collection of tumors from patients with the same UVs and the study of the patterns of genomic abnormalities in those tumors. Depending on the results, it might be useful to add genome-wide SNP array profiling of tumors from MMR-UV carriers to the existing tools to further elucidate the pathogenicity of the MMR-UVs.

KRAS2 pre-screening in familial CRC

General practitioners and medical specialists should be alert to recognize cases suspected for a hereditary cause of CRC. Several guidelines are available for this purpose; these include a positive family history, the age of onset, or the number and nature of polyps, e.g., adenomatous, hyperplastic, or hamartomatous (www.nav-vkgn.nl). As a supportive test, we studied the presence of the *MUTYH*-specific *KRAS2* c.34 G>T transversion in colon tumors in positive cases, followed by *MUTYH* hotspot analysis in FFPE tissues. The

sensitivity and specificity of the *KRAS2* c.34 G>T test combined with the *MUTYH* hotspot analysis is high for the detection of bi-allelic mutation carriers, although the exact figures cannot be calculated because we do not have access to the complete *MUTYH* sequence of all patients. We concluded that this test can reliably identify patients with (atypical) MAP. Therefore, we recommend *KRAS2* c.34G>T somatic pre-screening, followed by *MUTYH* hotspot mutation analysis if the result of the former is positive. If heterozygous hotspot *MUTYH* mutations are identified, a complete germline *MUTYH* mutation screening should be carried out if possible. Immediate *MUTYH* hotspot mutation analysis is a practical alternative in patients with >10 adenomas, or in cases of multiple CRCs in one generation for which only FFPE tissue is available (Chapter4). To this end, we developed a simplified *KRAS2* mutation detection procedure in archival tissue for codons 12 and 13. Furthermore, this *KRAS2* mutation analysis might be rather beneficial as it was recently shown that the presence of somatic *KRAS2* mutations leads to a negative response upon treatment with EGFR inhibitors in colon and lung cancers. This highlights the need for *KRAS2* mutation analysis to predict the response to treatment [9,10].

CHEK2

The *CHEK**1100delC allele has been proposed as a low-penetrance cancer susceptibility allele for breast cancer, and carriers appear to have a twofold increase in breast cancer risk [11]. The incidence of the 1100delC mutation was suggested to be higher among breast cancer families with CRC than in those without CRC, identifying a hereditary breast and colorectal cancer (HBCC) phenotype [12]. The incidence of the *CHEK2**1100delC mutation in familial and non familial CRC patients was 1.3% and 2.9%, respectively, which is not significantly higher than the European population frequency of 1.1–1.4%. With an estimated range of 1.3–1.6%, the frequency in the Dutch population seems similar [12]. Results suggest that the *CHEK2**1100delC mutation may not be significantly associated with familial CRC or with CRC risk in the population, although a very low-penetrance effect on CRC could not be excluded [13,14]. From our study, we conclude that homozygosity for the *CHEK2**1100delC mutation is not lethal in humans and does not lead to a severe clinical phenotype. CHEK2 protein abrogation is seen in cases with the *CHEK2**1100delC allele but not with the *CHEK2* germline variants, R117G, R137Q, R145W, I157T, and R180H in familial CRC. Other studies reported the correlation between two *CHEK2* variants (1100delC and I157T) and CRC; Sanchez *et al*. demonstrated that *CHEK2**1100delC is not of clinical relevance for Lynch syndrome and HBCC Spanish families [15], and de Jong *et al*. concluded that the frequency of the *CHEK2**1100delC genotype was not significantly increased in unselected CRC patients or in selected CRC patients diagnosed before age 50. However, after stratifying unselected CRC patients according to defined genetic risk, a significant trend of increasing frequency was observed [16]. In a study of Swedish CRC patients, the frequency of *CHEK2**1100delC was not significantly increased [17]. Based

on the research to date, the role of the *CHEK**1100delC allele in familiar CRC seems to be limited, which excludes it as a candidate allele for testing in the clinical genetic context in families with clustering of CRC. Two studies showed that *CHEK2* I157T is associated with an increased risk of CRC. Kilpivaara *et al*. observed the association in both familial and sporadic CRC patients. Furthermore, they found support for the role of *CHEK2* I157T as a susceptibility allele for multiple cancer types [18]. Cybulski *et al*. concluded that the I157T mutation increased the risk of CRC in the population. In addition, they suggested that truncating mutations may confer a lower risk or no increase in risk for CRC [19].

