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HNPCC,
Molecular and Clinical Dilemmas

Anja Wagner

HNPCC,
Molecular and Clinical Dilemmas

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Onvergeeflijk is het, dat de mensen dromen hebben
en ze niet waarmaken!
En toch, hoop vervliegt elke dag en overal
In elke kamer. Ieder bed.
Op mijn reizen heb ik dit duidelijk gezien:
Iedere ontmoeting, elk gesprek brengt mensen verder af
van wat zij willen.
Zij vertrouwen niet op wat zij zijn,
maar op hoe zij worden gezien.

Arthur Japin

Voor mijn ouders

In herinnering aan Ondiene en aan mijn patiënten die stierven aan kanker

Contents

Aims and outline of the thesis 9

Chapter 1. Introduction

1.1 Hereditary Non Polyposis Colorectal Cancer (HNPCC) 13
1.2 History 13
1.3 The HNPCC genes and their protein products 15
1.4 Candidate genes 19
1.5 The HNPCC gene mutation spectra 20
1.6 Molecular basis of tumour initiation and progression in HNPCC 21
1.7 Cancer risks 31
1.8 HNPCC tumours: histopathologic features 34
1.9 Microsatellite Instability (MSI) 34
1.10 Prognosis 35
1.11 Screening 36
1.12 Prevention 40
1.13 Differential diagnosis 41
1.14 Molecular diagnostics of HNPCC 43
1.15 Selection for mutation analysis 47
1.16 Genetic counselling 52

Chapter 2. Molecular genetic studies in HNPCC

2.1 Molecular Analysis of Hereditary Non-Polyposis Colorectal Cancer (HNPCC) in the USA: High Mutation Detection Rate among Clinically Selected Families and Characterisation of an American Founder Deletion in the *MSH2* Gene. *Am J Hum Genet* 2003:72;1088-1100. 57

2.2 A Founder Mutation of the *MSH2* Gene and Hereditary Nonpolyposis Colorectal Cancer in the United States. *JAMA* 2004:291;718-724. 73

2.3 A 10-Mb Paracentric Inversion of Chromosome arm 2p Inactivates *MSH2* and is Responsible for Hereditary Non Polyposis Colorectal Cancer in a North-American Kindred. *Genes Chromosomes Cancer* 2002:35;49-57. 83

Chapter 3. Clinical studies in HNPCC

3.1 Atypical HNPCC owing to *MSH6* germline mutations: analysis of a large Dutch pedigree. *J Med Genet* 2001:38;318-322. 97

3.2 Cancer Risk in Hereditary Nonpolyposis Colorectal Cancer Due to *MSH6* Mutations; Impact on Counselling and Surveillance. *Gastroenterology* 2004:127;17-25. 105

3.3 Genetic testing in hereditary non-polyposis colorectal cancer families with a *MSH2*, *MLH1*, or *MSH6* mutation. *J Med Genet* 2002:39;833-837. 117

3.4 Long term follow-up of HNPCC gene mutation carriers; compliance with screening and satisfaction with counselling and screening procedures. *Accepted for publication in Familial Cancer* 2005:4. 125

Chapter 4. Discussion 141

References 146

Summary 163

Samenvatting 166

List of tables and figures 169

Curriculum vitae 170

List of publications 171

Aims and outline of the thesis

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is the main inherited predisposition to colorectal cancer. Major features of HNPCC are colorectal and endometrial cancers. Tumours of the ovaries, stomach, small bowel, biliary tract, urinary tract, skin and brain occur at lower frequencies. Mutations in at least 4 different mismatch repair (MMR) genes are responsible for HNPCC, namely *MSH2* and *MLH1* in the majority of cases and, more rarely, *MSH6* and *PMS2*. In previous studies, *MSH2* and *MLH1* mutations were found in 45-64% of the families with HNPCC[73].

In **Chapter 2**, we questioned whether unresolved HNPCC families were due to a lack of sensitivity of *MSH2* and *MLH1* mutation detection techniques, or to mutations of *MSH6* or other genes. To this aim, we thoroughly investigated a cohort of 59 US HNPCC families, clinically selected by Prof. Henry Lynch, for the presence of point mutations and genomic rearrangements in the *MSH2*, *MLH1* and *MSH6* genes. We identified a North American founder mutation, a deletion of *MSH2* exons 1-6, common to 12% of the cohort. The birthplace of the eldest ancestor carrying this founder mutation could be traced back to 18th century Germany. Additionally we detected a10-Mb paracentric inversion inactivating the *MSH2* gene.

The clinical cancer phenotype associated with mutations of *MSH6* is less well defined when compared to *MLH1* and *MSH2*. In **Chapter 3.1** and **3.2**, we analysed a large Dutch pedigree and a cohort of 20 *MSH6* mutation positive families to calculate the cumulative age-specific risks of colorectal cancer and endometrial cancer, and compared the outcomes with the cancer risks in *MSH2* and *MLH1* mutation carriers. Based on the findings, we formulated *MSH6*-tailored screenings and preventative options.

The detection of a germline mutation in *MSH2*, *MLH1* or *MSH6* in an individual enables predictive genetic testing of at-risk relatives. In **Chapter 3.3**, we analysed the demand for genetic testing of members of 18 Dutch families with a known mutation in *MSH2*, *MLH1* or *MSH6*. Regular colonoscopy reduces the overall mortality by 65% in members of families with HNPCC [117]. It is therefore of utmost importance that mutation carriers adhere to surveillance protocols. We evaluated (**Chapter 3.4**) the impact of genetic testing on the adherence to cancer surveillance protocols. Simultaneously, we investigated the satisfaction with cancer screening and genetic testing procedures in 70 proven mutation carriers at the long term.

The studies described in this thesis contribute to the understanding of the molecular genetic aetiology of HNPCC, and add to evidence-based clinical care for HNPCC families.

Chapter 1.

Introduction

1.1. Hereditary Non Polyposis Colorectal Cancer syndrome (HNPCC)

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) or Lynch syndrome (MIM114500) is the most common genetic susceptibility for colorectal cancer. It accounts for 3-5% of all colorectal cancers in the Western world[73]. The HNPCC phenotype also includes other cancers, predominantly of the endometrium, but also ovarian, gastric, small bowel, biliary tract, urinary tract, skin and brain cancer may occur[5, 54, 158, 302, 303]. HNPCC is caused by germline mutations in the mismatch repair (MMR) genes *MSH2*, *MLH1*, *MSH6*, and *PMS2* (Table 1)[10, 26, 58, 152, 206, 218, 225]. The inheritance pattern is autosomal dominant, as shown by the 50% risk of children of an MMR gene mutation carrier of inheriting this predisposition to cancer. The Amsterdam criteria (Table 2) have been established by the International Collaborative Group on HNPCC (ICG-HNPCC) to allow clinical selection of HNPCC families. However, not all families fulfilling these criteria are bona fide HNPCC families. Viceversa, MMR gene mutations are also found in Amsterdam criteria negative families. Since loss of mismatch repair function causes microsatellite instability (MSI), a type of genetic instability in repetitive DNA sequences in the majority of HNPCC related cancers, this and aberrant immunohistochemical staining of MMR proteins are additional tools to identify HNPCC families on tumour material. As in the majority of cancer predisposition syndromes, (presymptomatic) diagnosis of HNPCC is of major importance for appropriate counselling, clinical surveillance and cancer prevention.

1.2. History

The first HNPCC family was reported in 1913 by A.S. Warthin (Figure 1)[324]. He described the family of his seamstress, known as Family G. Lynch et al. (Figure 1) described two additional HNPCC families and revisited family G in 1966 and 1971 respectively[185, 186].

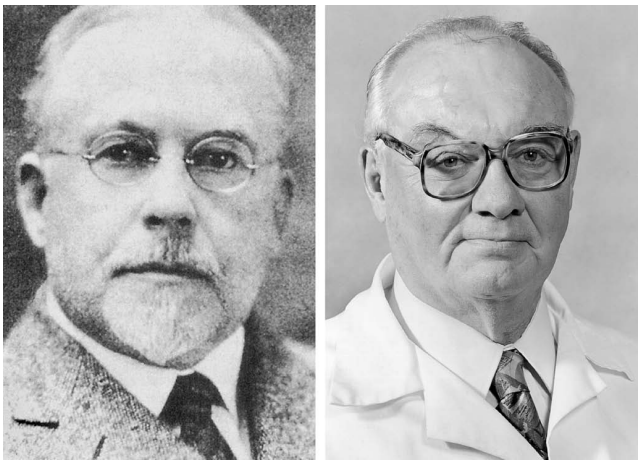

Figure 1.
A.S. Warthin (1866-1931), H.T. Lynch

Table 1.
Proven and "candidate" HNPCC genes.

Gene	Bacterial Homologue	Chromosome position	cDNA(kb)	Number of exons	HNPCC associated
MSH2	MutS	2p21	2,8	16	yes
MLH1	MutL	3p21-23	2,3	19	yes
MSH6	MutS	2p21	4,2	10	yes
PMS2	MutL	7p22	2,6	15	yes
PMS1	MutL	2q31-33	2.8	12	possibly
MLH3	MutL	14q24.3	4,7	12	probably not
MSH3	MutS	5q11-12	3,4	24	probably not
Exo1		1q42-43	3	14	probably not
TGFβR2		3p22	1,7	7	probably not

(www.expasy.org)

Table 2.
The Amsterdam criteria for the clinical diagnosis of HNPCC families[299,301].

Amsterdam criteria:	1: At least three relatives with colorectal cancer
	2: One should be first-degree relative of the other two
	3: At least two successive generations should be affected
	4: At least one should be diagnosed before age 50
	5: Familial Adenomatous Polyposis should be excluded
	6: Tumours should be verified by pathological examination
Revised Amsterdam criteria:	1: At least three relatives with an HNPCC-associated cancer (colorectal, endometrial, small bowel, ureter or renal pelvis cancer)
	2: One should be first-degree relative of the other two
	3: At least two successive generations should be affected
	4: At least one should be diagnosed before age 50
	5: Familial Adenomatous Polyposis should be excluded
	6: Tumours should be verified by pathological examination

Lynch recognised the autosomal dominant pattern of inheritance, and delineated HNPCC or Lynch syndrome. In 1986, autosomal dominant inheritance of colorectal cancer was also proven using segregation analysis in a cohort of 11 families[16].
International clinical criteria for HNPCC, the Amsterdam criteria, were formulated by the ICG-HNPCC in 1991 and subsequently updated in 1999 (Table 2)[299, 301].
In 1993 germline mutations in the MMR gene *MSH2* were found to be responsible for HNPCC (Table 1), followed by mutations in *MLH1*, *MSH6*, and *PMS2*[10, 26, 58, 152, 206, 218, 225].
The Bethesda guidelines to select HNPCC and HNPCC-like families for mutation analysis using MSI as a pre-screening tool were formulated in 1997 and updated in 2004 (Table 3)[248, 296]. International criteria for the diagnosis of MSI in colorectal cancer were formulated in 1998 (Table 4)[24]. More recently, immunohistochemical (IHC) analysis of the MSH2, MLH1, MSH6 and PMS2 protein in tumour sections has been added as an additional tool to direct mutation analysis[42, 43, 100, 160, 284]. Guidelines for screening of HNPCC risk carriers were proposed, and were proven to decrease overall mortality considerably[27, 117, 118, 238]. Since 1992, the Dutch Foundation for the Detection of Hereditary Tumours (Stichting Opsporing Erfelijke Tumoren/STOET) has been registering Dutch HNPCC family members and has played an important national and international role in evaluating the efficacy of cancer screening in HNPCC risk carriers. In 2001, the American Gastroenterological Association formulated recommendations on hereditary colorectal cancer and genetic testing[73]. Also, the Working Group on Oncogenetics of the Dutch Association of Clinical Genetics (Vereniging Klinische Genetica Nederland/VKGN) formulated guidelines for genetic testing, counselling and surveillance of HNPCC and HNPCC-like families[201].

1.3. The HNPCC genes and their protein products

As mentioned above, four MMR genes are known to date to cause HNPCC: *MSH2* and *MLH1* are responsible for the majority of the classical cases, whereas mutations in *MSH6* and *PMS2* account for more atypical kindreds (Table 1)(Figure 3).
MSH2. The *MSH2* gene was the first human HNPCC gene to be cloned[58, 152]. It resides on chromosome 2p21 and is the human homologue of the bacterial mismatch repair gene MutS. The *MSH2* gene contains 16 exons, with a total cDNA length of 2.8kb. In 2000, the crystal structure of the MutS protein was elucidated[149, 220], providing novel insights in the MMR function of the MutS and MSH2 protein. MutS proteins form a dimer with the general shape of two "opposing commas" or "praying hands" (Figure 2).

Table 3.
The Bethesda guidelines to select for MSI testing of colorectal tumours[248,296].

Bethesda guidelines:	1: Individuals with cancer in families that meet the Amsterdam criteria
	2: Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers*
	3: Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age <45y, and the adenoma diagnosed at age <40y
	4: Individuals with colorectal cancer or endometrial cancer diagnosed at age <45y
	5: Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/scribiform**) on histopathology diagnosed at age <45y
	6: Individuals with signet-ring-cell-type colorectal cancer diagnosed at age <45y***
	7: Individuals with adenomas diagnosed at age <40y
	* Endometrial, ovarian, gastric, hepatobiliary, or small bowel cancer, or transitional cell cancer of the renal pelvis or ureter. ** Solid/scribiform defined as poorly differentated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland like spaces. *** Composed of >50% signet ring cells
Revised Bethesda guidelines:	1: Colorectal cancer diagnosed in a patient who is less than 50y of age
	2: Presence of synchronous, metachronous colorectal or other HNPCC associated tumours* regardless of age
	3: Colorectal cancer with the MSI-H histology** diagnosed in a patient who is less than 60y of age
	4: Colorectal cancer diagnosed in one or more first degree relatives with an HNPCC related tumour, with one of the cancers being diagnosed under age 50y
	5: Colorectal cancer diagnosed in two or more first- or second degree relatives with HNPCC related tumour, regardless of age
	* Endometrial, stomach, ovarian, pancreas, ureter, renal pelvis, biliairy tract, brain (usually glioblastoma), smal bowel tumour and sebacous gland adenomas and keratoacanthomas ** Presence of tumour infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation or medullary growth pattern

Table 4.
Selected markers for MSI analysis in colorectal cancer and criteria for the interpretation of MSI[24].

Reference panel	**Alternative microsatellite markers**			
BAT25	BAT40	D18S58	D13S175	D17S787
BAT26	BAT34C4	D18S61	D17S588	D7S519
D5S346	TGFßR2	D18S64	D5S107	D20S100
D2S123	ACTC(635/636)	D3S1029	D8S87	
D17S250	D18S55	D10S197	D13S153	
Interpretation	**5 markers analysed**	**>5 markers analysed**		
Number of markers displaying instability	>1	≥30%	MSI-High (MSI-H)	
	1	<30%	MSI-Low (MSI-L)	
	0	0	MSI-Stable (MSS)	

Each MutS subunit contains 5 functional domains: domain I and IV are involved in DNA binding, domain V contains ATP-ase activity and links both MutS subunits, domain II and III connect the DNA binding and ATP binding domains of the MutS protein.

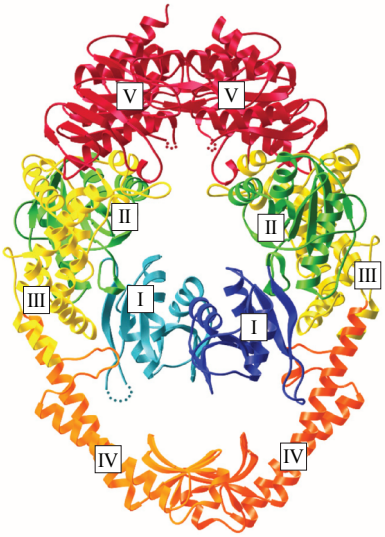

Figure 2.
The crystal structure of the MutS dimer:
Two MutS proteins are represented by ribbon diagrams in blue (domains I), green (domains II), yellow (domains III), orange (domains IV) and red (domains V). Domain I and IV are involved in DNA binding, domain V contains ATP-ase activity and links both MutS subunits, domain II and III connect the DNA binding and ATP binding domains of the MutS protein. In mismatch repair the DNA helix is positioned through the gap between domains I and IV.

Analogue to MutS, *MSH2* encompasses DNA binding domains, a domain containing ATP-ase activity, and a domain for protein-protein interaction with two other MutS-related proteins, MSH6 and MSH3. Accordingly, the MSH2 protein forms heterodimeric complexes of MutS-related proteins, MSH2-MSH6 (hMutS-α) and MSH2-MSH3 (hMutS-β), to bind DNA at distinct but overlapping spectra of mismatches[7, 110]

Apart from its role in the repair of somatic mutations, the *MSH2* gene is likely to encompass additional functions. Martin et al.[195] described a significantly increased frequency of chromosomal aberrations in sperm cells derived from *MSH2* mutation carriers, suggesting a role for mismatch repair in meiosis. Possibly, this is due to interactions of *MSH2* with repair pathways involved in chromosomal recombination. Accordingly, Villemure et al.[308] reported compromised homologous repair in *MSH2* deficient tumour cell lines. Also, in *Msh2*-mutant mouse embryonic stem cells homologous recombination between non-isogenic DNA strands is highly enhanced[281].

MLH1. The *MLH1* gene was recognised to cause HNPCC by Bronner et al.[26] and Papadopoulos et al. in 1994[225]. It is the human homologue of the bacterial MMR gene *MutL* and resides on chromosome 3p21-23. This gene contains 19 exons, with a total cDNA length of 2.3 kb[88]. The aminoacid sequence of MLH1 encompasses a DNA binding domain, an ATP-ase activity domain and domains for protein-protein interaction. During mismatch repair, MLH1 forms heterodimers with other MutL-related proteins, PMS2, MLH3, and

PMS1[156, 161, 223]. The binding of a hMutS heterodimer to a mismatch triggers ATP-dependent steps that allow interactions with the hMutL heterodimers and completion of the repair process.

As for *MSH2*, the *MLH1* gene is likely to play additional roles in meiosis. In yeast, a complex of Mlh1 and Mlh3 is involved in meiotic recombination, possibly by stabilising the Holliday junctions[17].

MSH6. In 1995 Palombo et al.[222] and Drummond et al.[53] described a complex that binds GT-mismatches. This complex appeared to consist of the MSH2 protein and an unknown 160 kDa protein, they called GTBP (GT binding protein). One year later, both the group of Miyaki[206] and Akiyama[10] described germline mutations in the GTBP gene, now known as the *MSH6* gene, in HNPCC-like families. The *MSH6* gene maps close to *MSH2* on chromosome 2p21, and it encodes for a MutS-related protein homologous to MSH2. It is likely that *MSH2* and *MSH6* are the result of an ancient duplication event of the MutS gene of higher eukaryotes. The *MSH6* gene encompasses 10 exons, with a total cDNA length of 4.2 kb. As for *MSH2,* it includes 2 DNA-binding domains, ATP/GTP-binding sites, and a PWWP-domain for protein-protein interaction. The MSH6 protein forms heterodimers with MSH2 that specifically recognise GT mismatches.

PMS2. *PMS2* is located on chromosome 7p22 and contains 15 exons for a total cDNA length of 2.6 kb. Its protein product is a MutL homologue and forms a heterodimer with MLH1 during the mismatch repair process. Apart from its protein-protein interaction motifs, *PMS2* harbours sites for DNA-binding and an ATP-ase activity domain. To date, only few *PMS2* mutations have been reported in HNPCC patients. Nicolaides et al.[217, 218] described two distinct germline mutations of the *PMS2* gene in two unrelated HNPCC families. *PMS2* mutations were subsequently also found in HNPCC kindreds with central nervous system tumours, a condition also known as Turcot syndrome[45, 86, 205]. Two compound missense mutations of *PMS2* were detected in a Turcot patient without a family history of cancer, whereas a homozygous *PMS2* mutation was found in a family with brain tumours and café au lait spots, thus suggesting a recessive mode of inheritance[45, 46]. Several studies of in total more than 200 HNPCC and HNPCC-like families did not reveal any *PMS2* mutation[165, 169, 306, 319]. These data suggest that *PMS2* mutations are responsible for a small subset of HNPCC or HNPCC-like families, and are possibly preferentially associated with the Turcot variant of HNPCC. However, mutation analysis of *PMS2* has also been hampered by the presence of pseudogenes in the genome, so the current figures of the contribution of this gene to HNPCC may be an underestimate[46].

1.4. Candidate genes

A number of additional genes have been indicated as putative HNPCC genes. These include other members of the mismatch repair machinery like *PMS1*, *MSH3*, and *MLH3*, but also genes known to play important roles in cellular functions other than MMR like *TGFβRII*, the *SMAD* genes and *EXOI* (Table 1).

Nicolaides et al.[217, 218] described a germline nonsense mutation of the *PMS1* gene resulting in exon-skipping in a HNPCC family. No other *PMS1* mutations have been reported since. The *PMS1* gene maps to chromosome 2q31-33 and it encodes for a MutL-like protein that forms a heterodimer with MLH1 during mismatch repair. *PMS1* contains 12 exons for a total cDNA length of 2,8 kb.

The *MSH3* gene maps to chromosome 5q11-12 and encodes for a MutS –like protein. *MSH3* encompasses 24 exons and has a total cDNA length of 3.4 kb. MSH3 forms a heterodimer with MSH2 that recognises specific subsets of mismatches in DNA[325]. To date, *MSH3* mutations have only been found in somatic cells[107, 345].

The *MLH3* gene on chromosome 14q24.3 was identified and characterised by Lipkin et al. (2000).[161]. The gene contains 12 exons, and has a total cDNA length of 4.7 kb. As for PMS2 and PMS1, MLH3 complexes with MLH1 to form the third hMutL heterodimer involved in mismatch repair. Wu et al.[341] described nine missense mutations and one frameshift mutation in a cohort composed of 288 HNPCC-like families. Three of the index patients carrying the putative *MLH3* missense mutation were shown to carry an additional *MSH6* mutation and no *MLH3* mutations were detected in 39 Amsterdam criteria positive HNPCC families. Likewise, no pathogenic *MLH3* germline mutations were detected in three subsequent studies on 142 patients from families with familial colorectal cancer that tested negative for mutations in *MSH2* or *MLH1*[104, 163, 176]. Liu et al.[166] described a family with both an *MLH3* and *MSH2* missense mutations, both segregating with colorectal cancer in the corresponding kindred. Altogether, these findings suggest that germline mutations of *MLH3* are not likely to contribute to the development of HNPCC, or may at best represent cancer risks modifiers among carriers of mutations of the major MMR genes. Functional redundancy among hMutS (MSH2/MSH3 and MSH2/MSH6) and hMutL (MLH1/PMS2, MLH1/MLH3, and MLH1/PMS1) heterodimers may explain the differential role of *MSH2* and *MLH1* as main disease-causing genes in HNPCC when compared with other MMR genes.

The *Exonuclease 1 (EXO1)* gene encodes for an MSH2-interacting protein presumably involved in both mismatch repair and DNA recombination[258, 290]. Wu et al.[342] detected a splice site mutation in one out of 33 HNPCC families in addition to 13 missense substitutions of uncertain pathological significance in a cohort of 225 atypical HNPCC

families. About half of the tumours available displayed an MSI-High phenotype. Sun et al.[275] showed that two missense mutations of *EXO1*, E109K and L410R, interfere with the exonuclease activity, and three others, P640S, G759E and P770L, affect MSH2-binding. Jaghmohan-Changur et al.[116] tested a large series of European CRC patients and population controls to clarify whether EXO1 variants may indeed predispose to familial CRC. Several variants observed in patients were also observed in controls with similar frequencies, including the truncating variant described by Wu et al.[342]. Thus, no conclusive evidence was found for a role of *EXO1* as a colorectal cancer susceptibility gene.

Molecular studies in sporadic colorectal cancer have indicated that two distinct signal transduction pathways, TGF-β and Wnt signalling pathways, play rate-limiting roles during tumour initiation and progression. Therefore, specific members of these signalling cascades may be regarded as potential candidate genes for hereditary CRC syndromes. In 1998 Lu et al.[177] described a putative germline mutation in the *TGFβRII* gene encoding for the TGF-β type 2 receptor in a kindred with familial late-onset colorectal cancer. Mizuguchi et al.[208] showed evidence that this mutation is likely to be a rare polymorphism. Also, no germline mutations in this gene were detected in a cohort of 67 patients with colorectal cancer below age 55 years, and in a series of HNPCC families[305]. Hence, *TGFβRII* germline mutations seem not to be associated with HNPCC. Also, no germline mutations of three other TGFβ pathway genes, *SMAD2*, *SMAD3* and *SMAD4*, have been found in HNPCC families so far[249].

1.5. The HNPCC gene mutation spectra

A broad spectrum of germline mutations is characteristic of all HNPCC genes (mutation database on website: http://www.nfdht.nl). The majority of mutations in *MLH1* and *MSH6* are single nucleotide substitutions or small insertions and deletions[227, 315]. The pathogenicity of these mutations is not always straightforward. Nonsense mutations in *MLH1* as well as in other genes were shown to cause exon skipping, leading to several aberrant transcripts[273]. Also, missense mutations and polymorfisms, as well as intronic sequence variations, can be pathogenic by affecting splicing[167, 216]. Missense mutations can also influence the structure and/or function of the encoded protein. Several missense mutations were shown to affect protein-protein interaction of MSH2 with MSH3/MSH6, or the heterodimer function[83, 95]. Lipkin et al. described a *MLH1* missense mutation (D132H) causing susceptibility to MS-Stable colorectal cancer[164]. The establishment of the pathogenicity of a sequence variant still represents a main challenge as it often implies

analysis of large cohorts of affected and healthy individuals, cosegregation analysis of the alleged mutation with the disease phenotype within extensive, multi-generation pedigrees, and *in vitro* if not *in vivo* functional studies[55, 64, 216, 294].

As reported in chapter 2.1, we found that large genomic rearrangements significantly contribute to the HNPCC mutation spectrum[35, 232, 334]. Among the 4 MMR genes, *MSH2* has been shown particularly prone to genomic deletions and other genomic rearrangements[297, 334]. The *MSH2* locus on chr 2p21 contains a relatively high concentration of repetitive short interspersed elements (SINEs) like Alu repeats, which explain the high frequency of genomic rearrangements within this gene due to homologous but unequal recombination events[297, 334]. We detected a founder deletion in *MSH2*, responsible for a substantial part of the HNPCC in Mid-western American families[192, 315]. Several other founder-mutations in *MLH1* and *MSH2* have been described[33, 64, 65, 109, 210]. One of these (the Newfoundland exon 5 splice donor site mutation in *MSH2*) appeared to be a recurrent mutation also[49].

Green et al.[81] described a *MLH1* promotor mutation in a Newfoundland kindred. Germline mutations were also detected in the promotor region of *MSH2*[35, 262]. Notably, aberrant methylation of the *MLH1* promotor was found in normal tissue of patients with MSI-High tumours, indicative of a hereditary predisposition to aberrant promotor methylation[72, 80, 276]. *De novo* mutations of *MLH1*, *MSH2* and *MSH6* seem rare. Only one *de novo* mutation of *MSH2* has been described[143].

Biallelic germline mutations in the MMR genes have also been reported. Individuals homozygous or compound heterozygous for *MLH1*, *MSH2*, *MSH6* or *PMS2* mutations are at risk of childhood tumours, mainly haematological tumours, brain tumours and HNPCC related tumours[45, 46, 71, 200, 243, 320, 331]. We also diagnosed a boy being compound heterozygous for a frame shift and a missense mutation in *MLH1*. He developed a Wilms tumour and a glioblastoma at the age of 4 years. The majority of the MMR deficient patients have "café au lait spots", a main feature of neurofibromatosis type 1 (NF1). Also, other features of NF1 like neurofibromas or Lish noduli were occasionally reported. However, none of these cases fulfilled the clinical criteria for NF1. Mutational analysis of the *NF1* gene was performed in a child homozygous for a *MSH6* mutation[200], and was negative.

1.6. Molecular basis of tumour initiation and progression in HNPCC

HNPCC is caused by germline mutations in mismatch repair genes. The DNA mismatch repair process is best outlined for the bacteria Escherichia coli (E.coli). It repairs single base mispairs, small insertions and deletions caused by slippage of DNA-polymerases during DNA replication of repetitive stretches (mono-, dinucleotide, repeats

or other simple repetitive sequences). In E.coli, four proteins are essential for mismatch repair: MutS, MutL, MutH and MutU[209]. A MutS homodimer recognises and binds to the mismatch. It then forms a complex with a MutL homodimer, that (in the presence of ATP) juxtaposes the MutS and the MutH protein. MutH has endonuclease activity and recognises the newly replicated DNA because GATC sequences in this strand are transiently unmethylated. It binds the hemimethylated DNA at a GATC site and cleaves the unmethylated DNA strand thus introducing a single strand nick. Subsequently, UvrD helicase (MutU) unwinds the DNA thus allowing single strand exonucleases to make a gap of approximately 2 kb, from the nick past the mismatch. While the single strand binding protein stabilises the remaining DNA strand, DNA polymerase III fills in the gap. DNA ligase is required to link the repaired DNA to the pre-existing sequence[241]. Eukaryotic mismatch repair contains several mismatch repair pathways in which several MutS and MutL homologues are involved (Figure 3): MSH2, MSH3, and MSH6 are human homologues of MutS. MLH1, PMS1, PMS2, and MLH3 are homologues of MutL. No human MutH homologues have been detected so far.

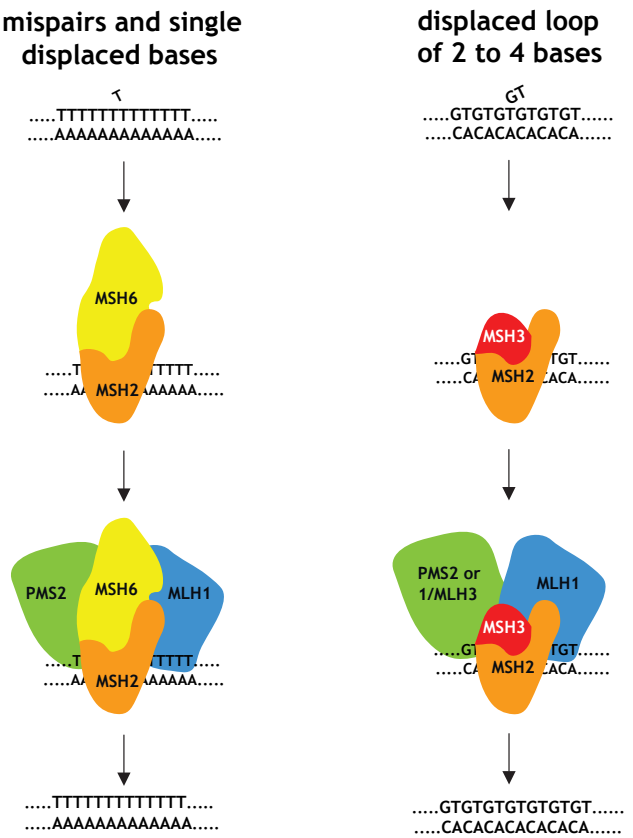

Figure 3.
Schematic representation of the eukaryotic mismatch repair (MMR) pathways:
Single mispairs and small base insertion/ deletions are preferentially recognised by MSH2/MSH6 heterodimers, whereas MSH2/MSH3 heterodimers recognise larger insertions/deletions.
After binding of the mismatch by MSH2/MSH6 or MSH2/MSH3, heterodimers of MLH1/PMS2, MLH1/PMS1 or MLH1/MLH3 are recruited.
Repair of the mismatch is now initiated.

A heterodimer of MSH2 and MSH6 (hMutS-α) recognises single base substitutions and small insertions/deletions-loops (IDL's; one to four nucleotides). After binding to mispaired DNA, this complex recruits a heterodimer of MLH1 and PMS2 (hMutL-α) and triggers mismatch repair. In addition to the mismatches recognised by hMutS-α, a heterodimer of MSH2 and MSH3 (hMutS-β) recognises larger IDL's (up to 12 base pairs). hMutS-β recruits hMutL-α or possibly a heterodimer of MLH1 and PMS1 or MLH3, again initiating mismatch repair[19, 121, 126, 138, 139, 193].

Apart from the repair of DNA-replication errors, the mismatch repair system is also involved in the repair of physical DNA damage, and in recombination and meiosis[17, 127, 129, 162, 255], either directly or through cross-talk with other repair systems. Yi Wang et al.[322] described a large (>2MDaltons) protein complex named BASC (BRCA1-associated genome complex), encompassing MSH2, MSH6, MLH1, PMS2, together with many other proteins involved in genomic recombination and repair (BRCA1, ATM, BLM, RAD50-MRE11-NBS1).

How does MMR deficiency contribute to tumor initiation and progression?

Cancer is a genetic disease involving mutations in multiple genes. The current concept of carcinogenesis is based on the assumption that a single cell may acquire a mutation that provides a selective growth advantage. From within the resulting clonal population a cell may acquire a second mutation, providing additional growth advantage, thus allowing further expansion. Repeated cycles of mutation followed by clonal expansion lead to a fully developed malignant tumour. Mutations of two classes of genes, proto-oncogenes, tumour-suppressor genes drive carcinogenesis[312].

Proto-oncogenes are involved in regulating proliferation and differentiation of normal cells. Mutations of proto-oncogenes result in a 'gain of function', and are dominant at the cellular level. Altered forms of these genes may evade cellular control and deregulate cell growth. Contrary to oncogenes, the function of tumour suppressor genes is to constrain cell growth. From this point of view, DNA repair genes involved in the maintenance of genome integrity, can be classified as tumour suppressor genes. Mutations of tumour-suppressor genes result in a 'loss of function', and are recessive at the cellular level as represented in the Knudson model (Figure 4)[134, 135]. This model was formulated based on observations on the incidence and distribution of sporadic and inherited retinoblastoma. Biallelic mutations of *RB1* cause retinoblastoma, an eye tumour. In the inherited form of retinoblastoma, the first 'hit' (mutation) is already present in the germline. For tumour formation, a second somatic mutation is needed. In view of the number of retina cells and the spontaneous mutation rate, a somatic mutation in at least one *RB1* allele in one retina

cell is highly likely to occur. In sporadic retinoblastoma, two independent somatic mutations must occur in the same cell.

The Knudson model illustrates how inherited and somatic mutations contribute to carcinogenesis and provides a rationale for the main clinical features of individuals with a genetic predisposition to cancer when compared with sporadic patients, e.g. age of onset, tumour multiplicity and multi-organ distribution.

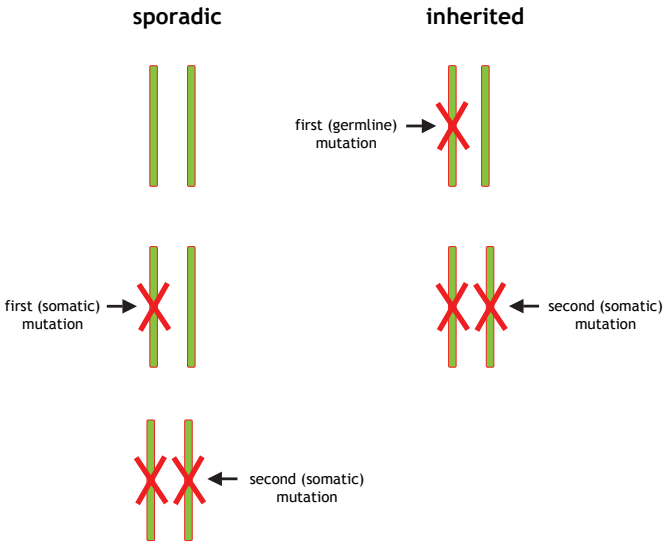

Figure 4.
Schematic representation of the Knudson model: The bars represent the alleles of a tumour suppressor gene, whereas the crosses symbolise mutations.
In sporadic tumours, all mutations are somatic, while in inherited tumours the first mutation is already present in the germline.

In addition to the inactivation of a tumour suppressor gene, 6-12 other regulatory genes must be activated or inactivated to allow progression towards malignancy[174]. Moreover, mutations at additional loci may be required to increase the chance of a second hit on a tumour suppressor gene. It has also been shown that haploinsufficiency, i.e. the gene dosage effect caused by a heterozygous mutation in a tumour suppressor gene may already trigger tumour initiation [60, 82].

Activation of oncogenes may result from chromosomal translocations, gene amplification or activating intragenic mutations. Inactivation of tumoursuppressor genes can be caused by point mutations, larger deletions or other genomic rearrangements, but also by epigenetic factors like methylation. Mutations can be acquired by replication errors, environmental agents, by normal reactive metabolites and, occasionally, by inheritance. The vast majority of the mutations that contribute to the development of cancer are somatic and are only present in the neoplastic cells of the patient. In contrast, germline mutations are present in all cells of the body including gametes, and thus may be passed to the next generation causing familial predisposition to cancer. Most cancer predisposing

genes are tumour-suppressor genes. In 2004, 291 genes involved in carcinogenesis have been reported. Ninety percent of these show somatic mutations whereas 20% are known to be mutated in the germline of hereditary cancer patients[70].

The MMR genes responsible for HNPCC when mutated in the germline, are classified as tumour suppressor genes. Loss of the wild type allele (loss of heterozygosity, LOH) has been observed in 44% of colorectal tumours from patients with a *MLH1* germline mutation[98]. Also, somatic mutations of *MLH1* occur[102]. LOH and somatic mutations of the wild type allele have been found less frequently in *MSH2*- than in *MLH1*-associated tumours[4, 98, 102].

Colorectal tumours progress through a series of clinical and histopathologic stages, ranging from normal epithelium to single crypt lesions (aberrant crypt foci) to small benign tumours (adenomatous polyps) and malignant cancer (carcinomas), the so-called adenoma-carcinoma sequence ("the Vogelgram", Figure 5). This stepwise progression results from a series of genetic changes that involve the activation of oncogenes and the inactivation of tumour suppressor genes[133, 310]. Current insights reveal that the single mutations among the adenoma-carcinoma sequence are indicative of the activation or inactivation of specific cellular regulatory pathways[312]. Signal transduction pathways involved in the development of colorectal cancer are Wnt/β-catenin, KRAS, TGF-β, and p53 pathways. A colorectal cell has to deregulate these signalling pathways to trigger adenoma formation and malignant transformation[40, 68, 78, 89, 151, 312]. The temporal order at which these mutations occur is also important: *APC* (triggering constitutive Wnt/β-catenin signalling) and *KRAS* mutations are generally involved in adenoma formation and growth, while mutations in the *p53* gene and in members of the TGF-β pathway are usually associated with malignant transformation.

Notably, although the general scheme of the adenoma-carcinoma sequence is common to hereditary and sporadic colorectal cancers, the somatic mutation profile of HNPCC-related tumours differs from other inherited and sporadic colorectal cancers at several points. In general, the vast majority of all colorectal cancers are characterised by aneuploidy and allelic losses (loss of heterozygosity, LOH), also referred to as chromosomal instability (CIN). Loss of mismatch repair function causes accumulation of DNA mismatches at an increased rate in coding and non-coding sequences, the so-called microsatellite instability (MIN/MSI). MIN tumours, both inherited and sporadic, are near-diploid. MSI is found in 12-18% of colorectal cancers[4, 114, 285] whereas HNPCC-related colorectal cancers show MSI in more than 90% of the cases[4, 67] As mentioned before, mutations of the MMR genes are present in the germline of HNPCC patients. In these cases, and in agreement with the Knudson hypothesis, the wild type allele is lost/inactivated by somatic mutations.

In contrast, more than 80% of sporadic MSI-High tumours are characterised by somatic hypermethylation of the *MLH1* promotor[103].

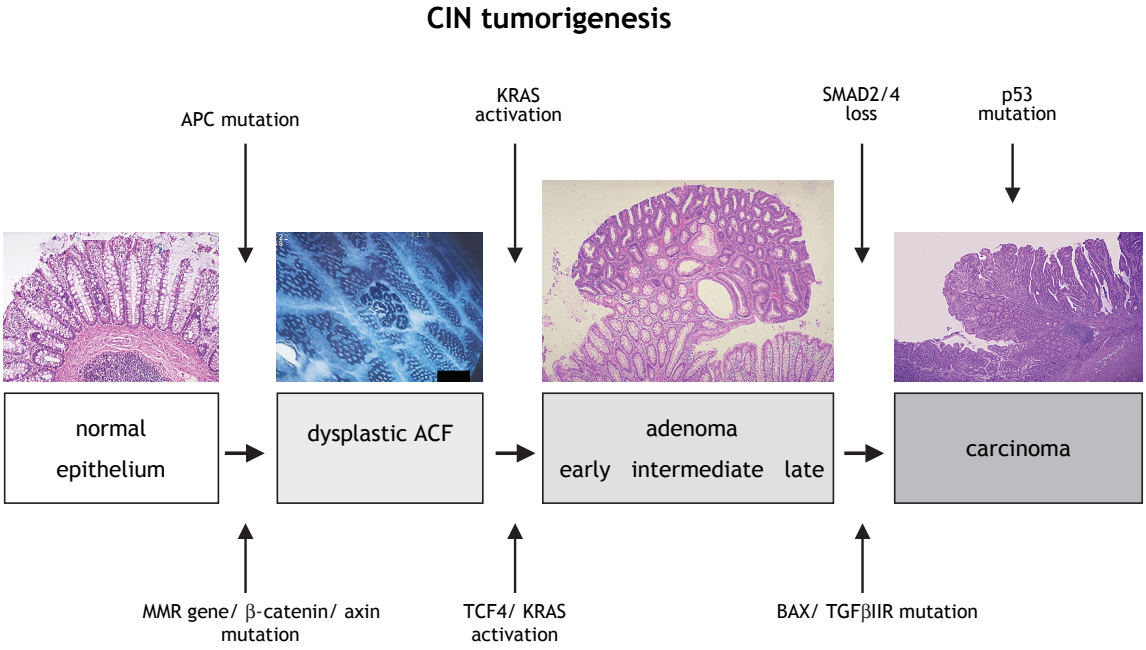

Figure 5.
The adenoma-carcinoma sequence:
The stepwise progression from normal epithelium to carcinoma results from a series of genetic changes that involve the activation of oncogenes and the inactivation of tumour suppressor genes. The different genes predominantly affected in CIN (upper part of the scheme) and MIN (lowers part of the scheme) tumours are depicted.

Members of the Wnt signal transduction pathway are mutated in both CIN and MIN tumours. The main tumour suppressing function of this signalling pathway is the regulation of β-catenin, a protein involved both in cell adhesion, when located at the cell membrane, and transcriptional regulation, when translocated to the nucleus (Figure 6). Several different extracellular Wnt ligands can bind and activate Frizzled and LRP6 receptors.

This ligand-receptor interaction prevents the formation of an intracellular multiprotein complex, the so-called 'destruction complex', composed of APC, β-catenin, GSK-3β, AXIN1 and AXIN2 (the latter also known as conductin). This complex earmarks β-catenin by Ser/Thr phosphorylation, thus triggering its ubiquitin-mediated proteolytic degradation[61]. In the absence of a functional APC protein, β-catenin accumulates in the cytoplasm and

eventually translocates to the nucleus where it acts as a transcriptional co-activator by associating with members of the T-cell factor/lymphoid enhancer (TCF/LEF) family. Downstream targets of the Wnt-signalling pathway are, among others, genes like *c-MYC* and *cyclinD1*, known to play a role in cell cycle regulation[61, 94, 283].

In the lower third of the colonic crypt, where intestinal stem and transient cells divide before migrating upwards and differentiate, Wnt/β-catenin signalling is responsible for stimulation of cell proliferation and inhibition of cell differentiation[132].

Loss of APC function constitutively activates signalling of β-catenin to the nucleus thus disturbing the equilibrium between proliferation and differentiation in the colonic crypt, and allowing clonal expansion, the first step in tumour formation. Specific activating β-catenin point mutations that render it resistant to proteolytic degradation are functionally equivalent to biallelic *APC* mutations[213].

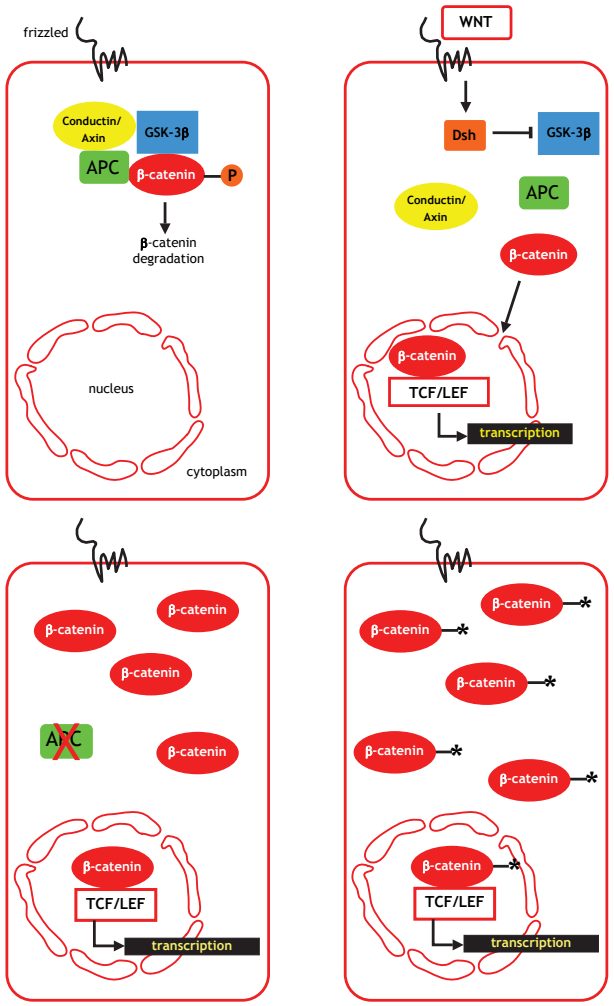

Figure 6.
A schematic representation of the Wnt-signalling pathway: The interaction of the WNT ligand with its receptor Frizzled prevents the formation of an intracellular multiprotein complex composed of APC, β-catenin, GSK-3β, AXIN1 and AXIN2 (the latter also known as conductin). This complex earmarks β-catenin by Ser/Thr phosphorylation, thus triggering its degradation. In the presence of a Wnt-signal β-catenin is not degradated and translocates to the nucleus, activating downstream targets.
In the absence of a functional APC protein or in the presence of a stabilising β-catenin mutation, β-catenin accumulates in the cytoplasm and eventually translocates to the nucleus where it acts as a transcriptional co-activator by associating with members of the T-cell factor/lymphoid enhancer (TCF/LEF) family.

Other members of the Wnt pathway such as AXIN1 have been found to be mutated in colorectal cancers with no *APC* mutations[170, 211, 270].Among CIN tumours, *APC* mutations are found in the vast majority of the cases, whereas gain-of-function β-catenin alterations are usually found in the minority of tumours with wild type *APC* [213,215, 267]. In MIN tumours, *APC* mutations are less common, though still detected at a considerably high incidence[142]. Somatic *APC* mutations were detected in 11 out of 19 (58%) MSI-High tumours from HNPCC patients, and were predominantly frameshifts within intragenic repeat sequences[105, 106]. In sporadic and HNPCC-related MSI-High colorectal tumours, mutations in β-catenin and in other components or downstream targets of Wnt-signalling occur more frequently[11, 170, 204, 260, 287].

Germline mutations of members of the Wnt signalling pathway underlie hereditary colorectal cancer syndromes. Germline *APC* mutations are responsible for Familial Adenomatosis Polyposis (FAP), a hereditary predisposition to the development of hundreds to thousands colorectal polyps (see section "Differential diagnosis"). *AXIN2* germline mutations were found in individuals with tooth agenesis and a predisposition to colorectal cancer[150].

In about 40% of both CIN and MIN colorectal cancers the *KRAS pathway* is activated by oncogenic *KRAS* mutations. KRAS belongs to the family of RAS proteins (KRAS, HRAS and MRAS) that are localised at the internal side of the cytoplasmatic membrane (Figure 7)[8, 151]. The activation in normal cells is triggered by the activation of growth factor receptors in the cell membrane. RAS is active when bound to GTP and inactive when bound to GDP.

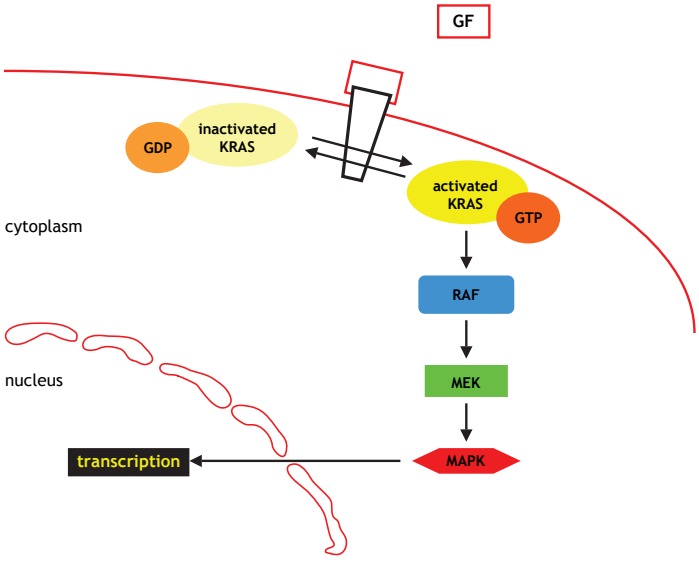

Figure 7.
A schematic representation of the KRAS-signalling pathway: Activation of growth factor receptors in the cell membrane activates RAS. Through other members of the pathway like RAF, MEK, and MAPK, RAS signalling results in the transcription of target genes.

GTP is normally dephosforylated to GDP by GAPs (GTP-ase activating proteins). Through other members of the pathway like RAF, MEK, and MAPK, RAS signalling influences cell shape, motility and growth. Also, RAS activation up-regulates vascular endothelial growth factor (VEGF), important in vascularisation of tumours[89]. *KRAS* mutations in colorectal adenoma and carcinoma lock KRAS in the GTP-bound form by interfering with its interaction with GAP. Mutations in the RAF gene *BRAF* are frequently found in sporadic but not in HNPCC related MSI-High tumours[48, 197].

The TGFβ-receptor pathway (Figure 8) is deregulated in both CIN and MIN tumours by mutations in different genes, respectively *SMAD2/4* and *TGFβIIR*[22, 151, 252]. A polyA tract in *TGFβIIR* represents a mutational hotspot in MSI-High tumours. Normally TGFβ binds TGFβ type 2 receptor (directly or via TGFβ type 3 receptor), which complexes with TGFβ type 1 receptor thus triggering its phosphorylation. TGFβ type 1 receptor phosphorylates SMAD2 or SMAD3, which bind to SMAD4. The heterodimer moves to the nucleus and induces transcription of specific target genes. TGFβ signalling induces cell cycle arrest in G1, differentiation, and apoptosis in normal cells.

Also, members of the TGFβ-receptor-pathway have been found to be mutated in the germline in men. In 1998 Lu et al. [177] described a germline mutation in the *TGFβ type2 receptor* in a family with familial late onset colorectal cancer. Intriguingly, Mizugucho et al.[208] showed *TGFβIIR* germline mutations to cause Marfan syndrome, a connective tissue disease. They also make likely that the mutation described by Lu et al. is a rare polymorphism. Germline mutations of *SMAD4* cause juvenile polyposis (see section "Differential diagnosis").

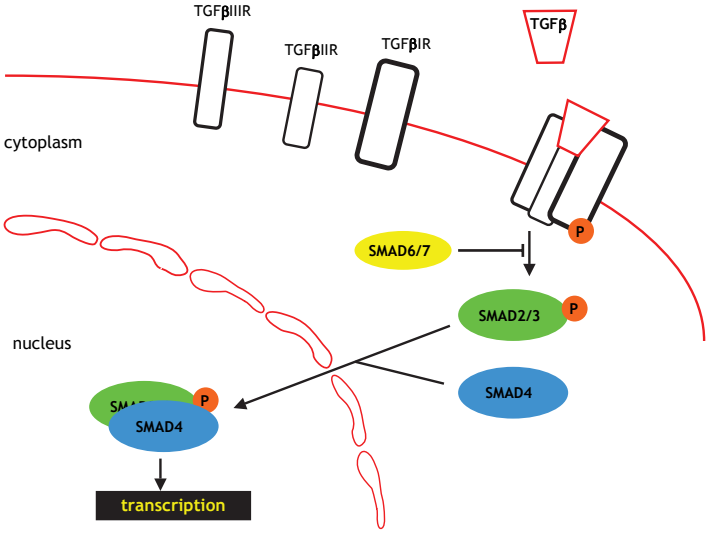

Figure 8.
A schematic representation of the TGFβ-signalling pathway: TGFβ binds TGFβ type 2 receptor (directly or via TGFβ type 3 receptor), which complexes with TGFβ type 1 receptor thus triggering its phosphorylation. TGFß type 1 receptor phosphorylates SMAD2 or SMAD3, which bind to SMAD4. The heterodimer moves to the nucleus and induces transcription of specific target genes.

The p53-pathway controls cellular responses to genotoxic damage, in particular apoptosis or cell cycle arrest to allow DNA repair (Figure 9). *TP53* mutations occur in 75% of CIN tumours, but not in MIN tumours. However, about 42% of MIN tumours carry mutations in the *BAX* gene[266], another member of the p53 pathway. P53 is normally activated by DNA damage (for example by hypoxia or ionizing radiation), aberrant growth signals (for example resulting from expression of the oncogenic *RAS* or *MYC*), chemotherapeutic drugs, UV-light, and/or protein-kinase inhibitors[311]. The p53 proteins form a tetramer able to bind DNA. The 5' side of the protein (the acid domain) can act as a transcription factor that can increase transcription of growth inhibiting genes like *BAX*, thus triggering apoptosis, or *p21*, causing G1 cell cycle arrest by inhibition of cyclin dependent kinases (CDK)[89, 151]. Also, p53 upregulates thrombospondin-1, an angiogenesis inhibitor, and is involved in the initiation of DNA repair[89].