PTPRJ

PTPRJ is one of the colon cancer susceptibility alleles identified in mouse studies. Frequent LOH of the *PTPRJ**1176 A>C allele was shown in human sporadic colorectal, breast [20], and lung tumors [21], and in human thyroid carcinomas [22]. Additionally, Ruivenkamp *et al*. concluded that LOH of the *PTPRJ**1176 A>C allele frequently occurs in the adenoma stage of sporadic human CRC [23]. Our study revealed that the importance of the newly identified *PTPRJ*- c.827A>C SNP appears to be limited in familial CRC. In 2006, one published report suggested that *PTPRJ* plays a role in early colon neoplasia by studying two *PTPRJ* microsatellite markers in 32 aberrant crypt foci [24]. In the recent association studies, the *PTPRJ*- c.827A>C SNP was not identified as a cancer susceptibility allele for CRC. However, this does not exclude the possibility that this SNP functions as a low-penetrant allele.

SNP typing of Lynch syndrome, MMR-UV, sporadic MSI-high and MAP tumors

We characterized chromosomal instability (physical loss, gain, and cnLOH) and microsatellite instability in carcinomas from Lynch syndrome patients with pathogenic MMR mutations, MMR-UV carriers, MAP patients, and patients with sporadic *MLH1* promoter hypermethylation. The profiles were distinct; in MSI-high carcinomas from Lynch syndrome patients with pathogenic mutations, copy number variation is rare. Genome-wide copy neutral LOH is also rare, and the only cnLOH detected is usually confined to the locus harboring pathogenic mutations in *MLH1, MSH2,* or *PMS2*. In MMR-UV cases and sporadic MMR deficiency, there is often a slight increase in chromosomal instability [25,26] (Chapters 3 and 4), whereas MAP carcinomas show many aberrant chromosomal regions. Interestingly, these regions are mostly affected by cnLOH. The latter is in contrast to sporadic colon cancer, where physical chromosomal loss is the main characteristic. The percentages of chromosomal amplifications in MAP and sporadic microsatellite stable colorectal carcinomas are comparable.

Co-segregation of *MSH6* and *MUTYH* germline mutations

The *MSH6* Lynch syndrome family in which family members are heterozygous or compound heterozygous for *MUTYH* germline mutations showed a remarkably mild clinical phenotype of an *MSH6/MUTYH* compound heterozygote mutation carrier (Chapter 6). Selection against MSH6 mismatch repair deficient cells might, at least in part, explain this phenotype, which is in line with Kambare *et al*., who suggested that BER and DNA MMR pathways are mutually exclusive. This suggestion implies that cells with abrogation of both pathways are not viable and undergo apoptosis [27]. We observed only one patient with the above-mentioned genotype. In the literature, combined germline defects such as *APC* plus an MMR mutation are described to be associated with an increased cancer risk or accelerated tumorigenesis [28,29]. *MUTYH* in addition to missense *MSH6* mutations are hypothesized to increase cancer risk [28]. A recent study does not find this association between *MUTYH* and *MSH6* UV and pathogenic germline mutations [30]. The number of patients is relatively low in the latter two studies, and no additional analysis was done in the family members of the identified patients. Additional experiments are now necessary to gain more understanding on the interaction of *MUTYH* and *MSH6*. We therefore obtained primary skin fibroblast cultures from the *MSH6*/*MUTYH* compound heterozygote mutation carrier and from her relatives carrying different combinations of *MSH6* and *MUTYH* mutations. In these cultures, DNA repair mechanisms will be analyzed for apoptotic responses, cell viability, and clonal survival in order to find support for the notion that abrogation of both MSH6 DNA mismatch and base excision repair in a cell can lead to apoptosis and a milder clinical phenotype. Furthermore, it would be of great value to study the fibroblasts from patients and their family members identified in the other studies to compare the difference between *MSH6*-UVs and pathogenic *MSH6* mutations in combination with mono-allelic *MUTYH* mutations.

Implications for the future

The contribution of molecular pathology in the identification of familial causes of CRC in the near future will be dual; it will play a role in diagnostic as well as research settings.

Tests that are readily applicable and straightforward (for example, MSI, additional MMR IHC, and *KRAS2* mutation analyses) will be extensively used in molecular pathology diagnostics.