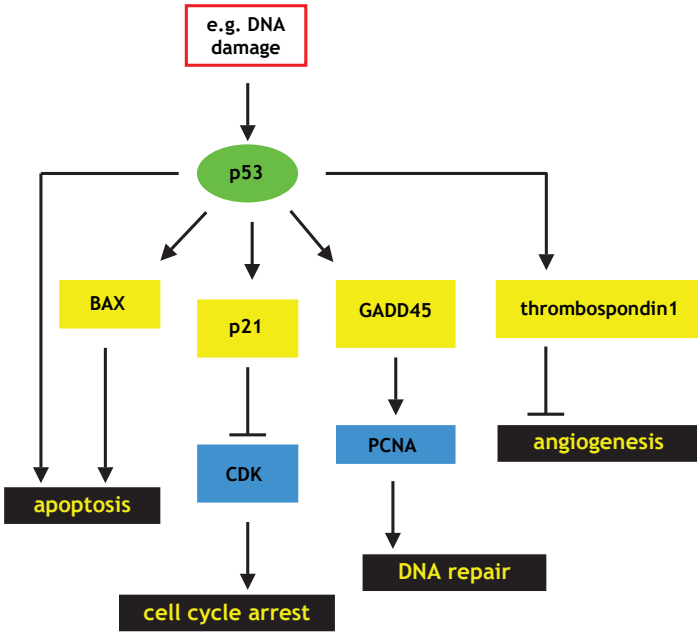

Figure 9.
A schematic representation of the p53-pathway:
P53 is normally activated by DNA damage, aberrant growth signals, chemotherapeutic drugs, UV-light, and/or protein-kinase inhibitors. The p53 proteins form a tetramer able to bind DNA. The 5' side of the protein (the acid domain) can act as a transcription factor that can increase transcription of growth inhibiting genes like *BAX*, thus triggering apoptosis, or *p21*, causing G1 cell cycle arrest by inhibition of cyclin dependent kinases (CDK). Also, p53 upregulates thrombospondin-1, an angiogenesis inhibitor, and is involved in the initiation of DNA repair.

TP53 germline mutations cause the Li Fraumeni syndrome, a cancer syndrome with childhood cancer (sarcomas and brain tumours), early onset breast cancer in addition to other tumours, among which, more occasionally, also colorectal cancer[207, 271].

By overcoming or modulating the different regulatory pathways as described above, tumour cells acquire the necessary qualities for local invasion and metastasis: autocrine growth signals (e.g. Wnt signalling and RAS pathway), insensitivity to growth inhibition (e.g. TGFβ pathway), resistance to apoptosis (e.g. MMR, p53 pathway), limitless

replicative potential (p53 pathway), sustained angiogenesis (e.g. p53 and RAS pathway), and tissue invasion and metastasis (e.g.Wnt signalling). In HNPCC, deregulation of the described pathways occurs predominantly through mutation of pathway members like *TGFβR2* and *TCF4*, that have been shown to encompass intragenic repeat sequences prone to replication errors normally repaired by MMR[228]. This accumulation of mutations at increased rate is often referred to as the mutator phenotype. Although the role of the mutator phenotype in tumour progression is generally accepted, different models have been advocated for the mechanisms underlying tumor initiation due to loss of MMR function. In a normal cell, excessive mutation load triggers apoptosis. MMR deficient cells have been shown to be resistant to apoptosis and the latter is more likely to represent the true selective advantage that enables the initial clonal expansion of MMR deficient cells. Accordingly, MMR genes have been shown to play a central role in 'sensing' the presence of DNA mismatches and the activation of either repair or apoptotic machinery[59, 127, 228].

The vulnerability of specific genes involved in carcinogenesis for MMR deficiency due to the repetitive nature of their coding sequences may at least partly explain the difference in tumor phenotypes in *MSH2*, *MLH1* and *MSH6* mutation carriers, the so-called genotype-phenotype correlation (see section "Cancer risks"). MSH6 is mainly involved in the recognition of single nucleotide mismatches, wheraes MLH1 and MSH2 are also important in the repair of larger mispairs or loops. Loss of the different MMR proteins is therefore likely to result in the accumulation of mispairs in respectively mononucleotide repeats and both mono- and multiple-nucleotide repeats. Regulatory genes in different tissues may be more or less prone to deficiency of a particular MMR pathway, depending on the nature of their repetitive sequences[228]. The observation of different MSI patterns in HNPCC related colorectal and endometrial cancer supports this theory[145].

1.7. Cancer risks

Many studies have been performed on cancer risks in HNPCC, though only a few have addressed proven *MLH1, MSH2* and *MSH6* mutations carriers. A summary of the results of these studies is presented in Table 5[5, 54, 101, 158, 302, 303, 314].

Colorectal cancer risk at age 70 years in *MLH1* and *MSH2* mutation carriers ranges between 65-100% in male and 30-83% in female carriers[5, 54, 158, 302, 303]. In the Netherlands, the Working Group on Oncogenetics of the Dutch Association of Clinical Genetics (Vereniging Klinische Genetica Nederland/VKGN) advises a lifetime risk of colorectal cancer of 60-90% for counselling purposes in high risk families. The mean age at diagnosis of colorectal cancer is younger when compared to sporadic cases (41-52 years vs.67 years) (Table 5)[5, 54,

[158, 302, 303]. The risk at age 70 years of male and female *MSH6* mutation carriers is 69% and 30% respectively, being significantly lower in female *MSH6* compared to male *MSH6* mutation carriers, and in *MLH1* and *MSH2* mutation carriers of both sexes. The age at diagnosis in both male and female *MSH6* mutation carriers is an average 5-10 years delayed when compared with *MSH2* and *MLH1* mutation carriers of both sexes (Table 5 and 6)[101, 314]. Only one third of sporadic colorectal cancer develops proximal to the splenic flexure, whereas approximately two thirds of HNPCC-associated colorectal cancers do. Multiple colorectal cancers occur frequently in HNPCC. Synchronous colorectal cancers have been reported at a frequency of 7.4% and 6.7% in *MLH1*- and *MSH2*- associated colorectal cancer, vs. 2.4% in sporadic colorectal cancer. The annual rate of a second colorectal cancer is 2.1% and 1.7% in *MLH1* and *MSH2* mutation carriers respectively, vs. 0.33% in sporadic patients[159, 180, 198, 199, 302].

Endometrial cancer risk at age 70 years ranges between 25-61% in female *MLH1* and *MSH2* mutation carriers. The risk in *MSH2* carriers has been suggested to be somewhat higher than in *MLH1* carriers[5, 54, 158, 302, 303]. The VKGN advices a lifetime risk of 30-40% for counselling purposes in high risk families. Female *MSH6* mutation carriers are at higher risk of endometrial cancer when compared to *MSH2* and *MLH1* mutation carriers (71% at age 70), though age at diagnosis is delayed by an average 5-10 years (Table 5 and 6)[101, 233, 314].

Other extracolonic tumours. Tumours of the stomach, ovary, urinary tract, small bowel (including Papilla Vateri), biliary tract, skin and brain are part of the tumour spectrum of HNPCC. Associations with other tumours are also incidentally reported, like pancreatic cancer[184, 199], laryngeal cancer[183], fibrous histiocytoma[264], prostate cancer [269], and breast cancer[245]. Of note, no consensus exists whether breast cancer is part of the HNPCC tumour spectrum[44, 214, 245, 304].

The cumulative risk at age 70 years of all extracolonic tumours (except endometrial cancer) usually does not exceed 10% among *MSH2* and *MLH1* mutation carriers (Table 5)[5, 54, 302, 303]. Vasen et al.[303] and Lin et al.[158] described a higher risk of extracolonic cancers in *MSH2* compared to *MLH1* mutation carriers.The risk of *MSH6*- associated other extracolonic tumours are at present largely unknown. The frequency of the extracolonic tumours in HNPCC must be evaluated in relation to their incidence rates in the general population[186, 226, 259]. Also, environmental factors and other genetic factors may influence the incidence of extracolonic tumours in HNPCC. Preliminary data suggest earlier ages at diagnosis of extracolonic tumours in HNPCC when compared to sporadic cases, in particular among *MLH1* and *MSH2* mutation carriers (Table 6). This phenomenon adds to clinical recognition of *MLH1* and *MSH2* mutation families.

Table 5.
Cumulative lifetime risks of colorectal (CRC), endometrial (EC), stomach (ST), ovarian (OV), urothelial cell (UR), small bowel (SMB), biliary tract (BIL) cancer and brain tumours (BT) in *MLH1*, *MSH2* and *MSH6* mutation carriers compared to the population risks[5,54,101,158,302,303,314].

Publication	Gene	Cumulative life time risk (%)								
		CRC		EC	ST	OV	UR	SMB	BIL	BT
		M	F							
Vasen et al. '96(a) n=240 (at 75 yrs)	*MLH1*	92	83	42						3.35
	MSH2			61						
Dunlop et al. '97 n=67 (at 70 yrs)	*MLH1* *MSH2*	74	30	42						
Lin et al. '98 n=105* (at 60 yrs)	*MLH1*	94	63							
	MSH2	96	39							
Aarnio et al. '99 n=360 (at 70 yrs)	*MLH1* *MSH2*	100	54	60	13	12	<4	<4	<4	<4
Vasen et al. '01 n=676** (at 70 yrs)	*MLH1*	65	55	25	2.1	3.4	1.3	7.2		0
	MSH2	75	55	37	4.3	10.4	5.4	4.5		1.2
Wagner et al. '01 n=34 (at 80 yrs)	*MSH6*	32								
Hendriks et al. '03 n=146 (at 70 yrs)	*MSH6*	69	30	71						
Population		5		1.5	1	1.5	<1	<0.1	<1	0.5

n=number of carriers;* 49 *MSH2* /56 *MLH1* mutation carriers;** 311 *MSH2*/356 *MLH1* mutation carriers

Table 6.
Mean age of onset (in years) of colorectal (CRC), endometrial (EC), stomach (ST), ovarian (OV), urothelial cell (UR), small bowel (SMB), biliary tract (BIL) cancer and brain tumours (BT) in *MLH1*, *MSH2* and *MSH6* mutation carriers compared to the age of onset of these tumours in the population[5,54,101,158,247,302,303,314].

Publication	Gene	Mean age of onset (YRS)							
		CRC	EC	ST	OV	UR	SMB	BIL	BT
Vasen et al. '96	*MLH1/ MSH2*	41-44	48-49						
Dunlop et al. '97	*MLH1/ MSH2*	47-50	54	59					
Aarnio et al. '97	*MLH1/ MSH2*			57					
Lin et al. '98	*MLH1/ MSH2*	45-52							
Rodrigues et al. '98	*MLH1/ MSH2*						49		
Aarnio et al. '99	*MLH1/ MSH2*				45				53
Vasen et al. '01	*MLH1/ MSH2*	43-44		68	43	50	54		56
Parc et al. '03	*MLH1/ MSH2*	43-48	46	49	40	49-53	45	43	63
Vasen et al. '01	*MSH6*	50							
Wagner et al. '01	*MSH6*	55	55						
Hendriks et al. '03	*MSH6*	56	55						
Population		67	55-65	60-70	>50	60-70	60	>50	>45

1.8 HNPCC tumours: histopathologic features

The main precursors of colorectal cancer are adenomatous polyps and flat adenomas[326]. In agreement with the underlying MMR genetic defect, polyps from HNPCC patients seem to progress to invasive cancer more rapidly than in sporadic or even FAP patients[119]. Specific pathologic characteristics of HNPCC colorectal tumours have been identified, but none of them are pathognomic: poor differentiation, presence of mucinous and signet cells, medullary features, peritumoural lymphocytic infiltration, Crohn's like reaction, and tumour infiltrating lymphocytes (TIL) mixed with tumour cells[181, 198, 346].
Most HNPCC-associated endometrial cancers are of the endometroid subtype[43].
Among other extracolonic HNPCC-associated neoplasms, gastric cancers are generally of the intestinal type[6], whereas ovarian cancers are adenocarcinomas, most commonly of the serous or mucinous type[5, 180]. With respect to tumours of the urinary tract, transitional cell carcinomas are associated with HNPCC, localised in the ureter and renal pelvis, though not in the bladder[265, 328]. Small bowel cancers are adenocarcinomas[180, 182, 247].
The skin tumours characteristic of the Muir-Torre allelic variant of HNPCC are predominantly sebaceous adenomas and adenocarcinomas. In 1995, Hamilton[86] recognised that HNPCC-related brain tumours are predominantly glioblastomas. The latter observation was subsequently confirmed by Vasen et al.[300] and Aarnio et al.[5].

1.9 Microsatellite Instability (MSI)

Microsatellite instability (MSI) is defined as the type of genomic instability associated with defective DNA mismatch repair in tumours. MSI provides an indication of the presence of genetic instability in a given tumour by comparing the size of a subset of simple repeated sequences occurring throughout the genome (mono-, di-, tri-, and, less frequently, tetranucleotide repeats) between normal and tumour DNA from the same individual. Slippage of DNA polymerases during replication of such simple sequence repeats often causes expansions and contractions of their alleles which are efficiently repaired by the MMR machinery. In MMR-deficient cells these errors are not properly corrected and accumulate at each cell division. Notably, MSI is rarely caused by processes other than defective mismatch repair, e.g. reduced replication fidelity by polymerase alterations, or imbalance in deoxynucleoside triphosphate pools[128].
MSI was initially described in a subset of colorectal cancers in 1993[4, 114, 285]. Initially, some authors used the term replication errors (RER), though in 1998 the National Cancer Institute Workshop on HNPCC recommended the use of the term MSI and established "MSI golden standards" that are currently employed in research and diagnostic laboratories worldwide[24] (see also chapter 1.14).

More than 90% of the HNPCC-associated colorectal cancers displays MSI[4, 67], compared to 12-18% of the sporadic tumours[114, 285]. MSI is also found in ~80% of adenomas of variable size from HNPCC patients[112]. With respect to MSI analysis of colorectal cancers, the NCI workshop recommended five most informative markers, and has formulated guidelines for MSI interpretation (Table 4 and chapter 1.14)[24].
No international criteria have been formulated for MSI analysis of endometrial cancer. However, based on studies with different microsatellite markers including those commonly used for colorectal cancer, at least 75% of the endometrial cancers from *MSH2* and *MLH1* mutation carriers displays MSI[43, 111, 246], compared to only 15-30% of the sporadic tumours[97, 246, 339]. Notably, *MSH6*-related endometrial cancers predominantly show instability at mononucleotide markers[43].
Insufficient data are yet available on MSI analysis of other HNPCC-related extracolonic tumours. However, in agreement with its molecular-genetic basis and based on studies with different microsatellite markers and on incidentally tested tumours in HNPCC families, MSI seems to represent the common denominator of virtually all HNPCC-related cancers: 75% of the HNPCC related gastric cancer displayed MSI vs 15-39% of sporadic gastric cancers[6, 51, 263, 278]; 5/5 HNPCC-related ovarian tumours were MSI-High vs. an overall frequency of 17% of MSI in ovarian tumours[38, 66, 111]; 21-31% of upper tract urothelial carcinomas exhibit MSI (Low and High) whereas incidentally tested HNPCC-related carcinomas of the same histological type were positive for MSI[21, 90, 314]; 3/3 HNPCC-related pancreatic ductal adenocarcinomas (Papilla Vateri) showed MSI vs. 26/100 sporadic tumours[343]. Entius et al.[56] detected MSI in 6 out of 10 sebacous gland tumours of possible Muir-Torre patients. Moreover, MSI is detected in a subset of early onset gliomas[124, 155]. Other tumour types thought not to belong to the HNPCC spectrum, like breast and other relatively common cancers, often display MSI in proven MMR mutation carriers, suggesting a role of MMR-deficiency in the development of these particular tumours[44, 245, 264, 269].

1.10 Prognosis

The prognosis of colorectal cancer patients from HNPCC families appears to be more favourable than that of sporadic CRC patients, with overall 5 years and 10 years survival rates of 65% vs. 44%, and 68% vs. 37%, respectively[181, 254, 327]. It has been hypothesized that this is caused by a higher sensitivity to chemotherapy of mismatch repair deficient tumours[99, 115]. However, a recent study did not show benefit of fluorouracil-based adjuvant chemotherapy in patients with MSI-High tumours[242]. Also, MMR-deficient cells are tolerant to alkylating chemotherapeutics. Another possible explanation for the increased survival in HNPCC-associated colorectal cancer is the

enhanced immune-response to the highly genetically unstable MSI tumours[250, 251]. Chang et al.[34] assumed that the abundant presence of immune cells in MMR-mutant tumours increases oxidative stress. Vulnerability of MMR-deficient cells to oxidative stress may cause cell cycle arrest and lead to a more favourable prognosis.
No difference in survival of endometrial cancer was found between HNPCC-related and sporadic patients[23].

1.11 Screening

An overview of the current screening advices in HNPCC and in other families reminiscent of HNPCC is presented in Table 7. In the Netherlands, members from these families are registered by the Dutch Foundation for the Detection of Hereditary Tumours (Stichting Opsporing Erfelijke Tumoren/STOET). This foundation sends timely notification to their gastrointestinal specialists to perform the colonoscopic screening and, reversely, receives information on the screening results. Hence, the foundation plays a central role in the optimisation of screening protocols for hereditary colorectal cancer families.
Colonoscopy is the technique of first choice for colorectal screening. It is a powerful tool in the detection and treatment of premalignant adenomas or early colorectal carcinomas in at-risk individuals. Regular colonoscopy was reported to reduce the colorectal cancer rate by 62%, and to decrease the overall mortality by about 65%[117, 118, 238]. These figure can be expected to improve with the development of high-resolution and -magnification techniques like magnifying endoscopy[141]. Currently, healthy MMR gene mutation carriers are advised to undergo a colonoscopy every 1-2 years from the age of 20-25 years onward[27, 87]. In female *MSH6* mutation carriers, the age to start colonoscopic screening may be postponed to 30 years[101]. In Amsterdam criteria positive families without a MMR gene mutation, colorectal screening advices are the same as for mutation positive families, and apply to all 1st-degree relatives of individuals diagnosed with an HNPCC related tumour and to all 2nd-degree relatives whose parent died at young age. Screening advices to affected relatives are weighted carefully, taking into account the prognosis due to the former tumour. Clear disadvantages of colonoscopy are the burden of this procedure for the patient, the risk of perforation of the colorectum (0.1%), the risk of bleeding, and the costs. Among 42 healthy Dutch MMR gene mutation carriers, 88% had colonoscopic screening every 1-2 years. In chapter 3.4, we discuss the appreciation of colonoscopy in these mutation carriers. In individuals with a contra-indication for colonoscopy, barium enema and/or faecal occult blood testing are, though less sensitive, alternatives[29, 337]. Testing for genetic markers TP53, BAT26, APC and KRAS in stool may represent a future non-invasive screening protocol to preselect patients for colonoscopy[9,

[50, 157, 268, 292, 293]. Virtual colonoscopy is also considered a potential non-invasive screening tool[79]. Using computer tomography (CT), 80-90% of the polyps larger than 6 mm can be detected. Because of the radiation load in CT scanning, magnetic resonance imaging (MRI) is a more patient-friendly alternative. However, since detection rates of smaller and flat polyps are rather poor in both CT and MRI, virtual colonoscopy is not likely to replace conventional colonoscopy in the near future. Moreover, when a polyp is detected by virtual colonoscopy, conventional colonoscopy has to be performed anyhow to allow polypectomy. No consensus exists on prophylactic colectomy in mutation carriers as a standard procedure, primarily because the combined colonoscopy and polypectomy are a highly reliable and effective surveillance and preventive strategy[137]. However, at time of diagnosis of colorectal cancer or polyps, subtotal colectomy may be considered in MMR gene mutation carriers[87, 187].

Yearly gynaecological examination is advised to female MMR gene carriers starting from the age of 30-35 years. This examination includes endometrial aspirate or vaginal ultrasound of the uterus and ovaries, and CA125 measurements in blood (CA 125 is a tumour marker of ovarian cancer)[27, 87, 298]. However, the value of this screening is disputed and adequate management of early symptoms remains very important[52, 244]. Prophylactic hysterectomy is not routinely offered, but in female carriers already scheduled for a colectomy and in female *MSH6* carriers, this procedure may be considered. In case of a family history of ovarian cancer, prophylactic oophorectomy may also be considered in view of the limited value of screening and the poor prognosis of this tumour type[87].

Advises for regular screening for other HNPCC related cancers are tailored based on the individual family history[27, 87, 298]. In families with two or more gastric cancers, gastroscopic screening is recommended from the age of 30-35 years every 1-2 years[27, 87]. Of note, gastroscopic screening had no beneficial effect in Finnish *MLH1* mutation carriers at a follow-up of 3-4 years[239]. However, limitations of this study are the size of the patient cohort, the genetic homology (the majority of the patients carried a Finnish founder mutation in *MLH1*) and the short follow-up period. Helicobacter Pylori may also represent a confounding factor. In HNPCC families with familial or early onset gastric cancer relatives should be analysed for H. Pylori infection. Urinary tract screening is also advised from the age of 30-35 years by cytological urine sediment analysis every 1-2 years. The sensitivity of this technique can be improved by molecular methods like microsatellite analysis and immunoassays on the urine sediment[37, 274], though this is not generally applied.

Table 7.
Screening advises in HNPCC(-like) families[27,29,30,73,201]. These advices apply to healthy first degree relatives of individuals with an HNPCC related tumour. Of note, if a parent died young of a not HNPCC related cause, a second-degree relative must be considered as first-degree.

Clinical criteria	Site	Technique	Age of Onset (yrs)	Interval (yrs)	Remarks
MLH1/MSH2/ MSH6 mutation carrier	colon	colonoscopy	20-25* or 5 yrs before earliest crc in family	1-2	Consider colectomy with ileorectal anastomosis at diagnosis of cancer or in case of multiple recurrent polyps. In female *MSH6* carriers the age of onset of screening may be postponed to 30 years.
	uterus	gynaecological examination & intravaginal ultrasound or endometrial aspirate	30-35	1	Consider prophylactic hysterectomy in *MSH6* mutation carriers.
	ovary	gynaecological examination & intravaginal ultrasound & CA125 in blood	30-35	1	Consider prophylactic oophorectomy if 2 or more relatives have ovarian cancer.
	stomach	gastroduodenoscopy	30-35	1-2	depending on family**
	small bowel	gastroduodenoscopy	30-35	1-2	depending on family**
	urinary tract	urine cytology & sediment	30-35	1	depending on family**

Clinical criteria	Site	Technique	Age of Onset (yrs)	Interval (yrs)	Remarks
MMR gene mutation negative/ unknown & AC positive	colon	colonoscopy	20-25	1-2	or starting 5 years before the youngest crc case in the family in crc-only families
	uterus	gynaecological examination & intravaginal ultrasound or endometrial aspirate	30-35	1	depending on family** (not if only crc occurs in the family)
	ovary	gynaecological examination & intravaginal ultrasound & CA125 in blood	30-35	1	depending on family**
	stomach	gastroduodenoscopy	30-35	1-2	depending on family**
	small bowel	gastroduodenoscopy	30-35	1-2	depending on family**
	urinary tract	urine cytology & sediment	30-35	1	depending on family**
1 first-degree relative with crc <50 yrs or 2 first-degree relatives with crc	colon	colonoscopy	45-50 or 5 years before youngest crc patient in family	5	
Others	colon	FOBT	50	1	on request Fecal Occult Blood Testing (FOBT) is less invasive but also less sensitive than other screening tools. Colonoscopy is the most sensitive tool.
		sigmoidscopy		5	
		sigmoidscopy & FOBT		5	
		double contrast barium enema		5-10	
		colonoscopy		10	

** If 2 or more relatives are affected with stomach, small bowel or urinary tract cancer (If only 1 relative is affected at an early age, screening the first degree relatives can be considered in view of additional riskfactors in this branch of the family).
crc = colorectal cancer; AC = Amsterdam criteria

1.12 Prevention

It is generally believed that diet has a profound impact on colorectal cancer risk. However, experimental evidence has been hard to obtain and it is unlikely that the colorectal cancer risks of MMR gene mutation carriers can be significantly modulated by dietary factors[131, 236]. No difference in meat consumption was seen in sporadic colorectal adenoma cases and HNPCC cases in the Netherlands[313]. A study on the prevention of polyps in HNPCC carriers by resistant starch supplements is in progress[28]. Nevertheless, a balanced diet with adequate fruit, vegetable and cereals intake, restricted alcohol consumption, no smoking, weight control, and regular physical exercise are reasonable advises.

Chemoprevention studies in HNPCC carriers are limited[309]. Studies on calcium carbonate were not conclusive[91]. A international study (CAPP2) on the effect of aspirin (alone and in combination with resistant starch) in HNPCC carriers is ongoing[28]. NSAIDs (non-steroidal anti-inflammatory drugs), including aspirin, have been consistently associated with a reduced risk of colorectal cancer[28, 236]. Aspirin inhibits the conversion of arachidonic acid to prostaglandins by cyclo-oxygenase 1 and 2 (COX1 and COX2). Because the adverse effects of long-term aspirin use are mainly caused by COX1 inhibition, novel NSAIDs, e.g. Celecoxib, were developed that selectively inhibit COX2. However, also selective COX2-inhibitors may increase the risk of cardiovascular events[213], making them unsuitable for chemoprevention purposes. Also, the spectrum of antineoplastic actions of NSAIDs is broad and includes inhibition of angiogenesis, induction of apoptosis, and cancer growth inhibition by interference with several signal transduction pathways[108, 288]. Both Celecoxib and Sulindac were reported to cause adenoma regression in patients with familial adenomatous polyposis (FAP)[41, 74, 91, 108, 288]. Also, a significant reduction in duodenal polyposis was seen in FAP carriers after treatment with Celecoxib[231]. However, no effect was observed in FAP carriers ranging from 8 to 25 years of age using standard doses of sulindac[75]. A study (CAPP1) on the effects of aspirin in FAP mutation carriers is about to report its results[28]. More insights in the molecular genetic mechanisms underlying tumour initiation and progression in HNPCC are expected to lead to the identification of new safe targets for tailor-made chemoprevention. For an overview of the status quo of chemoprevention in colorectal cancer see Hawk et.al.[92].

The mutator phenotype characteristic of HNPCC tumours causes frameshift mutations in a broad spectrum of genes leading to novel peptides that can be recognised by T cells and trigger an immune response. The characterisation of peptides specific for MSI-positive colorectal cancer cells fuels hope for the development of a prophylactic vaccine for HNPCC carriers[250].

1.13 Differential diagnosis

The Muir-Torre Syndrome. In 1966 Muir[212] described a syndrome with multiple primary tumours of the colon, duodenum, larynx, and skin. In 1968 Torre[291] presented an additional patient with multiple sebaceous adenomata and colon cancer. In the 1980's Lynch et al.[69, 188, 190] recognised the Muir-Torre syndrome as a clinical variation of HNPCC. Since then, germline mutations of *MSH2* and in a lesser extend of *MLH1* have been found in Muir-Torre families[140, 144].

The Turcot Syndrome. Another clinically defined syndrome that appeared to be an allelic variant of HNPCC is Turcot syndrome. In 1959 Turcot[295] described two cases (a brother and sister) with central nervous system tumours and polyposis coli. In 1995 Hamilton[86] recognised that this association could derive from two distinct types of germline defects: mutations in the APC gene that cause Familial Adenomatous Polyposis (FAP) and mismatch repair gene mutations (*MLH1* and *PMS2*). The brain tumours in FAP are predominantly medulloblastoma, while the HNPCC-associated brain tumours are glioblastoma[5, 300]. Wang et al.[321] demonstrated *NF1* somatic mutations in MMR-mutant cell lines, possibly providing a molecular basis for the development of brain tumours in a subset of MMR gene mutation carriers.

Familial Adenomatosis Polyposis (FAP) and Attenuated FAP (AFAP). In the majority of the cases, the clinical diagnosis of Familial Adenomatosis Polyposis is facilitated by the presence of hundreds to thousands colorectal adenomas. More than 95% of FAP patients carries a disease-causing *APC* mutation[147]. MMR gene defects play no apparent role in *APC* mutation negative FAP families[96].