In a research setting, molecular pathology will be an important player in determining the contribution to an increased cancer risk of the alleles that are presently identified with the analysis of disease susceptibility through whole genome association studies. In these studies, dedicated SNP profiles are identified that can predict higher chances

for certain disorders in individuals. Examples are the recent identification of susceptibility SNPs for breast cancer, CRC, and prostate cancer [31-38]. The CRC susceptibility SNPs published so far all have significant p values ($p < 10^{-7}$) although the odds ratio for each individual SNP is low. Based on a recently published paper on prostate cancer, one can speculate that a combination of five of these variants within one individual leads to an increased relative risk, although severe concerns were recently raised about the statistical analysis of these data [39-46]. The identification of these susceptibility SNPs is based on the common disease-common variant theory. Therefore, this approach is still unable to find rare susceptibility alleles in populations that include recessive alleles. Furthermore, the biological role of the now identified alleles is mostly unknown because these SNPs are most likely just tagging the true gene variants responsible. For all these reasons, the regions of interest are now sequenced for such causal variants [47]. How these responsible SNP variants contribute to an increased CRC risk should also be studied for example through molecular pathology in well-defined patient/tumor and control cohorts with available follow-up data.

The latest screening strategies for CRC in the general population focus on endoscopic surveillance above the age of 50, possibly in combination with fecal occult blood or fecal DNA testing. It will be interesting to see if and when the recently identified common disease/common variant SNPs and the true genetic variants that are linked to them will be implemented in CRC screening in the general population. If successful, generating these types of profiles for CRC susceptibility in the general population might be a very beneficial screening method, although ethical problems may be encountered.

Our recent experience showed that the role of tumor profiling in the search for as yet unidentified genetic causes of CRC is often met with skepticism. We now argue that the distinct tumor profiles that are found (chapters 3 and 5) are convincing examples that molecular pathology approaches might also be crucial for the characterization and possible elucidation of unresolved familial causes of CRC. We should not forget this critical example: the identification of *MUTYH* mutations in 2002 as the genetic cause for many unexplained polyposis patients, later named *MUTYH*-associated polyposis, came initially from tumor analysis.

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CHAPTER 10

Summary

In this thesis, molecular tools were applied to tumor tissues to identify individuals burdened with a genetic risk for colorectal cancer (CRC) and to generate insight into the tumorigenesis of familial CRC.

Chapter 1 gives a general introduction about the factors that determine the individual risk of CRC in the general population. A brief overview on colorectal tumorigenesis is given. Inheritable CRC syndromes and the contribution of low level genetic risk factors and environmental factors to CRC risk are also described.

Tumors from individuals with an early onset in addition to clustering of CRC in the family are analyzed for microsatellite instability and expression of the mismatch repair (MMR) genes (*MLH1*, *PMS2*, *MSH2*, *MSH6*) to identify Lynch syndrome and to give direction to possible additional germline mutation analysis. When a pathogenic mutation in one of the MMR genes is found, all family members can undergo testing for the presence of the identified germline defect. In **chapter 2**, the yield of microsatellite instability (MSI) analysis in families suspected for Lynch syndrome, for a group fulfilling the Bethesda criteria and a group that does not, was evaluated. We found that it would be better to include late onset families (three or more cases of CRC diagnosed at age >50 years) in the testing schemes and to raise the age at diagnosis of CRC from 45 to 50 years. In addition, we compared the results of immunohistochemical (IHC) staining and MSI analysis and assessed the additional value of PMS2 staining. Based on that part of the study, we recommend the inclusion of PMS2 staining in the panel of antibodies (MLH1, MSH2, and MSH6) to identify families eligible for mutation analysis.

In **chapter 3**, the patterns of genomic abnormalities of microsatellite unstable (MSI-high) CRC tumors from carriers of pathogenic germline mutations or unclassified variants (UVs) in MMR genes and tumors with methylation of the *MLH1* gene were studied. We identified different chromosomal aberrations in terms of frequency and distribution in the three MSI-high carcinoma groups, although these differences were subtle. Of interest was the increased number of chromosomal aberrations in colon carcinomas from MMR-UV carriers compared to pathogenic MMR mutation carriers and carcinomas with *MLH1* promoter hypermethylation. Apparently, chromosomal instability (CIN) was added to microsatellite instability in these MMR-UV cases during tumorigenesis.