An atypical variant of FAP, attenuated FAP (AFAP), characterised by reduced polyp multiplicity (between 5-10 and 100) and a delayed age of onset, is more reminiscent of the HNPCC phenotype[32]. AFAP is caused by specific *APC* mutations, usually located at the extreme 5' or in the 3' half of the gene[63], or by biallelic *MYH* mutations[15, 122]. The latter form is now referred to as MAP, *MYH*-associated polyposis. Wang et al.[318] described two patients with early onset colorectal cancer and respectively zero and three polyps, both homozygous for *MYH* mutations. In these cases family history and MSI/IHC tests are helpful to differentiate diagnoses. In case of a MSI stable phenotype, MMR-gene defects become unlikely and analysis of *APC* and *MYH* should be considered[15, 122].

Familial colorectal cancer. Colorectal cancer is a common type of cancer and random familial clustering of CRC cases is seen in about 15% of the families of patients with colorectal cancer. For example allelic variants of high risk colorectal cancer susceptibility genes can contribute to less penetrant forms of CRC predisposition[164]. The guidelines depicted in Figure 10 will facilitate differential diagnosis between bona fide HNPCC

families and those kindreds with a clustering of colorectal cancers due to other hereditary or non-hereditary factors. Detailed phenotypic characterisation will continue to be of great importance for the subclassification of the remaining unresolved colorectal cancer families. Until the moment additional molecular genetic markers will be available for most of the familial colorectal cancer syndromes, screening advises will mainly be based on accurate family history data (Table 7)[29, 30].

Crohn's disease and colitis ulcerosa. Inflammatory bowel disease is a known risk factor for colorectal cancer. *NOD2* has been identified as a low risk susceptibility gene for Crohn's disease[221]. The pathological features of HNPCC related colorectal tumours can include a Crohn's like reaction with tumour infiltrating lymphocytes (TIL) mixed with tumour cells[181, 198]. However, Crohn's disease and colitis ulcerosa are distinct clinical entities, which can easily be differentiated from HNPCC by symptoms and a detailed family history.

Hyperplastic polyposis, mixed polyposis and serrated adenomatosis. Hyperplastic polyposis is a loosely defined syndrome characterised by the occurrence of multiple hyperplastic polyps in the colorectum. Hyperplastic polyps are generally considered less prone to malignant transformation when compared with adenomatous polyps. However, dysplasia has been described in hyperplastic polyps and, especially in cases with high polyp multiplicity's, patients are at increased risk of colorectal cancer[93, 136, 153, 237].

A few families have been described with a combination of different types of colorectal polyps, atypical juvenile polyps, adenomas and hyperplastic polyps[256, 330]. These families and are classified as affected by Hereditary Mixed Polyposis Syndrome (HMPS). The disorder was mapped to chromosome 6q in 1996, though its genetic basis is yet to be elucidated[286].

Occasionally, polyps may display both hyperplastic and adenomatous features. These polyps are usually referred to as serrated adenomas. It has been claimed that these adenomas arise through a distinct MSI-Low pathway[120, 257].

As the above entities have overlapping clinical features with attenuated or atypical HNPCC and FAP cases, their diagnosis should always be excluded in families with hyperplastic, mixed or serated polyposis.

The hamartomatous polyposis syndromes. In juvenile polyposis, the Peutz-Jeghers syndrome, and the Cowden syndrome, colorectal hamartomatous polyps and cancer occur at increased frequency[76, 338]. Hamartomatous polyps can be pathologically distinguished from HNPCC-related adenomas. Hamartomatous polyposis are often considered as non-neoplastic. However, they may sometimes harbour dysplasia and their true neoplastic potential is yet unknown. Nevertheless, an increased cancer risk both within and outside the gastrointestinal tract exists in these syndromes. Other clinical features of

hamartomatous polyposis syndromes, like early age at diagnosis of juvenile polyposis, the mucocutanous pigmentations in Peutz-Jeghers syndrome and the tricholemmoma in Cowden syndrome, may help to differentiate them from HNPCC. Finally, mutation analysis of the respective causative genes (*SMAD4* and *BMPR1A* for juvenile polyposis, *LKB1/STK11* for Peutz-Jeghers syndrome, and *PTEN* for Cowden syndrome) may indisputably resolve these syndromes from HNPCC.

Hereditary breast-ovarian cancer. Germline *BRCA1* and *BRCA2* mutations cause an increased risk for breast and ovarian cancer[202, 340]. In familial ovarian cancer families both *BRCA1/BRCA2* susceptibility and HNPCC should be considered. Apart from mutation analysis of *BRCA1/BRCA2* and the MMR genes, MSI and IHC analysis may help to distinguish the two entities. However, data on MSI and IHC testing in ovarian cancer are limited.

Familial gastric cancer. Familial gastric cancer has been linked to two genetic predispositions, *E-cadherin* mutations and HNPCC[31]. Pathologically, *E-cadherin* mutations give rise to diffuse gastric cancer, while HNPCC-associated gastric cancer tends to be of the intestinal type. Once again, detailed family history, clinical data, and MSI/IHC tumour analysis can help to direct further molecular analysis and formulate a more accurate diagnosis[84, 230].

Neurofibromatosis type 1 (NF1). Though individuals with NF1 have an increased risk of colorectal cancer, the phenotype is generally easy to distinguish by the occurrence of café au lait spots, freckling and neurofibromas. However, children with biallelic MMR gene mutations develop features of neurofibromatosis type 1 in addition to other malignancies[45, 46, 71, 200, 243, 320, 331]. Hence, in children with features of NF1 and a family history of HNPCC-related tumours or a more recessive inheritance pattern, one should consider MMR gene mutation analysis.

1.14. Molecular diagnostics of HNPCC

The molecular diagnosis of HNPCC in a given kindred implies several steps and analytical procedures. Rather than directly applying mutation detection techniques to identify HNPCC-causing lesions in MMR genes, most diagnostic centres initiate their search by investigating tumour material (when available) to assess genetic instability and loss of specific MMR protein expression by MSI (microsatellite instability) and IHC (immunohistochemistry) analysis respectively. They allow confirmation of the MMR defect in the tumour (MSI and IHC) and the identification of a specific gene whose protein expression has been lost in the tumour (IHC). The latter gene will then represent the first candidate gene to be analysed for the presence of germline mutations in non-neoplastic

(usually blood) patient material. Here, each technical approach will be described separately.

Microsatellite instability (MSI) is defined as the type of genomic instability associated with defective DNA mismatch repair in tumours. Analysis of MSI is performed by PCR amplification of specific mono- and dinucleotide microsatellite repeats and by comparing the size of their alleles between normal and tumour DNA. Tumour-specific changes in allele sizes are indicative of genetic instability due to loss of MMR function. MSI analysis is usually performed on paraffin embedded tumour samples with or without microdissection of the parenchymal cells. The National Cancer Institute recommended five most informative markers with respect to colorectal cancers, and has formulated guidelines for MSI interpretation (Table 4)[24]. According to the degree of MSI detected, tumours are subdivided in high-frequency MSI (MSI-H), if two or more of the five markers show instability, and low-frequency MSI (MSI-L), if only one of the five markers shows instability. Tumours with no instability in any of the markers are considered to be microsatellite stable (MSS). The implications for MSI-L are not yet clear. It is suggested that if enough markers are tested, all colorectal tumours display some degree of instability[146]. The resolution between microsatellite high (MSI-H) and low frequency MSI (MSI-L) can only be accomplished if a greater set of markers (especially including mononucleotide markers like BAT40) is utilised (Table 4)[24, 296]. The use of additional mononucleotide repeats is also useful when dealing with HNPCC due to *MSH6* germline mutations, known to be associated with preferential microsatellite instability at mononucleotide repeats[43, 101]. The same NCI markers are also employed for the prediction of a MMR gene defect in endometrial cancers[43], though little data are yet available on their reliability for other HNPCC-related tumours.

Immunohistochemical analysis (IHC) is a rapid and inexpensive method for identifying MMR-gene alterations[160]. As for MSI, IHC is also performed on histological sections from paraffin embedded tumours with antibodies specifically raised against the main MMR proteins: MSH2, MLH1, MSH6 and PMS2[42, 43]. Absence of staining for a specific protein is likely to result from the 2nd somatic hit at the MMR gene where the germline mutation is present. Therefore, IHC represents a useful tool to direct mutation analysis and enhance cost-effectiveness of the mutation detection procedure. Normal staining of a given MMR protein, however, does not exclude the presence of a mutation of the corresponding gene[160, 317].

Tissue Microarrays (TMAs) represent a powerful novel technology for high-throughput analysis of protein expression in a large number of tissue (tumour) samples. Hundreds of tissue cores are arranged on a single slide, and then analysed by a single IHC reaction[123].

TMA's can significantly enhance the processivity of IHC, although their systematic use in a diagnostic setting has not been evaluated yet.

In order to detect mutations in genomic DNA, a large number of protocols are available[280]. However, since the comprehensive description of these diverse technologies is outside the scope of this thesis, I will here only briefly describe the HNPCC mutation detection protocols most commonly employed in our laboratory.

Denaturing gradient gel electrophoresis (DGGE) allows the rapid screening for single base changes in PCR-amplified genomic DNA[62]. The technique is based on the migration of double-stranded DNA molecules through polyacrylamide gels containing linearly increasing concentrations of a denaturing agent. The mobility of the DNA molecule is strongly retarded at the concentration at which the DNA strands with the lowest melting domain dissociate. This branched structure becomes entangled in the gel matrix and no further movement occurs. Complete strand separation is prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. The latter is usually accomplished during PCR amplification using a primer with a 5' tail consisting of a random sequence of approx. 40 GC. Single base substitutions, deletions and insertions are detected with high sensitivity by DGGE analysis[62]. For HNPCC mutation analysis, individual MSH2, MLH1 and MSH6 exons are amplified by PCR, with one of the two primers implemented with a 5' GC-clamp. The PCR product is then loaded on the denaturing gradient gel. Exons with altered patterns of migration on DGGE are subsequently sequenced to determine the nucleotide alteration[335, 336].

Mutation detection techniques such as DGGE detect "point mutations", i.e. single base substitutions and small (1-10 nt) deletions and insertions. However, larger genomic rearrangements such as deletions, duplications, insertions, and inversions also represent a frequent cause of hereditary conditions. To detect this type of genetic defect the implementation of other mutation analysis strategies is required.

Southern blot analysis is a rather old and cumbersome method but, to date, it still represents the most reliable and accurate approach to detect large rearrangements in total genomic DNA. High molecular weight DNA is usually extracted from peripheral blood lymphocytes though many other types of tissues can be employed. DNA is then digested by restriction endonucleases, fractionated according to the molecular weight of the resulting fragments by electrophoresis through agarose gels, and then transferred onto a nylon membrane. The latter are hybridized with (radioactively) labelled DNA probes that recognise specific single-copy restriction fragments among the complexity of genomic DNA[253].

Conform to our previously established protocol[334], Southern analysis of the *MSH2* gene is performed with XbaI, EcoRI, HindIII, NsiI, genomic DNA digests, followed by hybridization with three *MSH2*-specific probes (encompassing exons 1-7, 7-12, and 10-16 respectively). *MLH1* and *MSH6* Southern analysis is performed with XbaI, ApaI, HindIII and NsiI genomic DNA digests followed by hybridization with two *MLH1*- (exons 1-12 and 11-19) and two *MSH6*-specific probes (exons 1-4 and 5-10)[334]. As reported in chapter 2, the implementation of the Southern blotting procedure in the analysis of the MMR genes in HNPCC has resulted in the identification of a significant number of genomic deletions and other less common types of rearrangements[297, 315, 334]. Although Southern analysis is still the method of choice in a "discovery phase", i.e. to define the spectrum of genomic rearrangements at any given disease locus, its protocol is too cumbersome and time-consuming to be efficiently implemented in diagnostic laboratories. PCR-based methods for the detection of genomic deletions and duplications are nowadays available.

Multiplex Ligation-dependent Probe Amplification (MLPA) is based on quantitative multiplex PCR in order to determine the relative copy number of each exon within a gene of interest[77]. Two specific probes are hybridised with the denatured target DNA. If hybridised, the probes are ligated and PCR amplified. The amount of probe amplification product reflects the copy number of the target sequence. MLPA has been successfully applied for the detection of MMR genomic rearrangements in HNPCC patients[35, 36, 77, 189, 220, 232, 307, 315, 321]. However, there are some limitations. For example, rearrangements not encompassing the coding region will not be detected by MLPA unless specific PCR strategies are designed to cover extensive portions of the flanking regions where regulatory elements are likely to be located. Also, Southern analysis, when performed with at least two different restriction enzymes, allows accurate mapping of the extent of the rearrangement and the consequent design of PCR primers to specifically amplify the breakpoints and the rapid detection of the disease-causing deletion/insertion in other affected family members. MLPA does provide indication of the presence of a genomic deletion/duplication but not on the exact location of the breakpoint, especially when dealing with disease genes with very large introns. Moreover, inversions are not detected by MLPA as no quantitative change at the genomic DNA level is observed[113].

Other analytical approaches are available to aid molecular genetic investigations on specific cases, e.g. missense mutations of questionable pathogenicity, or MMR mutation-negative individuals belonging to Amsterdam positive families with MSI-H tumours and/or informative IHC assays.

Mono-Allelic Mutation Analysis (MAMA or conversion technology) represents a useful tool for the analysis of "masked" mutations (hard to detect by conventional techniques),

missense mutations, splicing errors and genomic alterations[216, 224, 344]. To allow RNA and protein expression analysis of putative disease-causing MMR alleles in a heterologous genetic background, somatic cell hybrids are obtained by fusing patient-derived cells (typically blood lymphocytes) with *Msh2*-deficient mouse cells (GMP Conversion Technologies Inc., Waltham, MA)[224, 344]. Somatic cell hybrids carrying the chromosome of interest can be identified by genotyping with dinucleotide repeat markers and are then selected for mono-allelic expression analysis, e.g. by northern or western blot analyses or, if *MSH2* is the gene of interest, by more functional assays taking advantage of the absence of endogenous *Msh2* expression in the recipient murine cells.
Additional functional assays can be performed in yeast based on interference of recombinant human MLH1 with the yeast MMR machinery[261]. Alternatively, yeast strains expressing mismatch repair genes mutations at codons homologous to 'suspected' substitutions found in HNPCC patients can be analysed by MMR assay that measures stability of mono- or dinucleotide repetitive tracts *in vivo*[55, 234]. Other functional assays make use of the ability of MLH1 and PMS2 or MSH2 and MSH6 to dimerize by co-immunoprecipitation analysis after transfection of mutant or wildtype *MLH1*, *PMS2*, *MSH2* or *MSH6* in *MLH1*- or *MSH2*-deficient colorectal cancer cells or in insect cells(Sf9)[25, 125, 219, 294].

In the Netherlands, diagnostic mutation analysis of the *MLH1*, *MSH2* and *MSH6* genes is provided by university-affiliated DNA laboratories. Each gene analysis usually takes 3 to 6 months. At present, *PMS2* testing is not offered in diagnostic settings. The costs per gene per 2004 amount to approximately ∈650. MSI and IHC analysis of HNPCC related tumours are offered by several pathology departments at variable costs. Costs for MSI, IHC, and mutation analyses are fully covered by health insurance companies. Diagnostic MMR gene testing is also offered by several laboratories in Europe on a non-profit basis (www.EDDNAL.com). Commercial tests are available in the United States (Myriad Genetics) and Europe (Gendia).

1.15 Selection for mutation analysis

The Amsterdam criteria, established by the International Collaborative Group on HNPCC (Table 2)[299, 301], are valuable guidelines for the clinical recognition of HNPCC. Families fulfilling these criteria have a high *a priori* risk (45%- 64%) to carry germline mutations in one of the two major MMR genes[73, 333]. Also families not fulfilling the Amsterdam criteria may harbour a MMR gene mutation, though chances are significantly lower (0%-47%)[73].

We showed that failure to detect a MMR gene mutation in Amsterdam criteria positive families could be explained by either (1) marginal fulfilment of the criteria in true-mutation negative families, or (2) incorrect patient selection (phenocopy) in mutation positive families, or (3) failure of mutation detection techniques in mutation positive families. Due to the relatively high incidence of colorectal cancer in the general population, families may fulfil the Amsterdam criteria by chance. Katballe et al.[130] calculated that the Amsterdam criteria I would be met by chance only in 1 out of 77 families with a young colorectal cancer patient (age of diagnosis <50 years). We detected a MMR mutation (mainly in *MLH1* and *MSH2*) in all families tested with 2 additional HNPCC-associated tumours apart from the Amsterdam criteria[315] and in all families fulfilling the Amsterdam criteria by the presence of a relative with a non-colorectal HNPCC-associated cancer, as formulated in the revised Amsterdam criteria. HNPCC-associated cancers become more common at older age. To minimise the risk of testing a phenocopy, the patient tested for MMR mutations should therefore preferentially be the youngest affected individual within the family. Using a comprehensive set of mutation detection techniques, we detected a *MSH2* or *MLH1* mutation in more than 90% of Amsterdam criteria positive families[315].

Families with a MMR gene mutation may fail to meet the Amsterdam criteria for several reasons, namely reduced penetrance of specific MMR mutant alleles (e.g. *MSH6* and *PMS2*), early death of relatives due to cancer unrelated causes, non-paternity, lack of family history data, and small family size. However, most of these families will eventually qualify as Amsterdam positive, after efforts have been made to expand the pedigree structure and to obtain additional medical information. Anamnestic data on family history should be verified whenever possible[203].

In addition to family history, MSI and IHC analysis have been proven useful tools for the selection for mutation analysis[18, 47, 148, 175, 282]. More than 90% of HNPCC-associated colorectal cancers display microsatellite instability[3, 67], in contrast to 12-18% of the sporadic colorectal cancers[114]. Reversely, Liu et al.[168] detected a MMR gene germline mutation in 73% of families with familial colorectal cancer and a MSI-High tumour in one of the patients. MSI as the only pre-screening tool for MMR gene mutation analysis in unselected colorectal cancer will result in a mutation detection rate of only 5-10%. Since MSI is an expensive and laborious technique, and in view of the many sporadic MSI-High tumours that would be detected, screening of all colorectal cancer by MSI analysis is unattractive as a pre-screen tool.

The Bethesda guidelines for selection of tumours for MSI analysis were formulated in 1997 and revised in 2004 (Table 3)[248, 296]. When compared to the Amsterdam criteria as a

clinical prescreen tool, the Bethesda guidelines improve the MMR gene mutation analysis sensitivity, but lowers its specificity as a considerable number of families with more atypical phenotypes are included [277]. However, these atypical families may harbour *MSH6* or *PMS2* mutations[42]. This pleads for a liberal selection of cases for MSI analysis as appreciated in the revised Bethesda criteria.

MSI analysis is preferentially performed on colorectal cancer of the youngest patient in the family. If no colorectal cancer is available, endometrial cancer, or other HNPCC-associated cancers, or adenomas, are often tested. However, the interpretation of normal MSI results (MSS) in these latter settings is more difficult and has to be scored as non-conclusive.

Immunohistochemical (IHC) analysis of MMR proteins has earned a place as a pre-screening tool for MMR gene mutation analysis[42, 43, 100, 160]. Of note, positive IHC staining does not exclude the presence of a MMR gene mutation, as certain mutation types do not interfere with the translation of a protein detectable by IHC[317]. Also, many sporadic colorectal cancers show a decreased MLH1 expression due to promotor hypermethylation. MSI and IHC patterns generally show a high degree of concordance[38, 39, 47, 160, 240, 317]. Berends et al. indicate that IHC analysis might be the best prescreen tool in colorectal cancers diagnosed under the age of 50 years[20].

Few studies have been carried out to evaluate the concordance between the presence of a *MLH1*, *MSH2* or *MSH6* germline mutation, and the MSI and/or IHC status of the corresponding tumours[20, 39, 47, 100, 317, 323]. The results of these studies are summarised in Table 8. In total, 98 out of 106 tumours from mutation carriers (92%) tested as MSI-High, 89 (84%) revealed aberrant MLH1, MSH2 or MSH6 staining patterns, whereas 87 (82%) had both. Eleven mutation carriers (10%) only displayed MSI with no IHC staining aberrations. Two patients (2%) only displayed aberrant IHC and no MSI. Therefore, a total of 13 mutation carriers (12% of all tumours) displayed either only MSI, or aberrant IHC staining in their tumours. Three of the mutations in these 13 carriers were of unknown significance. By combining MSI and IHC analysis, 100 out of 106 (94%) mutation carriers were detected; the 6 mutations unnoticed by MSI or IHC analysis were of unknown significance. Sixteen mutation negative index patients (33%) displayed both MSI and IHC aberrations, suggesting failure of mutation detection techniques. In 82 tumours from MMR gene mutation carriers in which at least MLH1 and MSH2 IHC-staining results were available, a strong correlation was found between aberrant staining and the presence of a germline mutation: 29 tumours from 30 *MLH1* mutation carriers were MLH1-negative (no nuclear staining) but positive for MSH2; 39 out of 42 tumours of *MSH2* mutation carriers showed no MSH2 staining and were positive for MLH1. Also, all the 10 tumours available

from *MSH6* mutation carriers displayed no MSH6 staining but were positive for MLH1 and MSH2. Among those cases tested for all three MMR proteins, MSH6 staining was often negative in tumours from *MSH2* mutation carriers and occasionally in *MLH-* related tumours.

To date, a combined approach of family history, MSI and IHC analysis is generally recognised as the most evidence-based and cost-effective way to select patients for MMR gene mutation analysis[42, 47, 175, 240, 296]. A practical flowchart of the steps involved in the selection of individuals with multiple or early onset HNPCC-associated tumours for MMR gene mutation analysis, and of families with some clustering of HNPCC-associated tumours, is shown in Figure 10.

Wijnen et al.[336] proposed a statistical approach for the selection of families for MMR gene mutation detection. They devised a logistic model for estimating the likelihood of a *MLH1* or *MSH2* mutation based on clinical data (Table 9). They recommended mutation analysis for any individual with a likelihood over 20% of carrying an MMR gene mutation. Loukola et al.[175] proposed to perform MSI analysis on tumours of individuals with a likelihood of more than 5% according to the algoritm developed by Wijnen et al.

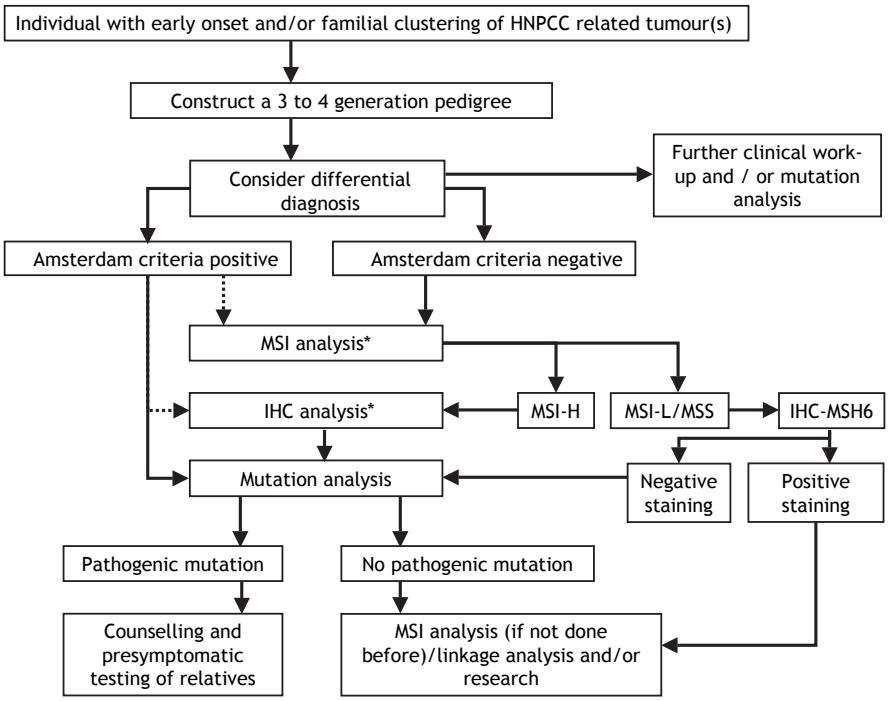

Figure 10.
A practical flowchart for the management of individuals with multiple or early onset HNPCC-associated tumours, and/of families with clustering of HNPCC-associated tumours. * The more "classic" the family, the less additional value has MSI and/or IHC, however IHC can be used to direct mutation analysis.

Table 8.
Comparison of MSI and IHC analysis in 154 tumours of 119 families analysed for *MLH1*, and *MSH2* mutations[20,39,47,100,317,323]. In all studies except in that of Debniak et al. the international markers for MSI analysis[24] (sometimes enhanced with BAT40) were used. Debniak et al. used 10 markers including 3 international markers and BAT40. Hendriks et al. and Berends et al. also performed IHC for MSh6 and mutation analysis of *MSH6*.

	Mutation		Normal IHC (%)	Aberrant IHC (%)	Total (%)
MSI-H/L (%)	positive	*MLH1*	8 (25)	24 (75)	32
		MSH2	1 (4)	26 (96)	27
		MSH6	2 (5)	37 (95)	39
		Subtotal	11 (11)	87 (89)	98 (92)
	Negative		8 (33)	16 (67)	24
	Subtotal		*19*	*103*	*122 (79)*
MSS (%)	positive	*MLH1*	3 (75)	1 (25)	4
		MSH2	3 (100)	0	3
		MSH6	0	1 (100)	1
		Subtotal	6 (75)	2 (25)	8 (8)
	Negative		23 (96)	1 (4)	24
	Subtotal		*29*	*3*	*32 (21)*
Total			**48**	**106**	**154**
All (%)	positive	*MLH1*	11 (31)	25 (69)	36
		MSH2	4 (13)	26 (87)	30
		MSH6	2 (5)	38 (95)	40
		Subtotal	17 (16)	89 (84)	106 (69)
	Negative		31 (65)	17 (35)	48 (31)
Total			**48 (31)**	**106 (69)**	**154**

Table 9.
Logistic regression model for selection of families for *MSH2* and *MLH1* mutation analysis[336].

Including Amsterdam criteria I	
Log Odds = L $L = 1,4 + (-0.1)V_1 + 1,7V_2 + 2,4V_3$	V_1 = mean age at diagnosis of all crc patients in the family
	V_2 = equal 1 if at least 1 relative has ec; otherwise it equals 0
	V_3 = equals1 if family meets ACI; otherwise it equals 0
Including Amsterdam criteria II	
Log Odds = L $L = 2,34 + (-0,1)V_1 + 0,25V_2 + 1,6V_3 + 0,96V_4$	V_1 = mean age at diagnosis of all crc patients in the family
	V_2 = number of patients with crc
	V_3 = number of patients with both crc/ec
	V_4 = equals1 if family meets ACII; otherwise it equals 0
Excluding Amsterdam criteria I and II	
Log Odds = L $L = 2,79 + (-0,1)V_1 + 0,33V_2 + 1,45V_3$	V_1 = mean age at diagnosis of all crc patients in the family
	V_2 = number of patients with crc
	V_3 = number of patients with both crc and ec
Mutation analysis of *MSH2* and *MLH1* is performed if $e^L/(1-e)^L \geq 0.2$ (probability of finding *MLH1* or *MSH2* mutation ≥20%)	

crc = colorectal cancer ec = endometrial cancer ACI/II = (Revised) Amsterdam criteria

1.16. Genetic counselling

Appropriate counselling is a prerequisite before genetic testing for HNPCC takes place, in view of the medical, psychological and social consequences for mutation carriers and their relatives. With respect to genetic diseases, the World Health Organization defines counselling as the 'provision of accurate, full and unbiased information in a caring, professional relationship that offers guidance, but allows individuals and families to come to their own decisions'[332]. General guidelines for cancer susceptibility testing were formulated by the American Society of Clinical Oncology in 1996 and updated in 2003[1, 2].