To identify *MUTYH*-associated polyposis (MAP) families that do not fulfill the clinical criteria for *MUTYH* germline mutation screening, we studied the feasibility of implementing a *KRAS2* c.34 G>T pre-screening method followed by an *MUTYH* hotspot mutation analysis in **chapter 4**. *KRAS2* c.34 G>T is found in 60% of MAP carcinomas but is infrequent in consecutive series of CRC. We tested formalin-fixed paraffin-embedded (FFPE) tumor tissues from individuals who presented with <10 adenomas or familial mismatch repair proficient colorectal carcinomas with <10 concomitant adenomas for somatic *KRAS2* mutations and for three Dutch hotspot *MUTYH* germline mutations (p.Tyr165Cys, p.Gly382Asp and p.Pro391Leu). We identified bi-allelic mutation carriers

with this approach. Therefore, we recommend performing the *KRAS2* c.34G>T somatic pre-screening and, if the result is positive, a subsequent *MUTYH* hotspot mutation analysis. When heterozygous hotspot *MUTYH* mutations are identified, a complete germline *MUTYH* mutation screening should be carried out if possible. Immediate *MUTYH* hotspot mutation analysis was a practical alternative in patients with >10 adenomas or in cases of multiple CRCs in one generation for which only FFPE tissue was available.

In CRC, there are two classical pathways that direct tumorigenesis: microsatellite instability (MSI or MIN) with near-diploidy and CIN. In MAP, the pathway involved in tumorigenesis remains unclear; both aneuploidy in adenomas as well as near-diploidy in carcinomas have been reported. In **chapter 5**, we analyzed 26 MAP carcinomas using SNP arrays. The high prevalence of copy neutral loss of heterozygosity (cnLOH) detected in those MAP carcinomas suggests a relationship between mitotic recombination and base excision repair (BER) deficiency, although further research into this possible relationship is required.

In the inherited MAP and Lynch syndrome, somatic mutations occur due to a loss of the caretaker functions that BER and MMR genes have, respectively. In **chapter 6**, a branch of a Lynch syndrome family in which *MSH6* and *MUTYH* germline mutations co-segregate was studied. One patient carried three mutations (1x *MSH6*, 2x *MUTYH*) and had an extremely mild clinical phenotype with only a few adenomas so far. We concluded that our data support the notion that abrogation of both MSH*6* DNA mismatch repair and base repair might be mutually exclusive in humans.

It is essential that candidate CRC predisposing genes appearing in the literature are verified in well-defined familial CRC cohorts and unexplained familial CRC cohorts. To improve efficiency, we studied the use of two high-throughput methods to analyze candidate CRC genes (chapters 7 and 8). In **chapter 7**, we describe the importance of the newly identified *PTPRJ**1176 A>C allele that appears to be limited to familial CRC. We concluded that MassEXTEND LOH analysis (using Sequenom's MassARRAY RT software) was a sensitive, high-throughput, and cost-effective method to screen SNP loci for LOH in FFPE tissues. In **chapter 8**, we concluded that homozygosity for the *CHEK2**1100delC mutation is not lethal in humans and does not lead to a severe clinical phenotype and that the loss of CHEK2 protein expression observed in familial CRC is not caused by the *CHEK2* germline variants, R117G, R137Q, R145W, I157T, and R180H. Furthermore, we concluded that immunohistochemistry on tissue microarrays is a valuable pre-screening method. The disadvantage of this technique is that the genetic alterations in the tumors must by definition lead to protein abrogation, and an antibody against the target of interest must also be available.

Chapter 9 contains concluding remarks and implication for the future. Molecular pathology has a high potential for playing an active role in identifying individuals with CRC

predisposing syndromes in a diagnostic setting as well as in studying tumorigenesis of CRC in a research setting.

Tests such as MSI, additional MMR IHC (chapter 2), and *KRAS2* mutation analyses (chapter 4), which are readily applicable and straightforward, are now extensively used in our daily molecular pathology diagnostics.

In the research setting, molecular pathology will be an important player in study the contribution to an increased CRC risk of the susceptibility alleles that are being identified. Furthermore, we now argue that the distinct tumor profiles that are found (chapters 3 and 5) are convincing examples that molecular pathology approaches are also crucial in the characterization and elucidation of unresolved familial causes of CRC.

CHAPTER 11

Nederlandse samenvatting

Curriculum vitae

List of additional publications

Nederlandse samenvatting

Dikke darmkanker (colorectaal carcinoom, CRC) is de op een na meest voorkomende doodsoorzaak in de westerse wereld. In Nederland worden elk jaar circa 11000 nieuwe gevallen gediagnosticeerd. Ongeveer de helft zal hieraan overlijden. Voor de algemene bevolking is het risico om CRC te ontwikkelen gedurende het leven bij benadering 4%, dus 1:25 personen krijgt darmkanker. Aan de oorzaak van CRC kunnen meerdere factoren ten grondslag liggen, enerzijds erfelijke hoog en laag risico factoren, anderzijds omgevingsfactoren inclusief de rol van de individuele levensstijl.