The counselee who initially seeks genetic advice generally fulfils a central role in the counselling of the family. He/she is asked to contact affected relatives for their participation in MSI/IHC and/or MMR gene analysis. Screening advises for relatives are initially communicated through the initial counselee. It is the task of the genetic counsellor to assist and advice the counselee in this process. Also, the implications of not finding a MMR gene mutation in a family must be understood by the counselee prior to testing.

In families with a known mutation in *MLH1*, *MSH2* or *MSH6*, genetic testing is offered to all affected and healthy adult risk carriers. Since the cancer risks in HNPCC start rising from about 20-25 years of age, genetic testing is not offered before adulthood. The use of genetic testing in families with a known MMR gene mutation has been studied by us and others[12, 154, 316]. Though the studies were performed in different settings, the test rate in individuals with *a priori* risk of 50% of carrying a mutation varied from 57 to 75%. If the counselee is affected by an HNPCC-related tumour, the risk of carrying the mutation is high though not 100%, and is therefore worth confirming by DNA analysis.

The recommended protocol for genetic testing includes three counselling sessions, a pre-test session, a session during which blood sampling is performed, and the test disclosure session [13, 154, 179]. Generally, this counselling procedure is judged as sufficient (see chapter 3.4)[13]. Of note, Dutch mutation carriers indicated that they would appreciate a regular update on new developments or scientific progress in the field of HNPCC, in addition to this protocol (see chapter 3.4).

Clearly, the absence or presence of a mutation is of considerable medical significance. Healthy individuals not carrying the mutation can be dismissed from medical surveillance, sparing them from invasive screening and reducing health-care costs[272]. Importantly, mutation carriers can benefit from medical surveillance programs. In 42 healthy Dutch HNPCC carriers, the compliance for colon screening increased from 31% before testing to 88% after testing (see chapter 3.4).

Genetic testing may also have a considerable psychological impact. Knowledge of having a cancer predisposition increases cancer worry. Several studies showed that most tested individuals are able to cope with a positive test result on the short-term, particularly in HNPCC[12, 13, 171]. After a short period of increased distress after the test result disclosure, the distress generally declines to levels comparable with those of non-carriers at one year follow-up[14]. Like in non-carriers, the ability to cope with a positive test result, is closely related to the ability to cope with problems overall, and to pre-test distress[171]. Also, on the long term the majority of Dutch mutation carrier was able to cope with being a gene carrier (chapter 3.4). In our study, about 10% of the mutation carriers experienced cancer worry on the long term. This was significantly associated with a high perceived risk of colorectal cancer. Regret of being tested occurred in 6 of 70 mutation carriers (9%) tested at our department (chapter 3.4). Notably, in 4 of these 6 mutation carriers, serious cancer events had happened in the family since their personal test disclosure. Events in the family are also known to influence perceived cancer risk[196].

Since the majority of the unaffected at risk relatives will receive a negative test result[329], most test applicants are relieved from fear. However, also non-carriers might experience adverse effects of testing. Especially if non-carriers show high pre-test distress and less effective coping strategies, they are at increased risk of post-test psychological distress and complex emotions summarised as "survivors guilt"[14, 171, 178, 289]. Non-carriers might feel guilty because they do not have the predisposition while close relatives have. This can have a large impact on family relationships; people even may become alienated from their family[178]. The relief of personal risk may also activate unresolved grief, and a few non-carriers will continue to have cancer fear[172].

Genetic testing may have considerable social consequences. First of all at the family-level, where several conflicts may arise. To search for the familial germline MMR mutation, co-operation of an affected relative often needs to be sought. Relatives may be unwilling to co-operate, depending on prior relationships and also on personal views. Sometimes unaffected relatives put too much pressure on their affected relatives to obtain their co-operation. Different coping strategies of relatives may disturb relationships. Informing children and family members about a genetic cancer risk is generally experienced as a burden, especially if more distant family members are concerned. There is no legal duty to inform relatives about a MMR gene mutation in the family, but most family members and professionals feel a moral duty to do so[57]. Peterson et al.[229] interviewed 39 members of 5 mutation positive HNPCC families. All participants had suspected a hereditary predisposition for cancer in their family prior to the identification of the HNPCC gene mutation. The pattern of communicating the news was

the same as the pattern used to communicate other non-urgent family news. The information was experienced as private but not secret and discussed first with the nuclear family members. Individuals who are encouraged by their relatives to seek genetic counselling seem to opt for genetic testing more frequently[85, 229].

Another important issue is the (fear of) employment and insurance discrimination. There is large international variation in legislation concerning individuals with a genetic predisposition to cancer. In the Netherlands, employers or insurers are forbidden by law to exclude individuals with a genetic predisposition for cancer from jobs or health insurance. For life or disability insurances, no questions about genetic predispositions may be asked by insurance employees for insurances below a certain limit (∈160.00 for life insurance and ∈32.000 in the first year of a disability insurance and ∈22.000 the following years). To our experience, possible future employment and insurance discrimination is an increasingly important reason to postpone or refrain from testing. Of 10 Dutch healthy HNPCC carriers opting for life or disability insurance or mortgage, 4 mentioned some trouble (see chapter 3.4). This is confirmed by Hadley et al.[85] who found potential effect on health insurance as the most important reason to refrain from testing in 50% risk carriers in 15 American families.

Chapter 2.

Molecular genetic studies in HNPCC

2.1.

Molecular Analysis of Hereditary Non-Polyposis Colorectal Cancer(HNPCC) in the USA: High Mutation Detection Rate among Clinically Selected Families and Charachterisation of an American Founder Deletion of the *MSH2* Gene

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Molecular Analysis of Hereditary Nonpolyposis Colorectal Cancer in the United States: High Mutation Detection Rate among Clinically Selected Families and Characterization of an American Founder Genomic Deletion of the *MSH2* Gene

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The identification of germline mutations in families with HNPCC is hampered by genetic heterogeneity and clinical variability. In previous studies, *MSH2* and *MLH1* mutations were found in approximately two-thirds of the Amsterdam-criteria–positive families and in much lower percentages of the Amsterdam-criteria–negative families. Therefore, a considerable proportion of HNPCC seems not to be accounted for by the major mismatch repair (MMR) genes. Does the latter result from a lack of sensitivity of mutation detection techniques, or do additional genes underlie the remaining cases? In this study we address these questions by thoroughly investigating a cohort of clinically selected North American families with HNPCC. We analyzed 59 clinically well-defined U.S. families with HNPCC for *MSH2*, *MLH1*, and *MSH6* mutations. To maximize mutation detection, different techniques were employed, including denaturing gradient gel electrophoresis, Southern analysis, microsatellite instability, immunohistochemistry, and monoallelic expression analysis. In 45 (92%) of the 49 Amsterdam-criteria–positive families and in 7 (70%) of the 10 Amsterdam-criteria–negative families, a mutation was detected in one of the three analyzed MMR genes. Forty-nine mutations were in *MSH2* or *MLH1*, and only three were in *MSH6*. A considerable proportion (27%) of the mutations were genomic rearrangements (12 in *MSH2* and 2 in *MLH1*). Notably, a deletion encompassing exons 1–6 of *MSH2* was detected in seven apparently unrelated families (12% of the total cohort) and was subsequently proven to be a founder. Screening of a second U.S. cohort with HNPCC from Ohio allowed the identification of two additional kindreds with the identical founder deletion. In the present study, we show that optimal mutation detection in HNPCC is achieved by combining accurate and expert clinical selection with an extensive mutation detection strategy. Notably, we identified a common North American deletion in *MSH2*, accounting for ~10% of our cohort. Genealogical, molecular, and haplotype studies showed that this deletion represents a North American founder mutation that could be traced back to the 19th century.

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC [MIM 114500]) is the most frequent autosomal dominant predisposition to the development of colorectal cancer. It is caused by germline mutations in human homologues of the bacterial mismatch repair (MMR) genes MutL and MutS: *MSH2* on chromosome 2p16, *MLH1* on 3p21, *MSH6* on 2p15, and *PMS1* and *PMS2* on 7p22 (Fishel et al. 1993; Bronner et al. 1994; Nicolaides et al. 1994; Akiyama et al. 1997; Miyaki et al. 1997). The identification of germline mutations in families with HNPCC and HNPCC-like disease is hampered not only by this locus heterogeneity but also by the clinical variability among families with HNPCC. Apart from a lifetime risk of colorectal cancer of ~80% (Vasen et al. 1996; Aarnio et al. 1999), individuals with an MMR gene mutation are characterized by an increased risk of tumors of the endometrium, stomach, small intestine,

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pancreas, hepatobiliary system, urinary tract, ovary, brain, and skin (Lynch 1999). The vast majority of HNPCC-causing mutations have been reported in *MSH2* and *MLH1* (Peltomaki and Vasen 1997). Accordingly, mutations in these genes give rise to the "classical" HNPCC phenotype (Wijnen et al. 1997; Giardiello et al. 2001), whereas *MSH6* mutations have been described in families with more atypical HNPCC (Kolodner et al. 1999; Wijnen et al. 1999; Wu et al. 1999; Wagner et al. 2001). Only a few *PMS2* germline mutations have been described so far (Hamilton et al. 1995; Liu et al. 2001).

Identification of HNPCC-causing MMR gene mutations enables at-risk relatives to be informed about their cancer risks and to benefit from intensive surveillance programs that have been proven to reduce their overall mortality by 65% (Jarvinen et al. 2000). To optimize mutation analysis in families with HNPCC and HNPCC-like disease, several clinical criteria have been formulated. Of these, the Amsterdam criteria, established by the International Collaborative Group on HNPCC (Vasen et al. 1991, 1999), are now recognized as the gold standard in HNPCC clinical selection. Previously, *MLH1* or *MSH2* mutations have been found in only 45%–64% and 0%–47% of the Amsterdam criteria–positive and –negative families, respectively (Giardiello et al. 2001). Since >90% of HNPCC colorectal cancers display microsatellite instability (MSI) (Aaltonen et al. 1994; Fujiwara et al. 1998), in contrast to 12%–18% of the sporadic colorectal cancers (Ionov et al. 1993; Thibodeau et al. 1993), the "Bethesda guidelines" include MSI as an additional selection tool for MMR gene mutation analysis (Rodriguez-Bigas et al. 1997). However, although the application of the Bethesda guidelines does improve the mutation analysis sensitivity, it also results in a remarkable reduction of its specificity compared with the Amsterdam criteria, since a considerable number of families with more atypical phenotypes are included (Syngal et al. 2000). The value of immunohistochemical (IHC) analysis of MMR proteins as a valuable additional selective tool has been shown in several studies (de Leeuw et al. 2000; Lindor et al. 2002; Hendriks et al. 2003), although positive IHC staining is observed in the presence of certain mutation types (Wahlenberg et al. 2002).

The fact that germline *MSH2* and *MLH1* mutations cannot be identified in a still-considerable proportion of the families with "classical" HNPCC may be explained by the involvement of additional HNPCC genes. *MSH6, PMS2,* and the more recently described *MLH3* and *EXO1* genes may represent candidate disease-causing genes for *MSH2*- and *MLH1*-negative families (Wu et al. 2001*a,* 2001*b*). However, preliminary data indicate that germline mutations in these genes are more likely to either result in atypical HNPCC phenotypes and are rare in families with "classical" HNPCC (*MSH6* and *PMS2*) or represent only mild genetic modifiers (*EXO1* and *MLH3*) (Hamilton et al. 1995; Kolodner et al. 1999; Wijnen et al. 1999; Wu et al. 1999, 2001*a,* 2001*b*; Wagner et al. 2001; Jagmohan-Changur et al. 2003). By means of more-sensitive mutation detection protocols (conversion technology or monoallelic mutation analysis [MAMA]), Yan et al. (2000) showed involvement of *MSH2* or *MLH1* in all the families with "classical" HNPCC analyzed. This suggested that clinically well-defined HNPCC is caused by *MSH2* and *MLH1* alone and that our efforts should be focused on developing new and/or more-thorough mutation detection approaches. To this end, we analyzed a cohort of families with clinically very well defined HNPCC and HNPCC-like disease from the United States for the presence of *MSH2, MLH1,* and *MSH6* germline mutations, by using different techniques to optimize mutation detection. The results presented here not only provide a comprehensive spectrum of the molecular basis of HNPCC in the United States but also underscore the need for clinical selection for a cost-effective screening of MMR genes.

Patients and Methods

The Henry Lynch HNPCC Cohort

The study included a total of 59 extended families selected for having conditions reminiscent of HNPCC by H. Lynch at the Department of Preventive Medicine and Public Health, Creighton University. The process of genetic counseling employed in the management of these families has been described elsewhere (Lynch et al. 1999). Forty-nine families fulfilled the Amsterdam criteria. Of the 10 Amsterdam criteria–negative families, 9 were characterized by two first-degree relatives with an HNPCC-related tumor (colorectal, endometrial, urinary tract, or small bowel cancer), with one relative diagnosed before the age of 50 years. Only one family did not meet the criteria because of the later (50 years) age at onset of the HNPCC-related tumors. However, one patient from this family presented with multiple HNPCC-related tumors. The majority of the families (80%) encompasses five or more generations, whereas, in the remaining cases, four generations were recorded. More family characteristics are listed in table 1. For each family, the youngest affected relative available was selected for mutation analysis, with only three exceptions: in two families, only a single relative with colorectal carcinoma in situ was available for mutation analysis; in a third one, only a nonpenetrant individual was available. Of the selected probands, 24 had colorectal cancer diagnosed before the age of 50 years (and 17 at the age of 45 years or younger). Eight probands had endometrial cancer (six diagnosed before the age of 50 years), and

Table 1

Clinical Features of the 59 Families with HNPCC Studied in this Article

	Colorectal Cancer	Endometrial Cancer	Other HNPCC-Related Tumors[a]
Mean no. per family	7.4	1.9	2.1
Mean age at onset, in years (range)	45.2 (19–85)	46.4 (31–77)	52.8 (27–76)

[a] Ovary cancer, stomach cancer, urinary tract cancer, and small-bowel cancer.

one had small bowel cancer at age 29 years. Twenty-three probands had multiple HNPCC-related tumors (including sebaceous adenomas).

Eleven families with HNPCC selected for this study and screened only for the founder mutation were diagnosed at the Ohio State University's James Cancer Hospital, Columbus, Ohio. Among these, five were Amsterdam-positive and, in the remaining six families, there were features highly suggestive of HNPCC, including early onset and/or multiple primary cancers in the proband, one or two first-degree relatives with endometrial cancer, or multiple second-degree relatives with colorectal or endometrial cancer. An additional cohort of 128 patients with colorectal ($n = 85$) or endometrial ($n = 43$) cancer from Ohio was studied. These patients represented all MSI-positive cases (98 MSI-high and 30 MSI-low) from a cohort of 716 unselected, consecutive, newly diagnosed patients with colorectal ($n = 482$) or endometrial ($n = 234$) cancer in the Columbus area, collected in 1998–2000.

Denaturing Gradient Gel Electrophoresis (DGGE)

DNA from at least one affected relative of every kindred was analyzed. Genomic DNA was obtained from peripheral blood lymphocytes. Initial DNA analysis was performed by DGGE and direct sequencing, as described elsewhere (Wijnen et al. 1998*b*, 1999). In short, *MSH2*- and *MLH1*-specific exons were amplified by PCR and were analyzed by GC-clamped DGGE. Exons with altered patterns of migration on DGGE were sequenced, to determine the nucleotide alteration. *MSH6* was analyzed by DGGE in families negative for pathogenic mutations in *MSH2* or *MLH1*.

Southern Blot Analysis

Southern analysis of *MSH2*, *MLH1*, and *MSH6* was performed in the families negative for a pathogenic point mutation after DGGE analysis, according to our previously established protocol (Wijnen et al. 1998*a*). Analysis of *MSH2* was performed with *Xba*I, *Hin*dIII, *Nsi*I, and *Eco*RI genomic DNA digests, followed by hybridization with three overlapping cDNA probes (encompassing exons 1–7, exons 7–12, and exons 10–16). Analysis of *MLH1* was performed with *Xba*I, *Apa*I, *Hin*dIII, and *Nsi*I digests hybridized with two specific cDNA probes encompassing exons 1–12 and exons 11–19; *MSH6* Southern analysis was performed with *Xba*I and *Apa*I digests, followed by hybridization with two cDNA probes encompassing exons 1–4 and exons 5–10.

Characterization of the Founder Deletion in MSH2

Molecular Analysis.—An apparently identical deletion encompassing exons 1–6 of the *MSH2* gene was identified by Southern analysis in seven apparently unrelated families. Primers were designed around the approximate location of breakpoints inferred from the restriction map of the deleted chromosome. The 3′ primer (F3) was located in intron 6 of the *MSH2* gene (5′-AAGCATCACAGTTACTGTTG-3′), whereas the 5′ primer (R3) was derived on the basis of genomic sequences ~2 kb upstream of exon 1 (5′-GCTGAATTAG-GTTTTGGAAC-3′). Through use of these primers, a deletion-specific PCR product of ~1,700 bp was obtained by long range PCR (Roche Molecular Biochemicals). This product was partly sequenced using a third primer (R2) located ~1 kb upstream of exon 1 (5′-TTTCAATCTGTCGCCCACGC-3′). These primers were also employed to screen two Ohio cohorts.

Genealogical Studies.—Genealogical data were obtained from the records of the Mormon Church (FamilySearch Internet Web site) and the Offices of Vital Statistics, as well as from cemeteries' records and patient recollection.

Haplotype Analysis.—To determine the genetic history of the deletion chromosome, chromosome 2 haplotypes were derived from several affected and unaffected individuals by PCR amplification of CA repeat markers proximal and distal to the *MSH2* gene. The employed chromosome 2 markers were as follows: tel-D2S119-D2S288-D2S391-*MSH2*$^{IVS10+12g\rightarrow a}$-CA1-D2S123-D2S378-cen. Primers and PCR conditions were as described elsewhere (Gyapay et al. 1994; Wijnen et al. 1994; Hutter et al. 2000). Also, a monochromosomal somatic cell hybrid (see below), containing the founder exon 1–6 deletion, was derived from Ohio family 726NM, to facilitate the study of the haplotype of the mutation-carrying chromosome.

MSI, IHC, and MAMA

MSI and IHC analysis was performed on tumor samples from one index patient negative for *MSH2*, *MLH1*,

or *MSH6* mutation (family 101), as well as from one index patient (family 125) with a missense mutation in *MSH2*. MSI analysis was performed with the National Cancer Institute–recommended markers (Boland et al. 1998), complemented by markers BAT40, MSH3, and MSH6 (Hendriks et al. 2003). IHC was performed with antibodies against MSH2, MLH1, and MSH6, as described elsewhere (de Leeuw et al. 2000). MSI status in the Ohio cohorts was determined with the NCI-recommended markers. Both MSI-high and -low cases were included.

We have applied MAMA of the alleged mutated allele isolated in a murine background (Papadopoulos et al. 1995; Yan et al. 2000). However, because of the limited availability of appropriate patient material from the 59 families with HNPCC, only few selected cases were analyzed by MAMA, mainly to provide proof of feasibility for this technical approach: the paracentric chromosome 2p inversion (family 140) (Wagner et al. 2002), a deletion of exons 1–13 of *MLH1* (family 103), and one family negative for mutations in *MSH2, MLH1,* or *MSH6* (family 143). In brief, somatic cell hybrids containing only one chromosome 2p or 3p allele from the index patient were generated by fusing peripheral blood lymphocytes or lymphoblastoid cell lines within an *Msh2*-deficient murine epithelial cell line (GMP Conversion Technologies) (Papadopoulos et al. 1995; Yan et al. 2000). Somatic cell hybrids were genotyped by CA repeat markers around the *MSH2* and *MLH1* genes on chromosomes 2p and 3p, respectively, and were selected for MAMA. Total RNA and protein samples were isolated from the different hybrid cell lines. RT-PCR using random hexamers (Fermentas) was performed, followed by a standard PCR using human-specific primers for *MSH2* (forward primer 5′-ATATCATGGAACCAGCA-3′ and reverse primer 5′-TACCTTCATTCCATTACT-GG-3′), *MLH1* (forward primer 5-GCCATTGTCACA-GAGGATAA-3′ and reverse primer 5′-CGAAGGAGTG-GTTAAGCAAC-3′) and *MSH6* (forward primer 5′-T-CGGTAGCGCCTGCTGCCCC-3′ and reverse primer 5′-TAAGTTGTGCCTACCTCCA-3′). In addition, western blot analysis was performed on protein lysates obtained from the somatic cell hybrid lines through use of a rabbit polyclonal antibody raised against amino acids 402–737 of human MSH2 (Smits et al. 2000).

Results

Point-Mutation Analysis

A total of 37 germline mutations was detected in *MSH2, MLH1,* and *MSH6* by DGGE analysis, including seven mutations of uncertain pathologic significance (six missense mutations and one in-frame 3-nt deletion) (table 2). In another three families, both a missense and a pathogenic mutation were detected (table 3). Most point mutations (23 of 37) were detected in *MLH1*. In three independent families, an identical nonsense mutation in *MLH1* exon 12 leading to exon skipping was found (Stella et al. 2001).

Table 2

Summary of Results of the Point Mutation Analysis in Our Cohort

Gene and Mutation Type	No. of Point Mutations in[a]		
	AC-Positive Families ($N = 49$)	AC-Negative Families ($N = 10$)	Total ($N = 59$)
MLH1:			
Frameshift	8	0	8
Nonsense	2	1	3
Splice site	6	2	8
Missense	3	1	4
Subtotal	19	4	23
MSH2:			
Frameshift	4	0	4
Nonsense	3	1	4
Splice site	1	0	1
Missense	1	1[b]	2
Subtotal	9	2	11
MSH6:			
Frameshift	1	0	1
In-frame deletion	0	1	1
Missense	1	0	1
Subtotal	2	1	3
Total	30	7	37

Note.—In case of double mutations, only the assumed disease-causing mutation is reported.

[a] AC = Amsterdam criteria.

[b] A636P in *MSH2,* a pathogenic Ashkenazi Jewish founder mutation (Yuan et al. 1999; Foulkes et al. 2002).

Detection of Genomic Rearrangements

Southern blot analysis was performed in 29 of the 59 families with HNPCC, where no pathogenic point mutation was found. In total, 14 genomic rearrangements were detected, 12 in *MSH2* and 2 in *MLH1* (table 4). One of the deletions is a ±13-kb deletion 6 kb upstream of *MSH2* (family 177). All families with a rearrangement of *MSH2* or *MLH1* fulfilled the Amsterdam criteria. In seven families, an identical deletion of exons 1–6 of the *MSH2* gene was identified. The further characterization of this deletion is described below. Other deletions and types of rearrangement were also observed in the *MSH2* gene: a second but different deletion of at least 20 kb encompassing exons 1–6 (family 108), an ~2-kb exon 3 deletion (family 150), and a deletion of exons 3–8 of ~50 kb (family 136). The chromosome 2 paracentric inversion encompassing *MSH2* exons 8–16 found in one kindred (family 140) has been reported elsewhere (Wagner et al. 2002). In *MLH1*, two genomic deletions, en-

Table 3

Overview of the Individual Probands and Mutations Detected in the 59 Families

Family	AC[a]	Mutation in		
		MSH2	*MLH1*	*MSH6*
101	+			
102	+		243delAATG	
103	+		Δ exons 1–13 and E578G	
104	+		G67R	
107	+	IVS5+3a→t		
108	+	Δ exons 1–6		
109	+		733insAACA	
110	+		IVS5−1g→c	
111	+	840delA		
112	+		A29S	
113	+		466delC	
114	+			
115	−	A636P		
116	+	N583S	IVS7−1g→t	
117	+	Q298X		
118	+		K461X	
119	+		64insA	
120	+		Δ exon 6	
121	+		IVS6−2a→g	
122	+	Δ exons 1–6[b]		
123	+	Δ exons 1–6[b]		
124	+	Δ exons 1–6[b]		
125	+	T552P		
126	+	129delTC and S473L		
127	+		R226L (splice site)	
128	+		52insA	
129	+		V185L	
130	+	Δ exons 1–6[b]		
131	+	55delC		
132	+		IVS18+1g→a	
133			K461X	
134	+		IVS6−2a→g	
135	+		K461X	
136	+	Δ exons 3–8		
137	+		733insAACA	
138	+	Q885X		
139	−		IVS13+1g→t	
140	+	inv exons 8–16		
141	−	R389X		
142	−		IVS9+1g→a	
143	−			
145	+		168insA	
146	+			
147	+			
148	+	Δ exons 1–6[b]		
149	+	806insA		
150	+	Δ exon 3		
151	−			
152	+	Δ exons 1–6[b]		
153	+			1087insC
154	−			1013delCTT
155	+	Δ exons 1–6[b]		
157	+	R680X		
158	+	IVS3−3t→g		
177	+	Δ *5′ MSH2*		
178	−		G244D	
205	+		525delTT	
207	−			
208	+			M492V

Note.—Δ = deletion.

[a] AC = Amsterdam criteria.

[b] Founder genomic deletion encompassing exons 1–6 of the *MSH2* gene.

Table 4

Summary of Genomic Rearrangements in the 59 Families with HNPCC

Family	Genomic Rearrangement in *MSH2*	Genomic Rearrangement in *MLH1*
103		Deletion of exons 1–13
108	Deletion of exons 1–6	
120		Deletion of exon 6
122	Deletion of exons 1–6[a]	
123	Deletion of exons 1–6[a]	
124	Deletion of exons 1–6[a]	
130	Deletion of exons 1–6[a]	
136	Deletion of exons 3–8	
140	Inversion of exons 8–16	
148	Deletion of exons 1–6[a]	
150	Deletion of exon 3	
152	Deletion of exons 1–6[a]	
155	Deletion of exons 1–6[a]	
177	Upstream deletion[b]	
Total	12	2

[a] The founder genomic deletion encompassing exons 1–6 of the *MSH2* gene.

[b] An ~13-kb deletion, the 3′ breakpoint end of which maps 6 kb 5′ of *MSH2*. The pathogenicity of this deletion could not be investigated.

compassing exon 6 (~2.3 kb; family 120) and exons 1–13 (at least 42 kb; family 103), were identified. No *MSH6* genomic rearrangements were found among the 29 families (tables 4 and 5).