DNA is de essentie van erfelijkheid, de opslagplaats van alle informatie die nodig is om een mens "te bouwen". Het erfelijk materiaal is verankerd in de chromosomen (elke cel heeft 46 chromosomen; 23 chromosomen van vader en 23 van moeder). De informatie voor een kenmerk (bv. oogkleur) die aanwezig is op een chromosoom, wordt gen genoemd. De vorm (bv. blauw) waarin een bepaald gen zich manifesteert in het DNA van een chromosoom, wordt allel genoemd.

Dikkedarm tumoren zijn het gevolg van een reeks van opeen stapelende DNA fouten waarbij het normale darmslijmvlies uiteindelijk verandert in een kankerproces.

De tumorontwikkeling bij CRC kan worden verdeeld in twee verschillende richtingen, te weten; chromosomale instabiliteit (CIN) en microsatelliet instabiliteit (MIN, MSI). Chromosomale instabiliteit wordt gekenmerkt door variaties in het aantal kopieën van een chromosoom waaronder chromosoom (arm) vermeerdering, chromosoom (arm) verlies en zogenaamd kopieneutraal verlies van heterozygositeit. Microsatelliet instabiliteit wordt gekenmerkt door kleine DNA deleties en inserties in korte repeterende DNA-stukjes (bijvoorbeeld CGCGCGCGCGCGCG). Naast CIN en MIN kunnen ook zogenaamde epigenetische veranderingen plaatsvinden gedurende de tumorontwikkeling. Dit proces kenmerkt zich door methyleringsveranderingen, een modificatie van het DNA in de cellen zonder wijziging in de DNA sequentie volgorde. Deze veranderingen leiden tot wijzigingen in genfunctie.

Het spectrum van CRC kan in twee klinische groepen worden verdeeld: sporadisch en familiair CRC. Met sporadisch wordt bedoeld dat er geen andere gevallen van dikke darmkanker worden gevonden in de familie. De meeste patiënten (65-90%) behoren tot deze groep. Zij ontwikkelen CRC meestal op oudere leeftijd (gemiddeld 70 jaar). De tweede groep bestaat uit 10-35% van alle CRC gevallen hierbij zijn er meer CRC patiënten binnen de familie. Slechts een klein deel van deze groep kan worden verklaard door bekende hoog penetrante erfelijke syndromen zoals het Lynch syndroom (eerder HNPCC genaamd) en familiaire adenomateuze polyposis (FAP). Lynch syndroom betreft een erfelijke aandoening met vaak rechts in de buik gelegen dikke darmkanker en relatief weinig dikke darmpoliepen. Het syndroom ontstaat door erfelijke kiembaanmutaties in de genen *MLH1*, *MSH2*, *MSH6* of *PMS2*. FAP-patiënten hebben juist heel veel poliepen

(adenomen) met een hoge kans dat een of meerdere hiervan zich kwaadaardig ontwikkelen. Het ziektebeeld wordt voornamelijk veroorzaakt door kiembaanmutaties in het *APC* gen. Het is reeds voldoende om de ziekte te krijgen als deze mutatie van een van de ouders wordt geërfd (een zogenaamd autosomaal dominant overervings patroon) dan wel nieuw ontstaan is. Een deel van de patiënten met adenomateuze polyposis kan verklaard worden door *MUTYH*-geassocieerde polyposis (MAP). Het onderliggende lijden wordt verklaard door twee kiembaan mutaties in *MUTYH*, geërfd van beide ouders (een autosomaal recessief overervings patroon). Voor veel individuen met familiair darmkanker is de reden nog onverklaard. Het risico van personen uit onverklaarde darmkankerfamilies om gedurende het leven CRC te ontwikkelen in vergelijking met de algemene bevolking is > 2 keer verhoogd wanneer een individu een aangedane eerstegraads verwant heeft. Het risico is > 3 keer verhoogd in vergelijking met de algemene bevolking wanneer de eerstegraads verwant jonger is dan 50 jaar op het moment van diagnose. Mogelijk zijn enkele onverklaarde familierisico's aan nog niet geïdentificeerde erfelijke hoogrisicofactoren toe te schrijven. Een andere optie zou een combinatie van verschillende erfelijke laagrisicofactoren kunnen zijn. Van omgevingsfactoren, als de westerse leefstijl; alcohol consumptie, roken, dieet en vetzucht is bekend dat ze in enige mate bijdragen aan het risico op CRC.