Characterization of the MSH2 *Exon 1–6 Founder Deletion*

Molecular Analysis.—In seven patients, aberrant fragments of ~14 kb were apparent by Southern analysis after digestion with *Eco*RI and hybridization with the *MSH2* exon 7 probe (fig. 1*a*). Also, on the basis of decreased intensity of the wild-type fragment and additional hybridizations with exon 1–6 probes (data not shown), the presence of the aberrant *Eco*RI fragment was indicative of a genomic deletion of exons 1–6 of the *MSH2* gene. Digestion by *Bcl*I revealed an ~13-kb aberrant fragment and confirmed the presence of an identical rearrangement in these seven families. To further characterize this recurring North American deletion previously not found among HNPCC samples of European origin (Wijnen et al. 1998*a*), PCR primers were designed around the approximate location of breakpoints inferred from the restriction map of the deleted chromosome. The 5′ primer (R3) was based on genomic sequences ~2 kb upstream of exon 1, whereas the 3′ primer (F3) was located in intron 6 of the *MSH2* gene. Long range PCR with these primers resulted in a deletion-specific PCR product of ~1,700 bp, exclusively observed in carriers of the common deletion and not in deletion-negative controls (data not shown). Sequencing of the breakpoint revealed exactly the same nucleotide sequence in all seven families diagnosed with the exon 1–6 deletion by Southern analysis. The breakpoints are positioned within two *Alu* repeats (fig. 1*B* and 1*C*). Thus, the deletion is likely to have arisen through an *Alu*-mediated recombination. The presence of identical breakpoint sequences in all seven cases is suggestive of a founder mutation, since a frequently recurring recombination event would be likely to result in at least a few single-nucleotide differences.

Screening for the Founder Deletion.—An additional cohort of 11 clinically selected families with HNPCC from Ohio was investigated for the presence of the common deletion, through use of the PCR primers (R3 and F3) described above. Two additional deletions were detected (families 726NM and CG336). Notably, the same mutation was not found in a consecutive series (n = 128) of newly diagnosed MSI-positive patients with colorectal and endometrial cancer derived from a total of 716 Ohio patients studied for MSI.

Haplotype Analysis.—To confirm the common genetic origin of the deletion of exons 1–6, haplotype analysis was performed (fig. 2). With the exception of families 124 and CG336, the haplotype of the deleted allele was reconstructed in the seven remaining families, six from the Lynch cohort and one of the two Ohio kindreds (726NM) found to carry the identical *MSH2* deletion. A common haplotype is evident in all families, with the one exception of kindred 155, which shows, possibly as the result of mitotic recombination, novel alleles at markers DS288 and D2S119. Additional variation was

Table 5

Overview of the Results of the Complete Mutation Analysis of the *MSH2, MLH1,* and *MSH6* Genes in the 59 Families with HNPCC

Gene and Mutation Type	No. of Mutations in AC-Positive Families (N = 49)	AC-Negative Families (N = 10)	Total (N = 59)
MLH1:			
Point mutation	19	4	23
Genomic deletion	2	0	2
Subtotal	21	4	25 (42%)
MSH2:			
Point mutation	10[a]	2	12
Genomic deletion/inversion	12	0	12
Subtotal	22	2	24 (41%)
MSH6:			
Point mutation	2	0	2
In-frame deletion	0	1	1
Subtotal	2	1	3 (5%)
Total	45 (92%)	7 (70%)	52 (88%)

Note.—In the case of double mutations, only the assumed disease-causing mutation is reported.

[a] Including the splice-site mutation detected by MAMA analysis by Yan et al. (2000).

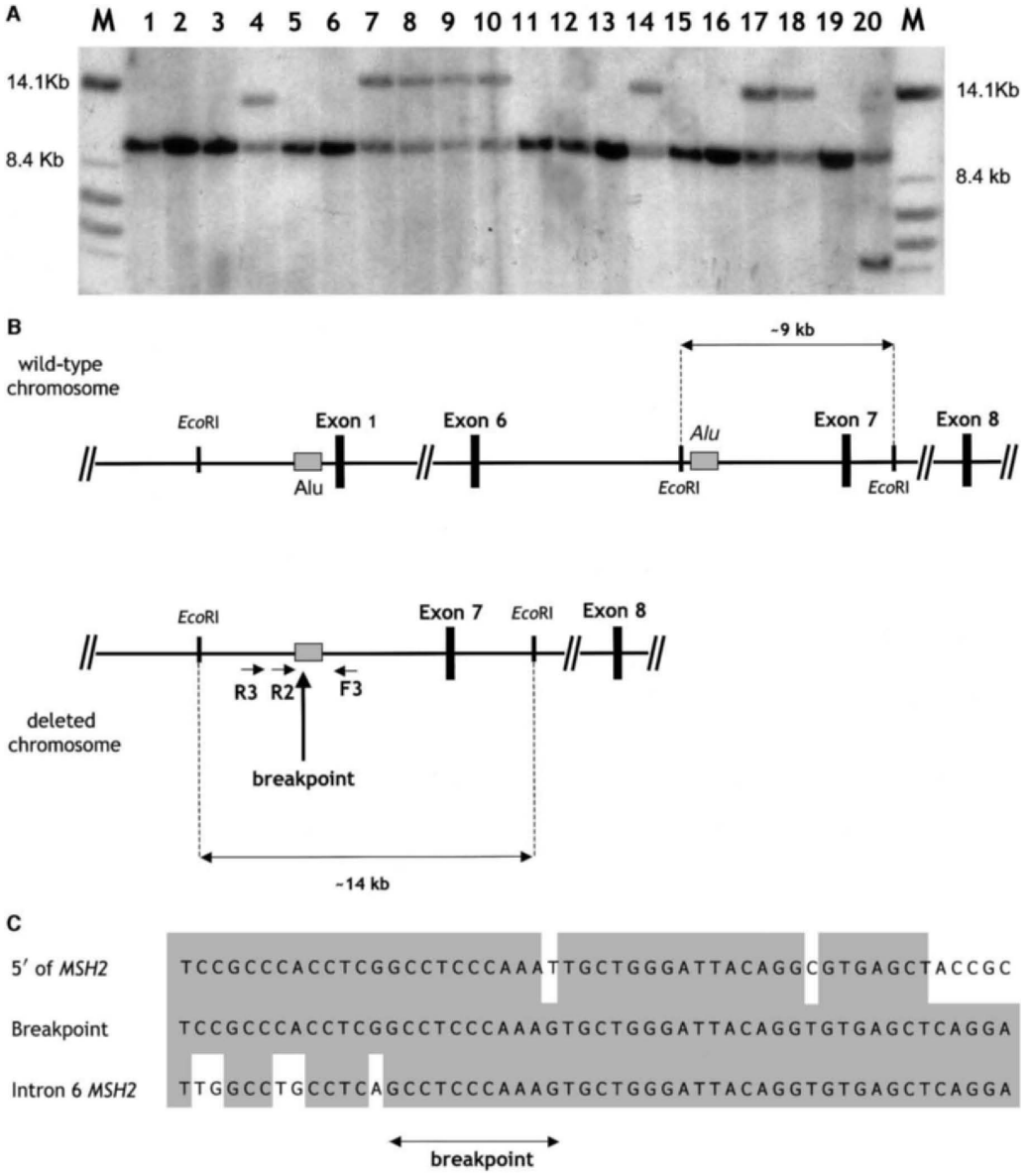

Figure 1 *A*, Southern blot analysis of genomic DNA samples from North American individuals with HNPCC, revealing a common rearrangement. Total genomic DNA was digested with *Eco*RI and was hybridized with the *MSH2* exon 7–specific probe. An aberrant ~14-kb band was observed in lanes 7, 8, 9, 10, 14, 17, and 18, corresponding to individuals from families 122, 123, 124, 130, 148, 152, and 155, respectively. Aberrant fragments of different sizes are also observed in lanes 4 (family 108, carrying a similar but distinct *MSH2* exon 1–6 deletion) and 20 (a previously identified carrier of an *MSH2* exon 6 deletion, employed here as positive control). Also note that the presence of additional bands is accompanied by a decreased intensity of the ~9-kb wild-type fragments encompassing exon 7. The latter was confirmed by hybridizations with exon 1–6 probes and by *Bcl*I digestion (data not shown). *B*, Genomic map of the normal and deleted *MSH2* gene. For the sake of clarity, only the *Eco*RI restriction sites, the *Alu* repeats involved in the recombination (*shaded boxes*), and the relevant *MSH2* exons (*blackened boxes*) are depicted. The normal (~9-kb) and aberrant (~14-kb) *Eco*RI restriction fragments also shown in panel *A* are indicated, as well as the primers (R3, R2, and F3) employed to selectively amplify the deletion breakpoint by PCR. *C*, Nucleotide sequence of the founder deletion breakpoint. Three distinct sequences are reported from top to bottom: the normal sequence flanking *MSH2* at the 5′ side, the deletion breakpoint, and the normal sequence of *MSH2* intron 6. The shaded boxes indicate the nucleotide homology between the two *Alu* repeats present in the 5′ flanking sequence and within intron 6 of *MSH2*. Because of the extensive homology, it is difficult to pinpoint the exact deletion breakpoint. The arrow indicates its most likely position on the basis of nucleotide homology.

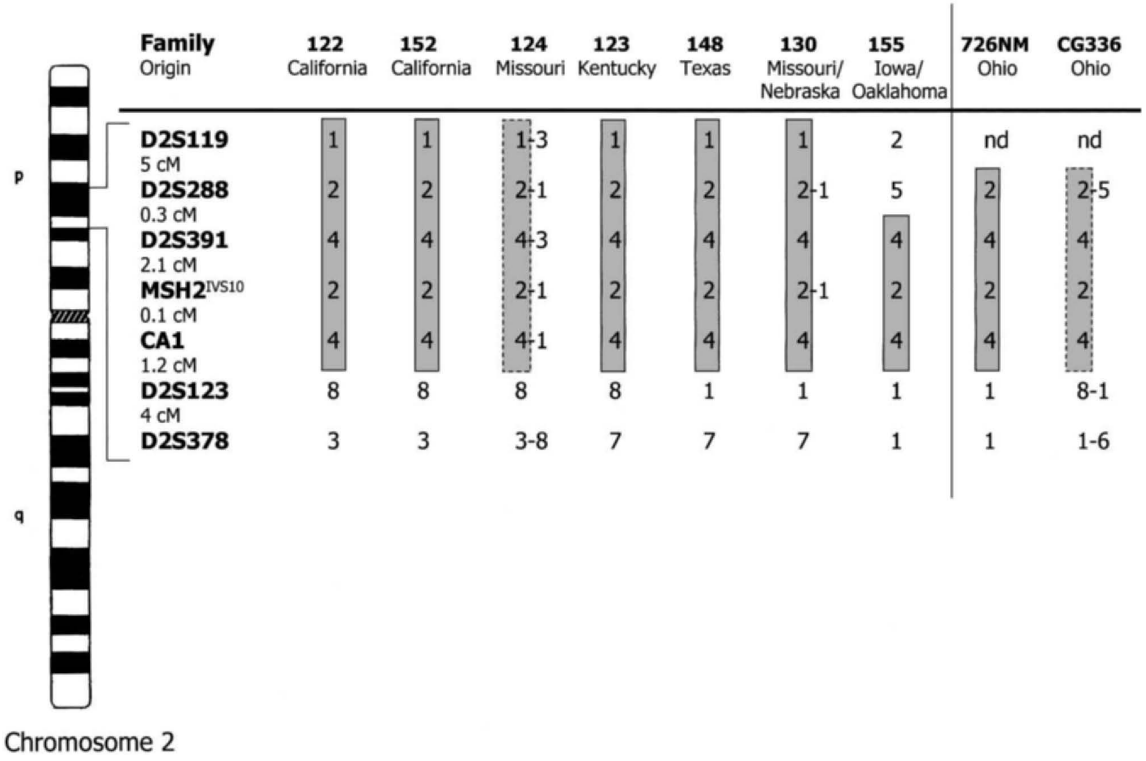

Figure 2 Haplotype analysis of the founder *MSH2* deletion chromosome. A total of seven polymorphic markers flanking *MSH2* on both the telomeric and centromeric side were employed. The haplotype encompassing the founder *MSH2* deletion could be reconstructed in most families, with the exception of family 124 from the Lynch cohort and kindred CG336 from the Ohio cohort, because of a lack of samples from informative relatives. The haplotype of family 726NM was derived from a somatic cell hybrid containing the isolated deletion chromosome in a rodent background. Shaded boxes indicate the alleged founder haplotype. Both alleles are indicated in those cases where the exact phase could not be derived.

observed exclusively at more-distant centromeric markers—that is, D2S123 and D2S378 (fig. 2).

Genealogical Studies.—By means of genealogical studies, a common ancestry could be traced for five of the nine families found to carry the *MSH2* exon 1–6 founder deletion. The alleged ancestor was born around 1814 in Alabama and was presumably of German origin. He married and became a Mormon. From Alabama, he and his family undertook a long journey to Utah, following the Mississippi River through Kentucky and Missouri. Some family members moved to southeastern Iowa, bordering Illinois, and then followed the Missouri River to Nebraska and Wyoming, eventually settling in Utah. However, the ancestor was excommunicated from the Mormon Church and moved, with an unspecified number of children, to California. Two of the families studied here (122 and 152) still carry the same ancestral name and live in California. The other families reside along the trail of their ancestors (A.B., A.W., P.F.F., S.C., Y.K., P.W., R.O., J.F.L., R.F., A.D., and H.T.L., unpublished data).

Alternative Approaches to Unresolved Cases

Even when applying the most thorough mutation detection strategy, unresolved cases—that is, mutation-negative samples or missense mutations of unknown pathogenic relevance—require additional analyses for their elucidation. In our study, we were confronted with a number of such cases. Unfortunately, the limited availability of blood or tumor samples hampered the application of additional approaches to some cases. However, a number of cases provided proof of feasibility for the general strategy (table 6).

In one of the families negative for *MSH2, MLH1,* or *MSH6* mutations (family 101), analysis of the corresponding colorectal cancer revealed an MSI-high phenotype and no expression of MSH2 by immunohisto-

Table 6

Families with Mutations of Unknown Significance and Families without Mutations

Family	AC[a]	Mutation	Test Result[b]		
			MSI	IHC	MAMA
101	+		High	Decreased expression MSH2	NP
104	+	G67R *MLH1*	NP	NP	NP
112	+	A29S *MLH1*	NP	NP	NP
114	+		NP	NP	NP
125	+	T552P *MSH2*	High	Normal expression MSH2, MLH1, MSH6	NP
129	+	V185L *MLH1*	NP	NP	NP
143	−		NP	NP	Normal expression *MSH2, MLH1, MSH6*
146	+		NP	NP	NP
147	+		NP	NP	NP
151	−		NP	NP	NP
154	−	1013delCTT *MSH6*	NP	NP	NP
177	+	D *5′ MSH2*	NP	NP	NP
178	−	G244D *MLH1*	NP	NP	NP
207	−		NP	NP	NP
208	+	M492V *MSH6*	NP	NP	NP

[a] AC = Amsterdam criteria.
[b] NP = not performed.

chemistry. This kindred was characterized by a classical clinical HNPCC phenotype that is likely the result of a genetic defect in the *MSH2* gene that was missed by the DGGE and Southern approaches. In one other individual found to carry an *MSH2* missense mutation of unknown pathogenic relevance (family 125), analysis of tumor DNA again revealed an MSI-high phenotype. IHC analysis of the corresponding tumor sections showed normal expression of MSH2, MLH1, and MSH6. Although it is possible that the mutant MSH2 proteins are functionally hampered by the missense mutations, at present we cannot determine the pathogenicity of this mutation (Wahlenberg et al. 2002).

MAMA, or conversion technology, represents a useful and promising approach for the analysis of missense mutations in *MSH2* (Papadopoulos et al. 1995; Yan et al. 2000). It has also proven to be a useful tool in the analysis of both splicing errors and genomic alterations (Nakagawa et al. 2002). Somatic cell hybrids containing the alleged mutated chromosome in a murine background can be analyzed by RT-PCR, northern, or western analysis, to ensure expression and stability of the human *MSH2* gene. We demonstrated proof of feasibility for this approach in the analysis of the pathogenicity of the chromosome 2p paracentric inversion (Wagner et al. 2002). Moreover, the chromosome 3 allele harboring the *MLH1* deletion of exons 1–13 (family 103) did not show any expression of this gene by RT-PCR after conversion (not shown). Conversion analysis of the chromosome 2 and 3 alleles of the index patient negative for mutations in *MSH2, MLH1,* and *MSH6* (family 143) displayed expression of the same genes by RT-PCR and western blot analysis of the somatic cell hybrids.

Discussion

The overall mutation detection rate in our study is remarkably high, compared with most previous studies (Giardiello et al. 2001). In 39 (80%) of 49 families fulfilling the Amsterdam criteria, and in 5 (50%) of the 10 Amsterdam criteria–negative families, a pathogenic mutation was found in one of the three MMR genes analyzed. When the mutations of uncertain significance (table 6) are included, the detection percentages rise to 92% (45 of 49) and 70% (7 of 10) for Amsterdam-positive and -negative families, respectively (table 5). These results are supportive of the extremely high *MSH2* and *MLH1* mutation detection rate among clinically well-selected HNPCC families. It is safe to assume that extension of the MAMA analysis to all the 59 index patients, improvement of the DGGE strategy, and further functional analysis of the nucleotide variants of as-yet-uncertain pathogenic significance would increase detection rate to close to 95%.

Apart from the more straightforward nonsense and deletion mutations that are expected to be disease-causing by nature, several missense mutations were detected in this study. One of these, the A636P mutation in *MSH2,* is a founder mutation in the Ashkenazi Jewish population and was proven to be pathogenic (Yuan et al. 1999; Foulkes et al. 2002). Two other missense mutations (G67R and V185L in *MLH;* families 104 and 129, respectively) were previously described to affect mismatch repair (Shimodaira et al. 1998; Ellison et al. 2001). In one index patient found to carry an *MSH2* missense mutation (T552P in family 125), tumor analysis revealed an MSI-high phenotype. As described by Foulkes et al. (2002), one possible approach to the anal-

ysis of the pathogenicity of missense mutations is the analysis of large cohorts of affected and healthy individuals. Moreover, cosegregation analysis of the alleged mutation with the disease phenotype within extensive, multigeneration pedigrees can provide strong support for the pathogenicity of the missense substitution. However, definitive proof or disproof of the pathogenicity of such mutations can be obtained only by implementing functional studies. The latter have been primarily performed in yeast (Ellison et al. 2001), although novel protocols based on mammalian expression systems have also been applied successfully (Nakagawa et al. 2002; Trojan et al. 2002; see below).

In a kindred with classical HNPCC that was negative for mutations in *MSH2, MLH1,* or *MSH6* (family 101), analysis of a colorectal cancer showed an MSI-high phenotype and loss of MSH2 protein expression by IHC, indicating that functional impairment of *MSH2*—for example, by a promotor mutation (Shin et al. 2002) or a splicing error due to a change within an intron—may underlie HNPCC in this family. In another kindred with classical HNPCC (family 158), a mutation was detected, by MAMA, by Yan et al. (2000). By using this technique, these authors found a splice-acceptor mutation in *MSH2* that was proven hard to detect by conventional mutation analysis (table 3) (Yan et al. 2000). One other family negative for mutations in *MSH2, MLH1,* or *MSH6* (family 143) was studied by MAMA. This family did not fulfill the Amsterdam criteria but was highly reminiscent of HNPCC, because of the presence of multiple related tumors in an affected individual and colorectal cancer in several individuals. The index patient from this family presented with colorectal cancer at age 56 years. Conversion analysis showed expression of the three MMR genes. Hence, the latter case may represent a bona fide carrier of a genetic defect at a locus other than the major MMR genes. Alternatively, the index patient may represent a phenocopy. This underscores the importance of testing individuals who are most likely, on the basis of clinical features, to carry the familial genetic defect—namely, the presence of HNPCC-related tumors and their age at onset (Wijnen et al. 1998*b*).

In total, only two mutations were found in *MSH6*. One is a frameshift mutation, and the other is an in-frame deletion of 3 nt of unknown significance (in families 153 and 154, respectively). Both families are characterized by patients with endometrial cancer and by a relatively late onset of colorectal cancer, in agreement with the atypical phenotypic features associated with *MSH6* mutations (Kolodner et al. 1999; Wijnen et al. 1999; Wu et al. 1999; Wagner et al. 2001). The relatively low incidence of *MSH6* mutations is interesting in view of the percentages (8% and 30% of the Amsterdam-positive and -negative kindreds, respectively) of the families with HNPCC in which no mutation could be detected. It is likely that genes like *MSH6* that cause an overlapping though distinct phenotype are responsible for a small subset of the families with HNPCC and for a larger proportion of the atypical kindreds.

The high detection rate in our study is partially due to the screening for and identification of larger genomic rearrangements. The latter enabled us to identify one common ~20-kb deletion, encompassing exons 1–6 of the *MSH2* gene, that was proven to represent a founder mutation in the North American population. The origin of this mutation could be traced back to the beginning of the 19th century. Nucleotide sequence analysis of the nine deletion breakpoints did not reveal any base pair difference, as would be expected from a recurring recombination event. Also, haplotype analysis of the seven of the nine families with HNPCC carrying the common deletion by CA repeat markers flanking the *MSH2* gene was in agreement with the founder hypothesis. In the majority of the cases, a region of ~8 cM is conserved. This is in agreement with a relatively young age of the founder deletion. Accordingly, although the alleged ancestor was of German origin, we were unable to find the same deletion in an extensive analysis of European patients with HNPCC (Wijnen et al. 1998*a*) and in a cohort of 89 German families with HNPCC (authors' unpublished data). As confirmed by the PCR screening of the small but clinically selected Ohio cohort, this mutation represents ⩾10% of the disease-causing mutations among the Midwestern white families with HNPCC. Further analyses are needed to determine the frequency of this founder in other U.S. populations. Notably, screening of the MSI-positive Ohio cohort (n = 128, 98 of whom were MSI-high and 30 of whom were MSI-low) for the presence of the founder *MSH2* deletion failed to detect any additional carrier. The latter finding does not affect the alleged frequency of the founder mutation, since it is known that a large fraction of the MSI-high cases represent sporadic tumors due to hypermethylation of the *MLH1* promoter. Moreover, a large fraction of sporadic CRC is known to be MSI-low (Halford et al. 2002). Therefore, this finding emphasizes the importance of clinical selection.

Seven additional genomic rearrangements were detected in our cohort. Five of the genomic rearrangements were in *MSH2*. Notably, in the case of family 177, the 13-kb deletion maps 6 kb 5′ of *MSH2*. Presumably, this deletion encompasses upstream regulatory sequences, although no evidence for its true pathogenicity could be demonstrated in this study. Also, in view of the high frequency of genomic rearrangements detected in the *MSH2* gene in our (data not shown) (Wijnen et al. 1998*a*) and other laboratories (Charbonnier et al. 2002), this locus may contain sequences prone to recombination. Indeed, genomic sequences immediately upstream of *MSH2* and some of its larger introns (in-

trons 6, 7, and 8; 13.3 kb, 15.6 kb, and 17.4 kb, respectively) do contain many repetitive sequences, like *Alu* and other repeats (from our own analysis by RepeatMasker [RepeatMasker Web Server]) (Charbonnier et al. 2002). These repeats are known to mediate recombination events between partially homologous sequences resulting in genomic deletions, insertions, inversions, or other more-complex rearrangements (Wagner et al. 2002). On the basis of our findings, mutation analysis must include screening for genomic rearrangements, since these represent a frequent cause of disease at the major MMR genes (in this study, 24% of the total HNPCC burden and 50% of the cases due to *MSH2*). The characterization of the breakpoints of the most common genomic rearrangements will allow the development of PCR-based screening protocols (present study) to circumvent more cumbersome and time-consuming methods such as Southern blotting.

Overall, accurate and expert clinical selection of the studied families greatly improves mutation detection, especially when combined with a thorough and extensive methodological approach. Thirty-four (69%) of the Amsterdam criteria–positive families in our cohort had two additional relatives with an HNPCC-related tumor, exceeding the Amsterdam criteria requirements. In all these families, an *MSH2* or *MLH1* mutation was detected. Also, a pathogenic mutation was detected in all families fulfilling the revised Amsterdam criteria, only one of which was in *MSH6*. This indicates that the fulfillment of the Amsterdam criteria by three patients with colorectal cancer is not necessarily an indication of the presence of an *MSH2* or *MLH1* mutation. This is probably because of the high frequency of colorectal cancer in the general population, often with relatively early age at onset, and because of other forms of familial clustering of colorectal cancer cases. The presence of additional relatives with HNPCC-related tumors considerably increases the chance of *MSH2* or *MLH1* mutation detection. Patients with multiple HNPCC-related tumors or with sebaceous tumors reminiscent of Muir-Torre syndrome were observed in mutation-positive families that did not fulfill the Amsterdam criteria. Conversely, we were unable to detect the disease-causing mutation in one family with "classical" HNPCC (family 101). A colorectal tumor sample of the index patient from this family displayed an MSI-high phenotype and loss of MSH2 expression.

In conclusion, improved clinical selection forms the basis of optimal mutation detection in HNPCC. Mutation analysis in "classical" HNPCC should focus on the two major MMR genes—that is, *MSH2* and *MLH1*. We underscore that, apart from point mutations, genomic rearrangements strongly contribute to the HNPCC mutation spectrum. MSI and IHC analyses will direct further mutation detection strategies in the mutation-negative families with classical HNPCC and in the more atypical HNPCC cases. This approach has also led to the identification of a common North American deletion in MSH2 accounting for ~10% of our cohort. Genealogical, molecular, and haplotype studies showed that this common deletion represents a North American founder mutation that could be traced back to the 19th century.

Acknowledgments

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Electronic-Database Information

The URLs for data presented herein are as follows:

FamilySearch Internet, http://www.familysearch.org/Eng/Search/frameset_search.asp

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for HNPCC)

RepeatMasker Web Server, http://ftp.genome.Washington.edu/cgi-bin/RepeatMasker

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2.2.