Dit proefschrift beschrijft de zoektocht naar analyses in de moleculaire pathologie die een rol kunnen spelen in het identificeren van individuen met een verhoogd risico op dikke darmkanker gebaseerd op onderliggende erfelijke oorzaken en die het mogelijk maken inzicht te verkrijgen in de tumorontwikkeling bij familiair CRC.
Het beschreven werk kan ruwweg worden verdeeld in:

1) Het toepassen van betrouwbare moleculaire analyses in formaline gefixeerd in paraffine ingebed (FFPE) weefsel. Dit laatste is bijzonder belangrijk omdat het gros van het tumor weefsel van familiaire dikke darmkanker patiënten alleen beschikbaar is als FFPE materiaal.

2) Karakterisering van tumor materiaal waardoor richting gegeven kan worden aan genetische test strategie en klinisch genetische besluitvorming maar tevens wordt ook inzicht verkregen in de tumorgenese in familiair dikke darmkanker (inclusief Lynch syndroom en *MUTYH* geassocieerde polyposis) en het bestuderen van de rol van de genen; *CHEK2* en *PTPRJ*.

De CRC tumoren van individuen die voldoen aan een aantal klinische criteria (jonge leeftijd dan wel meerdere individuen in de familie met CRC) dienen te worden geanalyseerd op microsatelliet instabiliteit en eiwitexpressie van de mismatch herstel (MMR) eiwitten (MLH1, PMS2, MSH2, MSH6). Deze analyses maken het mogelijk om het patiënten met Lynch syndroom te identificeren en richting te geven aan mogelijke aanvullende kiembaanmutatie analyses. Wanneer eenmaal een kiembaanmutatie in één van de MMR genen wordt gevonden, kunnen familieleden op de aanwezigheid van de geïden-

tificeerde mutatie worden onderzocht. De dragers van de mutatie kunnen vervolgens worden gescreend op darmslijmvlies afwijkingen zodat deze in een vroeg stadium van eventuele tumor ontwikkeling kunnen worden verwijderd. In **hoofdstuk 2** werd de opbrengst van microsatelliet instabiliteits (MSI) analyse geëvalueerd; enerzijds in families verdacht voor Lynch syndroom, anderzijds in een groep die niet aan de klinische criteria voldeden. Uit de resultaten bleek dat het beter is families met drie of meer gevallen van CRC gediagnostiseerd > 50 jaar oud, in de test schema's te includeren en de indicatie leeftijd voor MSI analyse op te trekken van 45 naar 50 jaar. Daarnaast werden de resultaten van de immunohistochemische (IHC) kleuringen vergeleken met die van de MSI analyse en werd de additionele waarde van de PMS2 kleuring bestudeerd. De PMS2 kleuring leidde tot de identificatie van een additionele 23% *MLH1* kiembaan mutatie dragers. Gebaseerd op dit deel van de studie was het advies, de PMS2 kleuring op te nemen in het panel van MMR antilichamen (MLH1, MSH2, MSH6) voor het identificeren van families die in aanmerking komen voor mutatie analyse.

In **hoofdstuk 3** werden de patronen van genomische abnormaliteiten van microsatelliet instabiele (MSI-high) FFPE carcinomen bestudeerd met "genoom omvattende enkel nucleotide polymorfisme arrays" (SNP arrays). Drie groepen MSI-high carcinomen werden samengesteld, de eerste groep bestond uit carcinomen van dragers van pathogene kiembaanmutaties in één van de MMR genen. De tweede groep was samengesteld uit carcinomen van niet geclassificeerde varianten (UVs) in de MMR genen, de derde groep bevatte carcinomen met sporadische hypermethylering van de *MLH1* promoter. Wij identificeerden verschillende chromosomale afwijkingen in de drie MSI-high carcinoma groepen, hoewel deze verschillen subtiel waren. Interessant was het verhoogde aantal chromosomale afwijkingen in carcinomen van MMR-UV dragers in vergelijking met pathogene MMR mutatie dragers. Blijkbaar, ontstond additionele CIN bij de aanwezige microsatelliet instabiliteit in deze MMR-UVs tijdens tumor ontwikkeling. Dit zou kunnen suggereren dat dergelijke additionele CIN voor tumorontwikkeling in gevallen met een *a priori* zwak mutator effect noodzakelijk is. Om deze bevinding beter te kunnen onderbouwen, lijkt het logisch de studie uit te breiden; meerdere carcinomen van patiënten met eenzelfde UV moeten verzameld worden en de genomische tumor patronen zullen moeten worden bestudeerd. Afhankelijk van de resultaten zal deze SNP array benadering nuttig kunnen zijn om naast de reeds gebruikte hulpmiddelen (MSI analyse, IHC van MMR proteïnen, segregatie analyse en functionele analyses) inzicht te verschaffen in de pathogeniciteit van MMR-UVs.