A Founder Mutation of the *MSH2* gene and Hereditary Nonpolyposis Colorectal Cancer in the United States

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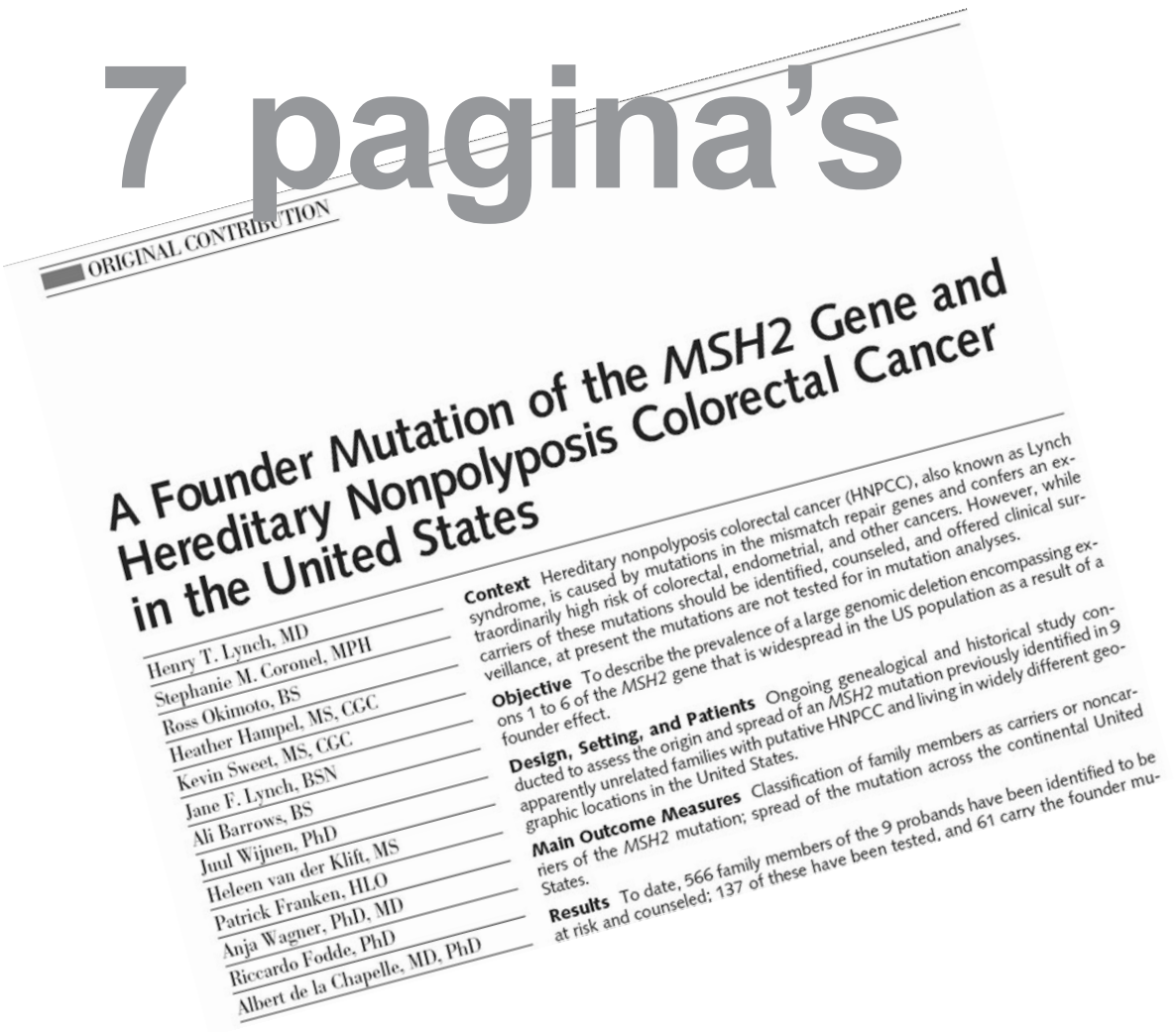

ORIGINAL CONTRIBUTION

A Founder Mutation of the *MSH2* Gene and Hereditary Nonpolyposis Colorectal Cancer in the United States

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Context Hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is caused by mutations in the mismatch repair genes and confers an extraordinarily high risk of colorectal, endometrial, and other cancers. However, while carriers of these mutations should be identified, counseled, and offered clinical surveillance, at present the mutations are not tested for in mutation analyses.

Objective To describe the prevalence of a large genomic deletion encompassing exons 1 to 6 of the *MSH2* gene that is widespread in the US population as a result of a founder effect.

Design, Setting, and Patients Ongoing genealogical and historical study conducted to assess the origin and spread of an *MSH2* mutation previously identified in 9 apparently unrelated families with putative HNPCC and living in widely different geographic locations in the United States.

Main Outcome Measures Classification of family members as carriers or noncarriers of the *MSH2* mutation; spread of the mutation across the continental United States.

Results To date, 566 family members of the 9 probands have been identified to be at risk and counseled; 137 of these have been tested, and 61 carry the founder mu-

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2004- vol 291, No 6
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2.3.

A 10-Mb Paracentric Inversion of Chromosome arm 2p Inactivates *MSH2* and is Responsible for Hereditary Non Polyposis Colorectal Cancer in a North-American Kindred

Genes Chromosomes Cancer 2002:35;49-57

A 10-Mb Paracentric Inversion of Chromosome Arm 2p Inactivates *MSH2* and Is Responsible for Hereditary Nonpolyposis Colorectal Cancer in a North-American Kindred

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Genomic deletions of the *MSH2* gene are a frequent cause of hereditary nonpolyposis colorectal cancer (HNPCC), a common hereditary predisposition to the development of tumors in several organs including the gastrointestinal and urinary tracts and endometrium. The mutation spectrum at the *MSH2* gene is extremely heterogeneous because it includes nonsense and missense point mutations, small insertions and deletions leading to frameshifts, and larger genomic deletions, the latter representing approximately 25% of the total mutation burden. Here, we report the identification and molecular characterization of the first paracentric inversion of the *MSH2* locus known to cause HNPCC. Southern blot analysis and inverse PCR showed that the centromeric and telomeric breakpoints of the paracentric inversion map within intron 7 and to a contig 10 Mb 3′ of *MSH2,* respectively. Pathogenicity of the paracentric inversion was demonstrated by conversion analysis. The patient's lymphocytes were employed to generate somatic cell hybrids to analyze the expression of the inverted *MSH2* allele in an *Msh2*-deficient rodent cellular background. The inversion was shown to abolish *MSH2* expression by both northern and western analysis. This study confirms that Southern blot analysis still represents a useful and informative tool to screen for and identify complex genomic rearrangements in HNPCC. Moreover, monoallelic expression analysis represents an attractive approach to demonstrate pathogenicity of unusual mutations in autosomal dominant hereditary conditions.
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INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome is a common autosomal dominant cancer syndrome caused by germline mutations in *MSH2* and *MSH6* on chromosome 2, *MLH1* on chromosome 3, and *PMS2* on chromosome 7 (Fishel et al., 1993; Bronner et al., 1994; Nicolaides et al., 1994; Akiyama et al., 1997; Miyaki et al., 1997). These genes are responsible for DNA mismatch repair (MMR) and are homologs of the *Escherichia coli MutS* and *MutL* MMR genes. Carriers of a germline mutation in one of these genes are at increased risk of developing colorectal, endometrial, and ovarian cancer, transitional cell carcinoma of the ureters and renal pelvis, gastric and small bowel cancer, brain tumors, and sebaceous skin cancer (Lynch, 1999). Clinically, HNPCC is defined by the Amsterdam criteria: at least three cases of pathologically verified colorectal, endometrial, transitional cell, or small bowel cancer should be present within two generations, one of which was diagnosed under the age of 50. Moreover, one patient must be a first-degree relative of the others and familial adenomatous polyposis (FAP) has to be excluded (Vasen et al., 1999).

In the vast majority of HNPCC families, germline mutations are found in *MSH2* and *MLH1* (Peltomaki and Vasen, 1997). These mutations are predominantly single nucleotide substitutions or small insertions or deletions. However, larger genomic deletions have also been recognized as a frequent cause of HNPCC (Wijnen et al., 1998a). Here, we describe the first HNPCC family caused by an inversion within the short arm of chromosome 2 disrupting the *MSH2* gene.

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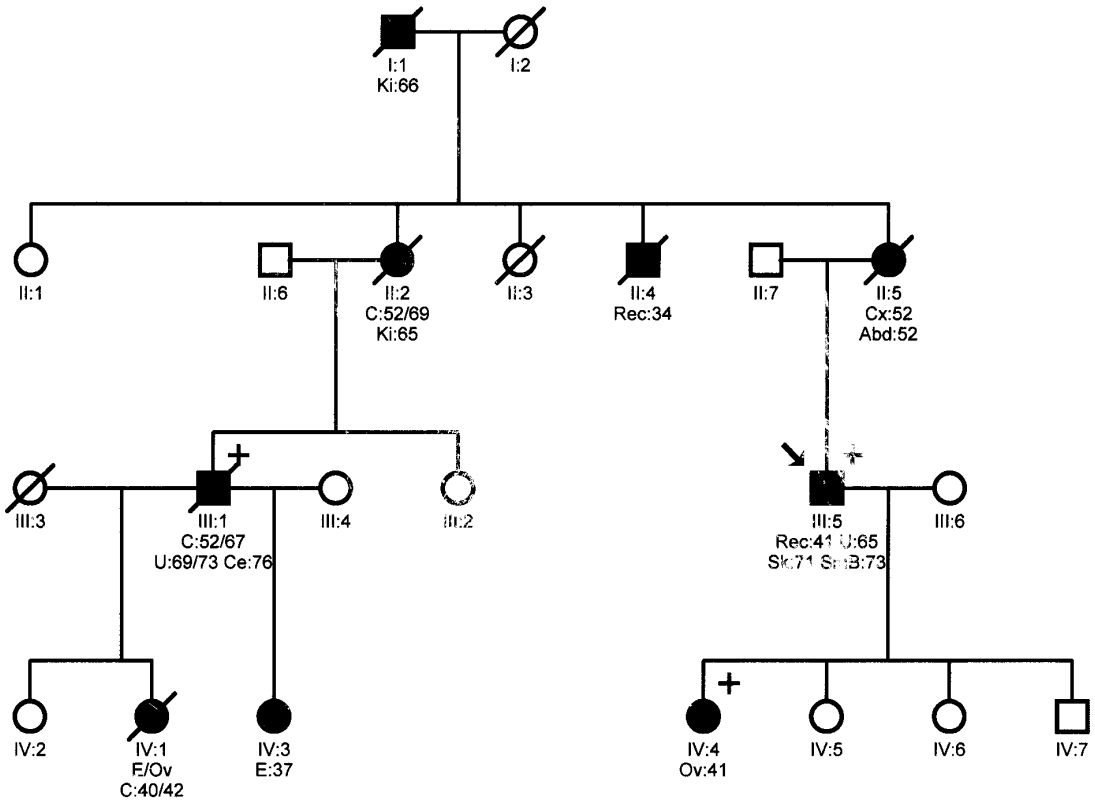

Figure 1. Pedigree of the North-American HNPCC family. Symbols: +, carrier of the paracentric inversion; C, colorectal cancer; Rec, rectal cancer; Ce, cecal cancer; E, endometrial cancer; Ov, ovarian cancer; U, urinary tract cancer; Ki, kidney cancer; SmB, small bowel cancer; Sk, skin cancer; Cx, cervical cancer; Abd, abdominal cancer. The index patient, III:5, is indicated with an arrow.

MATERIALS AND METHODS

The Family

The family described herein is Caucasian and is part of the cohort of North-American HNPCC families collected by Dr. Henry Lynch. The kindred fully comply with the clinical Amsterdam criteria for HNPCC (Fig. 1). The index patient is a 77-year-old male (III:5), who had a malignant rectal polyp at age 41, a transitional cell carcinoma of the left kidney at 65, and a small bowel carcinoma at 73. At the age of 71, he was also diagnosed with several sebaceous adenomas and squamous cell carcinomas. One of the index's three daughters developed an endometroid carcinoma of the ovary at age 41 (IV:4). His mother was diagnosed with cervical cancer and died of an abdominal carcinoma at 52 years of age (II:5). Her brother had rectal cancer at 34 and died at 36 (II:4). One of her sisters developed two colon adenocarcinomas at 52 and 69 years, respectively, and a kidney adenocarcinoma at 65 (II:2). She died at 72 years of age. Her son presented with adenocarcinomas of the colon at age 52 and 67, respectively (III:1). Furthermore, he was diagnosed with transitional cell carcinoma of the right ureter and bladder at 69 and 73 years. At the age of 76, he developed an adenocarcinoma of the cecum and died of cardiac disease at 77. Two of his daughters (from different marriages) also presented with HNPCC-related tumors (IV:1 and IV:3). There was no evidence of miscarriages in this family.

Denaturing Gradient Gel Electrophoresis (DGGE)

Mutation analysis of the *MSH2*, *MLH1*, and *MSH6* genes was performed by DGGE as previously described (Wijnen et al., 1998b, 1999).

Southern Blot Analysis

DNA was extracted from peripheral blood lymphocytes. Conforming to our previously established protocol (Wijnen et al., 1998a), Southern blot analysis of

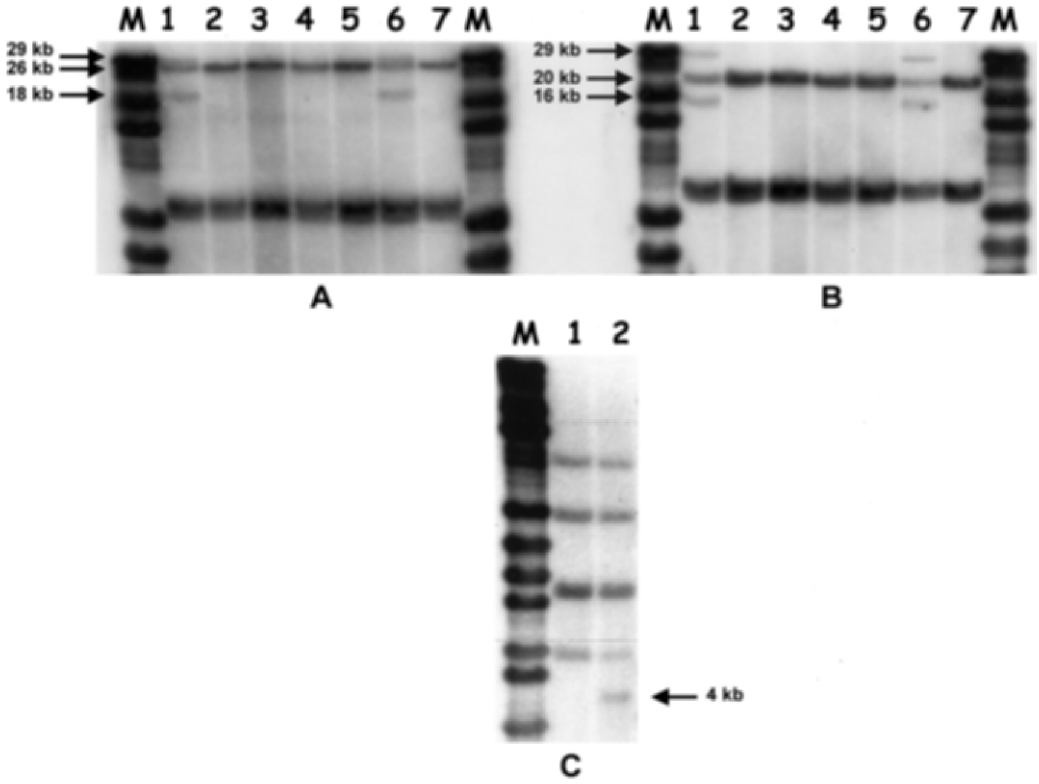

Figure 2. Southern blot analysis of the index patient and other affected and nonaffected relatives from the HNPCC family. *Xba*I (**A**) and *Bsp*I (**B**) digests hybridized with the 3a probe (encompassing *MSH2* exons 7–12). Lane 1: index patient (III:5); lanes 2–5: unaffected 50% risk carriers; lane 6: affected relative (III:1); lane 7: normal control. M indicates the molecular weight markers. The molecular weight of the specific *MSH2* bands is indicated. *Hin*cII (**C**) digests from a normal control (lane 1) and from individual III:5 (lane 2) were hybridized with the 3a probe.

MSH2 was performed with *Xba*I, *Eco*RI, *Hin*dIII, and *Nsi*I genomic DNA digests followed by hybridization with three *MSH2* probes: probe 5, encompassing exons 1–7; probe 3a, for exons 7–12; and probe 3b, for exons 10–16 (see also Fig. 2). In addition, digestion with the restriction endonucleases *Bsp* 1407I, *Hin*cII, and *Bcl*I was performed. The latter filters were hybridized with several exon-specific *MSH2* probes, including exons 6 to 9. *MLH1* and *MSH6* Southern analysis was performed with *Xba*I, *Apa*I, *Hin*dIII, and *Nsi*I genomic DNA digests followed by hybridization with two *MLH1* probes (encompassing exons 1–12 and exons 11–19) and two *MSH6* probes (encompassing exons 1–4 and exons 5–10) (Wijnen et al., 1998a).

Cytogenetic and Fluorescence In Situ Hybridization Analyses

Prometaphase and metaphase chromosomes were obtained from EBV-transformed lymphoblastic cells according to standard protocols (Dauwerse et al., 1992). Karyotyping was performed using GTG-banding (Seabright, 1971) and multicolor fluorescence in situ hybridization (MFISH). For MFISH, the COBRA (for COmbined Binary RAtio) labeling technique with p/q arm distinction was employed as previously described (Tanke et al., 1999; Wiegant et al., 2000).

Inverse Polymerase Chain Reaction (IPCR)

IPCR was performed as previously described (Fodde et al., 1990) (Fig. 3). A 5-μg sample of genomic DNA extracted from lympoblastoid cells was digested with *Hin*cII. The digested DNA was extracted by phenol-chloroform, ethanol-precipitated, diluted to a concentration of 1 μg/ml in ligation buffer (Fermentas, St. Leon-Rot, Germany), and ligated overnight at 16°C with 1 U/μl ligase (Fermentas).

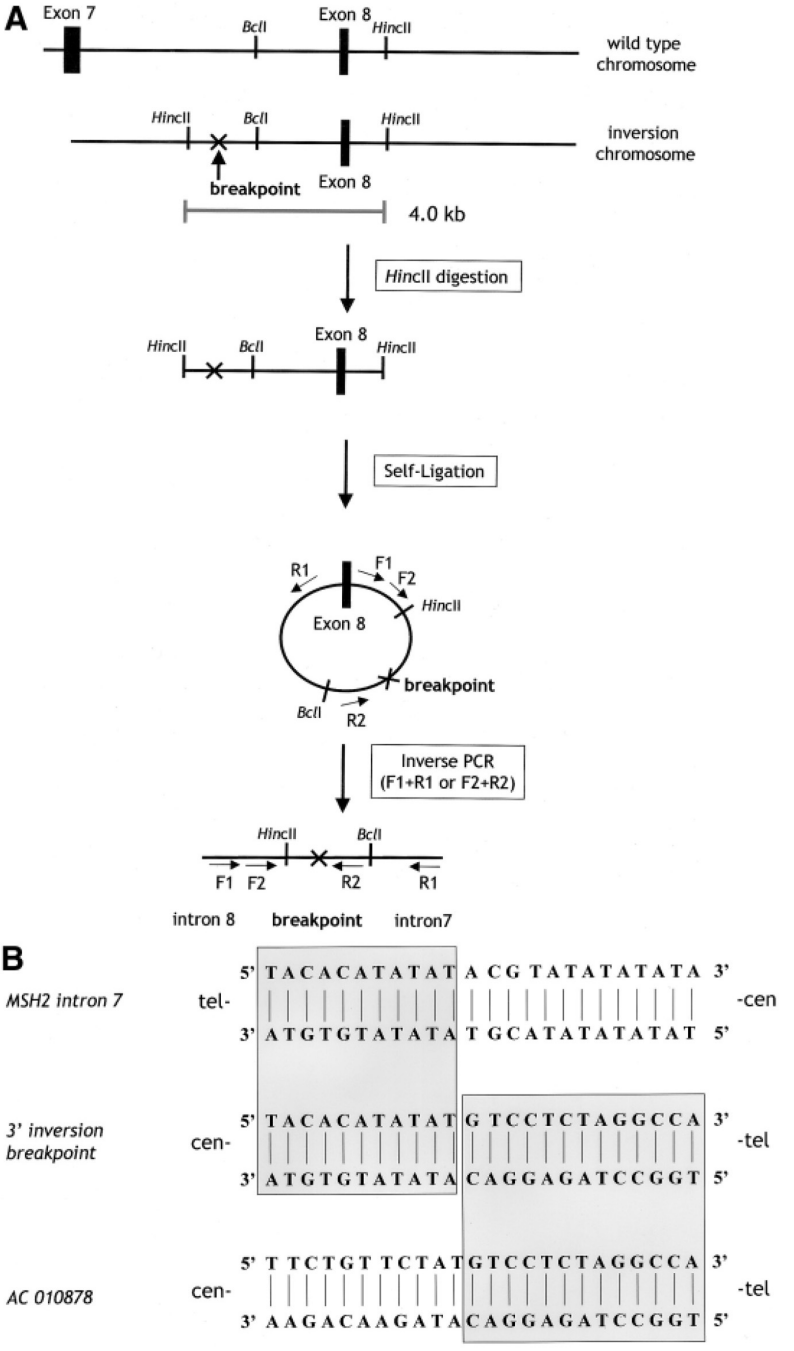

Figure 3. Cloning and characterization of the 3′ (telomeric) inversion breakpoint by inverse-PCR. **A:** The inverse-PCR procedure. Genomic DNA from the index patient (III:5) was digested by *Hin*cII. The latter result in an aberrant 4.0-kb fragment encompassing *MSH2* exon 8 and the 3′ breakpoint as indicated. The digested DNA was ligated as described under Materials and Methods. The ligated DNA was employed in a PCR reaction with primer sets F1/R1 and F2/R2, indicated by arrows. In both cases, only DNA from carriers of the paracentric inversion resulted in the expected inverse-PCR products. The latter encompass the 5′ end of *MSH2* intron 8, the original *Hin*cII site, the inversion breakpoint, and the 3′ end of *MSH2* intron 7. **B:** Both the sense and antisense strands of the sequences around the 3′ (telomeric) inversion breakpoint are depicted. The alignment shows (from top to bottom) the wild-type *MSH2* intron 7 sequence, the 3′ (telomeric) breakpoint obtained by inverse PCR, and the sequence of the contig AC010878, located 10 Mb 3′ (telomeric) to *MSH2*. The shaded boxes indicate the homologies among the three aligned sequences. The orientations of both the centromere and telomere relative to the reported sequences are indicated. The sequence of *MSH2* intron 7 is depicted in the reverse direction relative to the other two sequences.

A forward primer (F1) was designed at the 3′ end of exon 8 (5′-AGAAACAACTTTAGATATGG-3′), whereas the reverse primer (R1) was developed 3′ of the *Bcl*I site at position 13,392 within intron 7 (5′-AGACTTTGTAAAGCTACATTC-3′) (Fig. 3). High-fidelity PCR (Roche Molecular Diagnostic Systems, Alameda, CA) was performed with these primers. The IPCR product was analyzed by agarose gel electrophoresis. An approximately 2.0-kb band was observed in the index DNA but

not in the normal control. This band was excised and purified using a nucleotrap kit (Machery-Nagel, Düren, Germany). The purified product was cloned by TOPO-cloning (Invitrogen, La Jolla, CA) and sequenced using the same F1 and R1 primers. Based on the newly obtained sequences, new primers were designed, that is, a forward primer (F2) in intron 8 (5′-TCAAGCTATCCTCCGGCCTC-3′) and a reverse primer (R2) in intron 7 (5′-GGATGCAGTGAACAGGGAAC-3′) (Fig. 3). The IPCR procedure was then repeated with F2 and R2, and the patient-specific PCR product was sequenced.

This sequence also enabled us to develop additional primers around the 5′ (centromeric) breakpoint (Fig. 4): a forward primer (F3, 5′-TGCTGACATGGCCCATTCCTAC-3′) within the contig AC010878, and a reverse primer (R3, 5′-TCCCAGTTTAACTCTAGCAG-3′) in intron 7. PCR amplification with these primers resulted in a fragment of approximately 900 bp in the index patient (III:5) and in two other affected individuals (III:1 and IV:4) but not in a normal control (see also Fig. 4C). This fragment was again excised and purified by the nucleotrap procedure and sequenced using the PCR forward and reverse primers.

Monoallelic Expression Analysis

To allow RNA and protein expression analysis of the *MSH2* allele carrying the inversion, somatic cell hybrids containing the wild type and rearranged chromosome 2p from the index patient were generated in a *Msh2*-deficient murine background (GMP Conversion Technologies, Waltham, MA) (Papadopoulos et al., 1995; Yan et al., 2000). Somatic cell hybrids were genotyped by CA repeat markers around the *MSH2* gene on chromosome arm 2p and selected for monoallelic expression analysis. Total RNA and protein samples were isolated from the different hybrid cell lines, and northern and western blot analyses were carried out according to conventional procedures. Probe 3b (*MSH2* exons 10–16) was employed for the northern analysis, whereas a rabbit polyclonal antibody raised against amino acids 402–737 of human MSH2 was used for the western analysis (Smits et al., 2000).

RESULTS AND DISCUSSION

Southern Analysis

DGGE analysis of *MSH2, MLH1,* and *MSH6* did not reveal any mutation in the index patient. Therefore, Southern blot analysis was performed. *MLH1* and *MSH6* hybridizations did not show any aberrant fragments. After hybridization with the *MSH2* 5′ probe (encompassing exons 1–7), an aberrant band of approximately 30 kb was seen in the *Xba*I digest (not shown). Hybridization with the *MSH2* 3a probe (exons 7–12) revealed two aberrant fragments of 29 and 18 kb, respectively (Fig. 2A). Based on the decreased intensity of the wild-type 26-kb fragment encompassing *MSH2* exons 7 and 8, we assumed that the genomic rearrangement involved this segment of the gene. Hybridization with exon 7- and exon 8-specific probes indicated that the 29-kb band contained exon 7, whereas the 18-kb fragment encompassed exon 8 of *MSH2.* This was confirmed by digestion with *Bsp*1407I: in addition to the wild type 20-kb fragment containing exons 7 and 8, hybridization with both the 3′a-specific and the exon 7- and exon 8-specific probes revealed two fragments of 29 and 16 kb, encompassing exon 7 and exon 8, respectively (Fig. 2B). These results are indicative of a complex rearrangement encompassing both exon 7 and exon 8 in the mutated allele. The normal intensity of all other digestion fragments suggested that no other *MSH2* exon was involved in the rearrangement (data not shown). This also indicated that the genomic rearrangement was likely to be a translocation or inversion with a breakpoint in intron 7. The aberrant restriction site closest to the breakpoint appeared to be a *Hin*cII site at position 11,738 in intron 7 (Fig. 2C). *Bcl*I digestion showed no aberrant fragment after hybridization with an exon 8-specific probe, indicating that the breakpoint had to map 5′ to the *Bcl*I site located at position 13,392 within intron 7. Given that no other aberrant fragments were found after hybridization with any of the other *MSH2* probes, the other breakpoint had to map outside the *MSH2* gene.

Additional Southern blot analysis of individual III1, IV4, and several unaffected relatives showed co-segregation of the rearrangement with the disease in this family (not shown).

Cytogenetic and FISH Analyses

Karyotype analysis of the index patient by GTG-banding and PQ-COBRA-FISH did not show either any apparent translocation or an inversion concerning chromosome 2. This indicated that the alleged genomic rearrangement had to be either a small inter- or intrachromosomal translocation, or a paracentric inversion (data not shown).