Om MAP families te identificeren die niet aan de klinische criteria voldeden, en dus niet in aanmerking kwamen voor kiembaan *MUTYH* onderzoek, bestudeerden wij de haalbaarheid van het uitvoeren van een onderzoekmethode die een specifieke *KRAS2* mutatie (c.34 G> T) analyseert. Deze specifieke *KRAS2* mutatie wordt gevonden in 60% van de carcinomen van MAP patiënten en is zeldzaam in dikke darmcarcinomen van an-

dere origine. Bij detectie van de *KRAS2* mutatie in onze studie werd deze analyse gevolgd door een mutatie analyse van de drie meest voorkomende *MUTYH* mutaties in Nederland (*MUTYH* hotspots). In **hoofdstuk 4** hebben wij FFPE tumoren getest, van individuen met <10 adenomen of patiënten met een microsatelliet stabiele dikke darmtumor met <10 additionele poliepen (adenomen), op somatische DNA-veranderingen in *KRAS2* en op drie Nederlandse hotspot *MUTYH* kiembaanmutaties (p.Tyr165Cys, p.Gly382Asp en p.Pro391Leu). Wij waren in staat met deze benadering bi-allelische *MUTYH* mutatie dragers te identificeren. Daarom adviseren wij om dit onderzoek van tumoren op aanwezigheid van de *KRAS2* c.34G >T mutatie bij positiviteit te vervolgen met *MUTYH* hotspot mutatie analyse en deze combinatie van analyses te implementeren in de moleculaire diagnostiek voor het opsporen van mogelijk erfelijke vormen van darmkanker. Wanneer een heterozygote hotspot *MUTYH* verandering wordt geïdentificeerd, moet een volledig kiembaan *MUTYH* mutatie onderzoek worden uitgevoerd op DNA uit bloed. Tevens is de directe hotspot *MUTYH* mutatie analyse een praktisch alternatief voor patiënten met >10 adenomen of in gevallen van veelvoudige CRCs in één generatie waarvan slechts FFPE weefsel beschikbaar is.

Zoals eerder genoemd zijn er in CRC twee klassieke wegen die tot tumorontwikkeling leiden; CIN en MIN cq MSI. Het proces dat in *MUTYH*-geassocieerde polyposis (MAP) tot tumor formatie leidt is nog niet compleet ontrafeld. In **hoofdstuk 5** analyseerden wij 26 MAP carcinomen, gebruik makend van SNP arrays. Een hoge frequentie kopienummer neutraal verlies van heterozygositeit (cnLOH) werd gevonden in deze carcinomen. Dit betekent dat er verlies heeft plaats gevonden van een paternaal of een maternaal allel. Het allel dat overbleef is gedupliceerd zodat er weer twee allelen zijn. Dit proces wordt mitotische recombinatie genoemd. De bevindingen suggereren een relatie tussen mitotische recombinatie en deficiëntie van het base excisie herstel mechanisme (BER) waar het *MUTYH* gen deel vanuit maakt. Echter verder onderzoek naar deze mogelijke relatie is belangrijk.

In **hoofdstuk 6** werd een tak van een Lynch syndroom familie waarin zowel een *MSH6* als twee *MUTYH* kiembaan mutaties voorkomen bestudeerd. Eén patiënt droeg alle drie de veranderingen en had een uiterst mild klinisch beeld met tot op heden slechts enkele adenomen. Wij concludeerden dat een defect in zowel MSH6 mismatch herstel en base excisie herstel wellicht niet goed naast elkaar kan bestaan. Cellen die alle drie de defecten dragen hebben wellicht maar een kleine kans om te overleven.

Het is essentieel dat mogelijke genetische risicofactoren, die leiden tot een hogere kans op CRC beschreven in de literatuur, in goed gedefinieerde CRC cohorten met controle groepen worden geverifieerd. Wij kozen twee methoden met een hoge doorvoersnelheid om beschreven mogelijke genetische risicofactoren te bestuderen (**hoofdstuk 7** en **hoofdstuk 8**). In **hoofdstuk 7** onderzochten we de rol van *PTPRJ*- c.827A>C in familiair CRC met de MassEXTEND LOH analyse. PTPRJ behoort tot de eiwit tyrosine fosfatase

familie. Deze familie bestaat uit signaal moleculen die verschillende processen in de cel regelen waaronder celgroei, celdifferentiatie en er is gesuggereerd dat ze mogelijk functioneren als een tumoronderdrukker in darmkanker. Wij beschreven dat de impact van de nieuw geïdentificeerde *PTPRJ*- c.827A>C beperkt lijkt te zijn in familiaire CRC. Verder concludeerden wij dat de MassEXTEND LOH analyse (gebruik makend van Sequenom MassARRAY software) een sensitieve, kosteneffectieve analyse is met een hoge doorvoersnelheid om LOH van SNPs te bestuderen in gearchiveerd tumor weefsel.

In **hoofdstuk 8** werd de rol van de *CHEK**1100delC mutatie bestudeerd. CHEK2 speelt een rol in DNA herstel, in celdeling en celdood. Een immunohistochemische pre-screening met een CHEK2 antilichaam werd uitgevoerd op 564 tumoren afkomstig van familiaire darmkanker patiënten die op "tissue micro array's" (TMAs) waren samengevoegd. Wij identificeerden drie patiënten met een *CHEK**1100delC mutatie één van deze patiënten had twee *CHEK**1100delC mutaties, welke niet leidde tot een evident klinisch beeld. Tevens werd verlies van eiwitexpressie van CHEK2 in familiair CRC in een beperkt deel door de *CHEK**1100delC verklaard, het expressie verlies kon niet worden verklaard door *CHEK2* kiembaan varianten R117G, R137Q, R145W, I157T, en R180H. IHC pre-screening van TMAs is een betrouwbare pre-screenings methode voor *CHEK**1100delC. Het nadeel van deze methode met hoge doorvoersnelheid is dat de te onderzoeken mutatie moet leiden tot afschakeling van het betreffende eiwit en een antilichaam voorhanden moet zijn wil deze methode bruikbaar zijn.

Hoofdstuk 9 bevat concluderende opmerkingen over het onderzoek zoals beschreven in dit proefschrift en de invloed die het mogelijk in de toekomst zal hebben. De moleculaire pathologie heeft een hoog potentieel om een actieve rol te spelen in het identificeren van individuen met een erfelijke belasting op CRC. Tevens kan met moleculaire technieken tumorontwikkeling van CRC worden bestudeerd.

Testen zoals; MSI, additionele MMR IHC (hoofdstuk 2) en *KRAS2* mutatie analyse (hoofdstuk 4) die goed toepasbaar zijn en relatief eenvoudig te implementeren zijn worden nu intensief gebruikt in de dagelijkse moleculaire diagnostiek op pathologie-afdelingen.

Binnen het wetenschappelijk onderzoek zal de moleculaire pathologie een belangrijke schakel zijn bij het bepalen van de biologische rol van de allelen die op dit moment met associatie studies geïdentificeerd worden als veronderstelde ziekte gerelateerde allelen. Bovendien zijn de onderscheidende tumorprofielen die gevonden werden in de verschillende tumor groepen(hoofdstuk 3 en 5) overtuigende voorbeelden van het feit dat benaderingen met moleculaire pathologie cruciaal kunnen zijn in het karakteriseren en mogelijk oplossen van tot op heden onopgeloste oorzaken van darmkanker.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 6 november 1972 te Goirle. Na het behalen van een diploma aan de St. Canisius MAVO te Tilburg in 1990 werd in 1994 het diploma van het Middelbaar Laboratorium Onderwijs aan het toenmalige Spectrum College Breda te Breda behaald. Tijdens de afstudeerstage van het Hoger Laboratorium Onderwijs aan de Leidse Hogeschool te Leiderdorp werd onderzoek gedaan naar de rol van CD40-CD40 ligand interacties in muizen met experimentele autoimmuun encephalomyelitis (TNO Preventie en Gezondheid, Immunologische en Infectieziekten, Leiden, Prof. J.D. Laman). Na het behalen het HLO diploma in 1997 werd een VSB-beurs aan de auteur toegekend waarmee zij, in de functie van analiste, een jaar onderzoek deed naar B cel ontwikkeling in muismodellen (Basel Institute for Immunology, Basel, Zwitserland, Prof. J. Anderson and Prof. F. Melchers). Na terugkeer in Nederland werd gedurende 8 maanden als analiste gewerkt aan een onderzoek naar immunotherapie in muismodellen (Nederlands Kanker Instituut, Tumor Immunologie, Amsterdam, Prof. A.M. Kruisbeek). In mei 1999 trad de auteur als analiste in dienst van de afdeling pathologie van het LUMC in Leiden. Van januari 2003 tot mei 2008 werd het onderzoek beschreven in dit proefschrift aldaar uitgevoerd.

List of additional publications

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