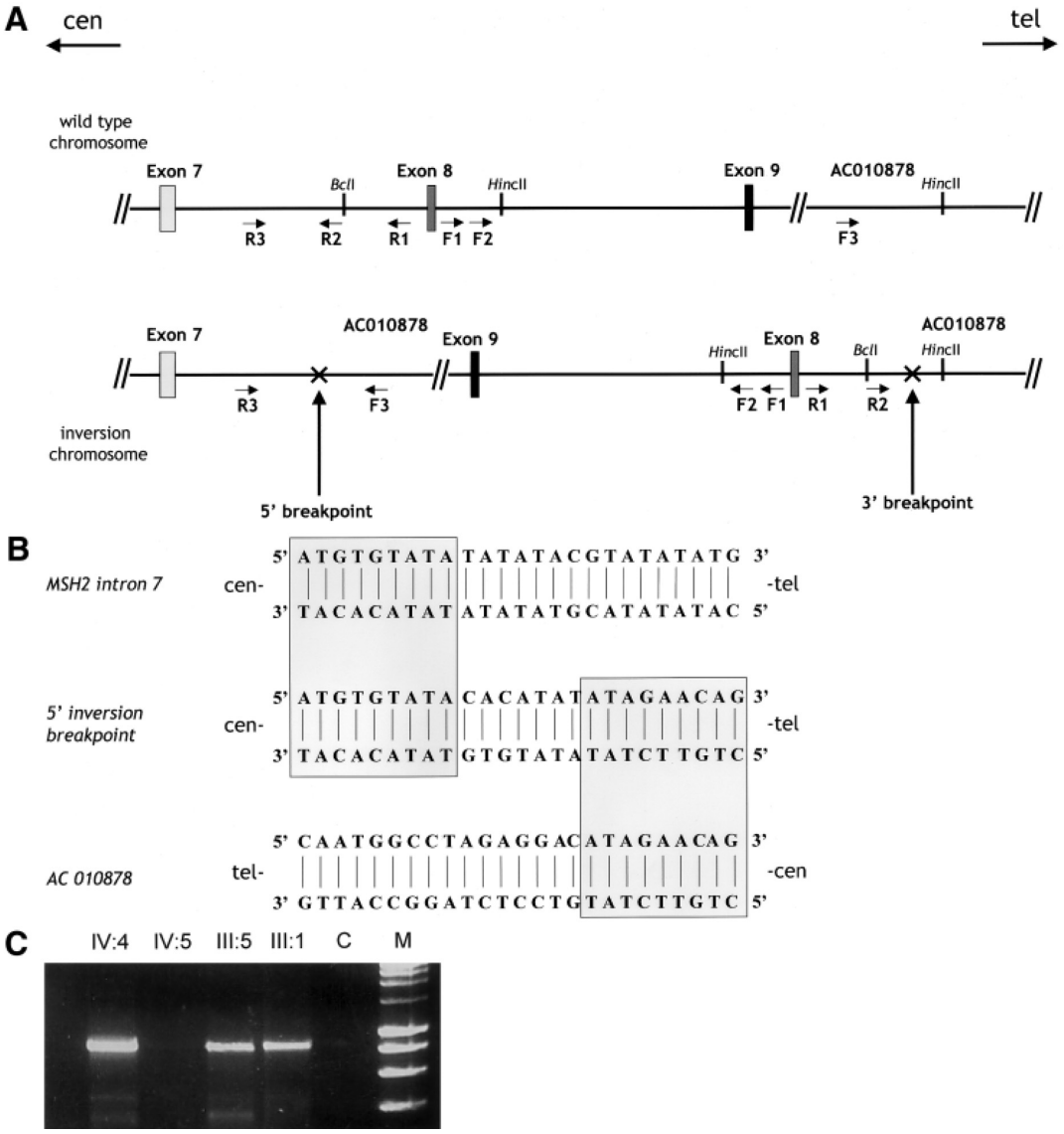

Figure 4. Cloning and characterization of the 5′ (centromeric) inversion breakpoint. **A:** Scheme of the wild-type and mutated *MSH2* alleles. Only the part of the chromosome 2 region involved in the paracentric inversion is shown. Shaded and black boxes indicate exons 7, 8, and 9 of the *MSH2* gene, respectively. Only the *Hin*cII and *Bcl*I restriction sites and the 5′ (centromeric) and 3′ (telomeric) breakpoints are depicted for the sake of clarity. AC010878 is the contig located 10 Mb 3′ of *MSH2*. The primer sets employed to amplify and sequence both breakpoints (F1/R1, F2/R2, F3/R3) are indicated by arrows. **B:** Both the sense and antisense strands of the sequences around the 5′ (centromeric) inversion breakpoint are depicted. The alignment shows (from top to bottom) the wild-type *MSH2* intron 7 sequence, the 5′ breakpoint obtained by PCR with primers F3 and R3, and the sequence of the contig AC010878, located 10 Mb 3′ (telomeric) to *MSH2*. The shaded boxes indicate the homologies among the three aligned sequences. The orientations of both the centromere and telomere relative to the reported sequences are indicated. The sequence of the AC010878 contig is depicted in the reverse orientation relative to the other two sequences. **C:** 5′ Breakpoint-specific PCR results obtained with primers F3 and R3 (see A) in affected (IV:4, III:5, III:1) and nonaffected (IV:5) individuals.

IPCR

To discriminate among the above possibilities, we set out to characterize the intron 7 breakpoint by IPCR (Fig. 3A). Based on the Southern blot analysis, *Hin*cII digestion results in a 4-kb fragment encompassing exon 8 and the breakpoint of the genomic rearrangement within intron 7 (Fig. 2C). The IPCR procedure enabled us to obtain an ap-

proximately 2-kb PCR product containing the 5′ end of intron 8, the *Hin*cII site used for digestion and ligation, 60 base pairs of an unknown sequence, the breakpoint, and the 3′ end of intron 7 (Fig. 3B). The IPCR product was not observed when genomic DNA from a healthy individual was subjected to the procedure. By use of the Basic Local Alignment Search Tool (BLAST) database of the National Center of Biotechnology Information, a perfect match was found between the unknown sequence and a contig (AC010878) located 10 Mb 3′ of *MSH2*. The latter finding indicated that the genomic rearrangement in this kindred is likely to be a 10-Mb inversion of exons 8 to 16 of the *MSH2* gene (Fig. 4A). To test this hypothesis, PCR primers (F3 and R3) were designed within AC010878 and in *MSH2* intron 7, to allow amplification and sequencing of the 5′ (centromeric) breakpoint of the 10-Mb inversion (Fig. 4A). The approximately 900-bp PCR product obtained using these primers encompasses sequences derived from AC010878 (in the reverse direction relative to the sequence found in the 3′ telomeric breakpoint sequence), the 5′ centromeric breakpoint, and 5′ intron 7 sequences (again, in the reverse orientation relative to the 3′ telomeric breakpoint sequence) (Fig. 4B). Moreover, the combination of these primers resulted in the 900-bp product only when DNA from the index patient or the other two available affected individuals (III:1 and IV:4 in Fig. 1) from the affected kindred were employed (Fig. 4C). This proved that the rearrangement in this family was indeed a paracentric inversion of about 10 Mb, including exons 8 to 16 of the *MSH2* gene.

Genomic rearrangements were previously described in *MSH2*, as well as in other cancer-susceptibility genes like *APC*, *MLH1*, *BRCA1*, *TP53*, *NF2*, and *STK11* (van der Luijt et al., 1995; Petrij-Bosch et al., 1997; Jenne et al., 1998; Slebos et al., 1998; Wijnen et al., 1998a; Puget et al., 1999; Legoix et al., 2000; Chan et al., 2001). These rearrangements may result from nonhomologous breakage and reunion events but also from homologous recombination between repetitive sequences dispersed throughout the genome, like Alu repeats (Nystrom-Lahti et al., 1995; Mauillon et al., 1996; Slebos et al., 1998). Also, genomic inversions have been described in hemophilia resulting from unequal alignments between small-sequence homologies (Lakich et al., 1993). Computer analysis (BLAST) of intron 7 of the *MSH2* gene revealed many repetitive sequences, among which were Alu and simple-sequence repeats (not shown). During our search for mutations in HNPCC families, we identified four genomic deletions of *MSH2* with a breakpoint within intron 7 (unpublished observations). However, because the deletion breakpoints in these families, including the one studied here, appear to be located at different sites along intron 7, no actual recombination hotspot can be pinpointed. We did not find Alu repeats at or in the flanking regions of the breakpoints of the inversion reported here. Hence, a nonhomologous breakage and reunion recombination event is the most likely cause of the genomic rearrangement observed in this family. Notably, during the recombination process, nine base pairs were lost from intron 7, whereas an additional seven base pairs were found at the 5′ (centromeric) breakpoint (Figs. 3B and 4B). The latter does not result from a PCR artifact, given that sequence analysis of the 5′ breakpoint of the affected individuals III:1 and IV:4 confirmed the presence of the 9-bp deletion and the 7-bp insertion (data not shown). To elucidate the recombination mechanism underlying the paracentric inversion, we searched the entire intron 7 and AC010878 sequences for the presence of the 7-bp insertion. Because of the presence of two dinucleotide repeats within these short sequences, several hits were obtained by BLAST in both genomic sequences. The latter does not allow us to infer the exact recombination mechanism leading to the present rearrangement.

Monoallelic Expression Analysis

To confirm the pathogenicity of the newly characterized genomic rearrangement, *MSH2* expression was analyzed by northern and western blots of somatic cell hybrids containing the wild-type and inversion chromosomes 2 from the index patient (GMP Conversion Technologies) (Papadopoulos et al., 1995; Yan et al., 2000). The murine cell line employed for this aim was *Msh2*-deficient and was therefore particularly suited to analyze expression of the human *MSH2* gene without the interference of the highly homologous and ubiquitously expressed mouse homolog. Neither *MSH2* mRNA nor MSH2 protein was detectable in the hybrid containing the rearranged chromosome arm 2p, whereas the full-length allele was observed in the hybrid containing the wild-type allele (Fig. 5). Hence, the paracentric chromosome arm 2p inversion represents a true null allele because it disrupts the *MSH2* gene and its expression.

In conclusion, we report a novel 10-Mb paracentric inversion of chromosome 2 responsible for HNPCC in a North American family. In agreement with previous reports (Wijnen et al., 1998a), this

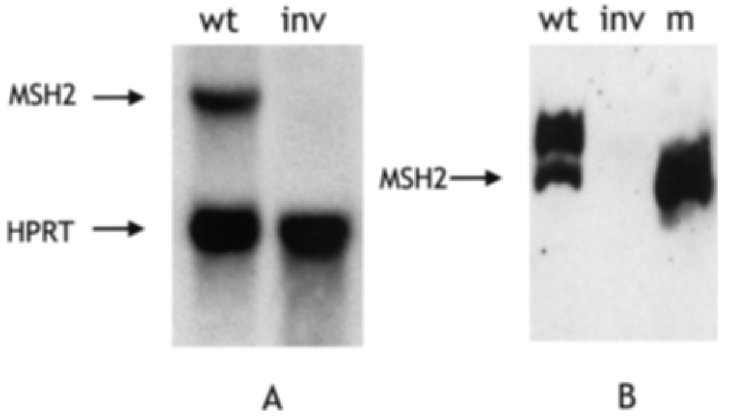

Figure 5. Northern and western analysis of somatic cell hybrids containing the wild-type and inversion chromosome 2p. Somatic cell hybrids were generated by GMP conversion technology from an EBV-lymphoblastoid cell line derived from the index patient. Individual hybrids were screened by CA repeat markers flanking the *MSH2* gene on chromosome arm 2p, to discriminate between those carrying the wild-type (wt) and inversion (inv) allele. One of each was employed for expression analysis by northern (**A**) and western (**B**) blot. Probe 3b (*MSH2* exons 10–16) was employed for the northern analysis together with an *HPRT* control probe, whereas a rabbit polyclonal antibody raised against amino acids 402–737 of human MSH2 was used for the western analysis (Smits et al., 2000). "m" indicates a mouse cellular lysate employed as control for the detection of the mouse Msh2 protein. The recipient mouse cell line employed to generate the somatic cell hybrids is $Msh2^{-/-}$. Therefore, the 100-kDa band corresponding to the human MSH2 protein is detectable only in the hybrid containing the wild-type *MSH2* allele.

study confirms that the *MSH2* gene is susceptible to genomic rearrangements such as deletions and inversions. In view of the latter, Southern blot analysis still represents a useful and informative tool to identify and characterize disease-causing complex genomic rearrangement in HNPCC families, and to facilitate presymptomatic diagnosis and patient care. Moreover, the generation of somatic cell hybrids containing the alleged mutant chromosome arm 2p allele in a *Msh2*-deficient murine cellular background greatly facilitates the analysis of its pathogenicity.

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Chapter 3.

Clinical studies in HNPCC

3.1.

Atypical HNPCC owing to *MSH6* germline mutations; analysis of a large Dutch pedigree

J Med Genet 2001:38;318-322

Short report

Atypical HNPCC owing to *MSH6* germline mutations: analysis of a large Dutch pedigree

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Abstract

Hereditary non-polyposis colorectal cancer (HNPCC) is the most common genetic susceptibility syndrome for colorectal cancer. HNPCC is most frequently caused by germline mutations in the DNA mismatch repair (MMR) genes *MSH2* and *MLH1*. Recently, mutations in another MMR gene, *MSH6* (also known as *GTBP*), have also been shown to result in HNPCC. Preliminary data indicate that the phenotype related to *MSH6* mutations may differ from the classical HNPCC caused by defects in *MSH2* and *MLH1*.

Here, we describe an extended Dutch HNPCC family not fulfilling the Amsterdam criteria II and resulting from a *MSH6* mutation. Overall, the penetrance of colorectal cancer appears to be significantly decreased ($p<0.001$) among the *MSH6* mutation carriers in this family when compared with *MSH2* and *MLH1* carriers (32% by the age of 80 *v* >80%).

Endometrial cancer is a frequent manifestation among female carriers (six out of 13 malignant tumours). Transitional cell carcinoma of the urinary tract is also relatively common in both male and female carriers (10% of the carriers).

Moreover, the mean age of onset of both colorectal cancer (*MSH6 v MSH2/MLH1* = 55 years *v* 44/41 years) and endometrial carcinomas (*MSH6 v MSH2/MLH1* = 55 years *v* 49/48 years) is delayed. As previously reported, we confirm that the pattern of microsatellite instability, in combination with immunohistochemical analysis, can predict the presence of a *MSH6* germline defect.

The detailed characterisation of the clinical phenotype of this kindred contributes to the establishment of genotype-phenotype correlations in HNPCC owing to mutations in specific mismatch repair genes.

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Keywords: hereditary non-polyposis colorectal cancer; *MSH6*

Hereditary non-polyposis colorectal cancer (HNPCC) is one of the most common autosomal dominant conditions predisposing to cancer. HNPCC carriers are at risk for the development of a broad spectrum of malignancies including tumours of the colon, endometrium, stomach, small intestine, hepatobiliary system, ureter, renal pelvis, and ovary.[1] HNPCC is caused by germline mutations in DNA mismatch repair (MMR) genes, predominantly *MSH2* on chromosome 2p16[2] and *MLH1* on chromosome 3p21.[3] In a minority of cases, mutations have been described in other MMR genes like *PMS1* and *PMS2*.[4] Recently, germline mutations in the *MSH6* gene on chromosome 2p15 were recognised as a frequent cause of atypical HNPCC, that is, not complying with the clinical "Amsterdam criteria".[5–9]

The involvement of MMR genes in HNPCC is reflected by the occurrence of instability of simple repetitive DNA sequences (microsatellite instability or MSI) in tumours from HNPCC patients. Accordingly, more than 90% of the colorectal tumours and at least 75% of the endometrial tumours from HNPCC patients display MSI.[10–12] In sporadic colorectal and endometrial cancer, MSI is found in about 15-30% of tumours.[13 14]

The first reports on *MSH6* germline mutations have indicated that the clinical phenotype of the affected families may differ from the "classical" HNPCC caused by *MSH2* and *MLH1* mutations.[5–9] The penetrance of colorectal cancer seemed to be reduced. However, endometrial cancer was likely to represent a more important clinical manifestation among female *MSH6* carriers. The reported mean age of onset of colorectal and endometrial cancer appeared to be delayed in families with *MSH6* germline mutations, 50 years and older compared to 44 (*MSH2*) and 41 (*MLH1*) years of age[15] and 53 years and older compared to 49 (*MSH2*) and 48 (*MLH1*) years, respectively.[7] Notably, MSI analysis of tumours from *MSH6* mutation carriers suggested a reduced penetrance of the MSI-H phenotype and preferential instability at mononucleotide repeats.[7–9 12]

Here, we report the clinical and molecular characterisation of a large HNPCC kindred

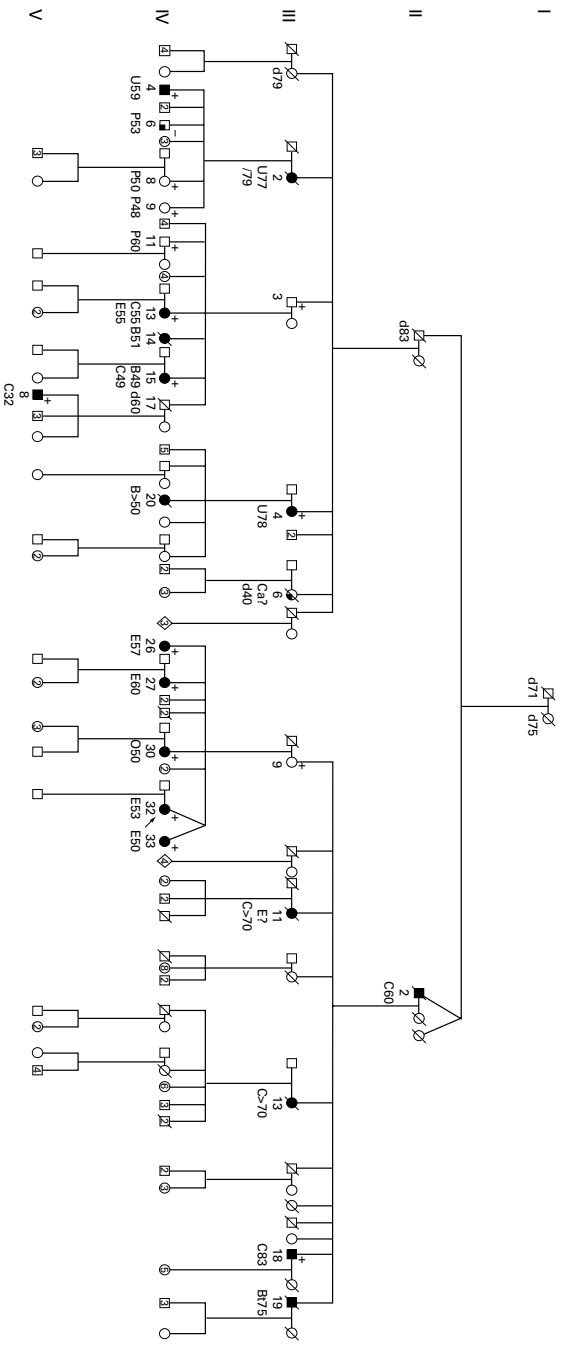

Figure 1 Pedigree of MSH6 family. C = colorectal cancer. E = endometrial cancer. U = urinary tract cancer. P = polyp. O = ovarian cancer. B = breast cancer. Bt = brain tumour. Ca? = cancer of unknown origin. + = MSH6 mutation positive. – = MSH6 mutation negative.

resulting from a frameshift mutation in the *MSH6* gene.

Patients

The proband, a 50 year old female, was referred to the Department of Clinical Genetics, Rotterdam by her gynaecologist in 1993. At that time, three of her six sisters were diagnosed with endometrial cancer at 50, 57, and 60 years of age. A fourth sister had developed ovarian cancer (endometroid adenocarcinoma) at the age of 50. The proband's father died at the age of 85 without any symptoms of HNPCC. Her mother underwent a hysterectomy for leiomyomata at 55 and was in good health at 92 years of age.

The differential diagnosis in this family included HNPCC. Mutation analysis of the *MLH1* and *MSH2* genes by PCR and DGGE in one of the affected sisters of the proband did not show any pathogenic mutation. In the meantime, the proband developed endometrial cancer at the age of 53, the fourth case of endometrial cancer in this sibship.

As soon as *MSH6* mutation analysis became available, mutation screening was performed and a frameshift mutation (del T codon 594) in exon 4 was identified.[7] The proband, her affected sisters, and mother were found to carry the same mutation.

At the request of the proband, family members were informed about the findings and were invited to contact the Department of Clinical Genetics for additional information and/or to be tested for the presence of the mutation.

We were also able to link the family to another kindred under investigation at the Department of Clinical Genetics at the Leiden University Medical Centre.

Mutation analysis

In total, DNA testing for the *MSH6* mutation was performed in 80 out of 132 living relatives with at least a 25% risk of being carrier of the *MSH6* mutation (fig 1), 27 males (out of 63, 43%) and 53 females (out of 69, 77%). Out of these 132 relatives, 11 were previously diagnosed with an HNPCC related tumour and all of them were tested. Two tested unaffected relatives were obligate carriers. Out of 75 carriers at 50% risk and 44 at 25% risk, 46 (61%) and 21 (48%) were tested, respectively (tables 1 and 2).

Ten of the 11 affected subjects were carriers of the familial *MSH6* mutation. The patient (IV.6) negative for the mutation was a 55 year old male, diagnosed with numerous tubulovillous adenomas at the age of 53. Since his clinical presentation was suggestive of familial adenomatous polyposis (FAP), though with a delayed age of onset, mutation analysis of the *APC* gene was performed. This did not show any alteration.

The *MSH6* mutation was confirmed in both unaffected obligate carriers.

Out of the 46 tested healthy subjects with a 50% risk for the mutation, 17 (37%) were found to carry the *MSH6* mutation; none of those with a 25% risk tested positive (table 2).

All 17 unaffected mutation carriers above the age of 25 years (five males and 12 females) were offered the generally accepted surveillance for HNPCC, namely colonoscopy every one to two years, yearly gynaecological examination with vaginal ultrasound and CA125 blood testing

Table 1 (Pre)malignant tumours in the extended Dutch MSH6 family

Patient	*Tumour*	*Age at diagnosis*	*MSH6 mutation*
II.2	Colorectal cancer	60	Obligate carrier
III.2	Transitional cell carcinoma right pyelum & transitional cell carcinoma left ureter	77 79	Obligate carrier
III.4	Transitional cell carcinoma	78	+
III.6	Unknown	40	Not tested
III.11	Endometrial carcinoma	?	Obligate carrier
	Colon carcinoma	>70	
III.13	Colon carcinoma	>70	Not tested
III.18	Colon carcinoma	83	+
III.19	Astrocytoma	75	Not tested
IV.4	Transitional cell carcinoma ureter	59	+
IV.6	Multiple adenomatous polyps of the colon	53	−
IV.8	Adenoma	50	+
IV.9	Adenoma	48	+
IV.11	Tubulovillous adenoma	60	+
IV.13	Endometrial & rectal carcinoma	55	+
IV.14	Breast cancer	51	Not tested
IV.15	Breast & colon carcinoma	49	+
IV.20	Breast cancer	>50	Not tested
IV.26	Endometrial carcinoma	57	+
IV.27	Endometrial carcinoma	60	+
IV.30	Endometroid adenocarcinoma ovary	50	+
IV.32	Endometrial carcinoma	53	+
IV.33	Endometrial carcinoma	50	+
V.8	Colorectal cancer	32	+

+ indicates the presence of the *MSH6* mutation.
– indicates the absence of the *MSH6* mutation.

Table 2 Results of mutation analysis

	Male			*Female*			*Total*
Pretest risk	100%*	50%	25%	100%*	50%	25%	
Tested	5	15	7	8	31	14	80
MSH6+	4	4	0	8	13	0	29
MSH6–	1	11	7	0	18	14	51
Not tested	0	19	17	0	10	6	52
Total	5	34	24	8	41	20	132

*Relatives with an HNPCC related tumour and obligate carriers.

(for female carriers), and yearly urine sediment testing and cytology. During the first screening the following diagnoses were made: a tubulovillous adenoma with dysplasia in the proximal rectum of a 60 year old male; an invasive ductal adenocarcinoma of the breast and an adenocarcinoma of the colon in a 49 year old female; a small tubular adenoma and endometrial hyperplasia in a 50 year old female; and an adenoma in a female aged 48. Of the remaining 13 carriers, four were under the age of 45 and four were above 70 years without any symptoms of the genetic predisposition. However, a 92 year old female underwent a hysterectomy because of leiomyomata of the uterus at the age of 55.

The family included five dead obligate mutation carriers, three females and two males. Three of them had been diagnosed with a HNPCC related tumour (table 1). One male reached the age of 83 without having developed any HNPCC related tumour.

We were not able to determine the *MSH6* status of five cancer patients in this family: one colorectal cancer (onset >70) in a female patient (III.13) whose 13 descendants tested negative for the mutation; one male with an astrocytoma (III.19) at the age of 75, three of whose four children did not carry the mutation; two breast cancer patients (IV.14, IV.20, onset >50); and one patient with cancer of unknown origin around the age of 40 (III.6).

In total, 34 mutation carriers (29 alive and five dead) were identified in this pedigree, 17 of whom developed a (pre)malignant tumour (table 1). Of the carriers with a malignancy, four had two primary HNPCC related tumours. Using the Kaplan-Meyer method, 7% of the carriers developed colorectal cancer by the age of 50 years and 32% by the age of 80 years (mean age of onset 55 years, ranging from 32 to 83 years). Of the female carriers, 52% developed endometrial cancer by the age of 80 and all of them were diagnosed above the age of 50 years. Three carriers had a papillary transitional cell carcinoma of the urinary tract (10% of the carriers).

Microsatellite instability (MSI) and immunohistochemical (IHC) analyses

We performed MSI analysis and/or immunohistochemistry with antibodies against MLH1, MSH2, and MSH6[12] on tumour samples derived from 12 subjects; in four cases, two independent tumour samples were tested (table 3).

Using the markers recommended by Boland *et al*,[16] MSI analysis showed variable patterns (table 3). However, all tumour samples derived from the *MSH6* carriers displayed instability of at least one mononucleotide marker when an extended set of markers, including BAT40, was used.

Immunohistochemical (IHC) analysis of the MSH6 protein in tumour sections showed no expression in any of the samples tested, with

Table 3 Microsatellite and immunohistochemical analysis in tumours from 12 affected relatives

	Subject															
	III.2		*III.4*	*III.13*	*III.18*	*IV.4*		*IV.6*	*IV.13*		*IV.15*		*IV.26*	*IV.27*	*IV.30*	*IV.32*
Tumour type	U	U	U	C	C	U	U	P	R	E	C	B	E	E	O	E
Microsatellite status	H	H	L	NT	H	H	L	S	H	L	S	H	H	L	H	S
Mononucleotide markers																
BAT25*	+	+	+	NT	+	+	−	−	+	−	−	+	+	−	+	−
BAT26*	NT	+	−	NT	+	+	+	−	+	+	−	+	+	+	+	−
BAT40	+	+	+	NT	+	+	+/−	−	+	+	+	+	NT	+	+	+
Dinucleotide markers																
D2S123*	+	NT	−	NT	−	−	NT	−	−	−	−	−	−	−	−	−
D5S346*	−	+	−	NT	+	−	−	−	+	−	−	−	−	−	−	−
D17S250*	+	NT	−	NT	+	−	−	−	+	−	−	−	−	−	−	−
Immunohistochemistry																
MLH1	NT	NT	+	++	+	+	NT	+	+	++	+	+	+	+	++	+++
MSH2	NT	NT	+	++	++	+	NT	+	−	+	+	+	++	+	+	++
MSH6	NT	NT	−	+	−	−	NT	+	−	−	−	−	−	−	NT	−

* = markers recommended by NCI for MSI analysis.[16] + = Unstable. − = Stable. NT = not tested/not conclusive. H = MSI high according to NCI recommendations.[16] L = MSI low according to NCI recommendations.[16] S = MSI stable according to NCI recommendations.[16] C = colon tumour. U = urinary tract tumour. E = endometrial tumour. O = ovarian tumour. R = rectal tumour. P = polyp. B = breast tumour.
Immunohistochemistry: +++ = strong staining, ++ = moderate staining, + = weak staining, − = no staining.

the exception of III.13 and IV.6. Patient III.13 could not be tested for either the presence of the germline mutation or for MSI, and is likely to represent a phenocopy, in view of the late age of onset (colon carcinoma at >70 years) and the failure to detect the *MSH6* mutation in her 13 descendants. We were able to perform MSI analysis in a single adenoma from patient IV.6 that did not display instability for any of the tested markers. MLH1 and MSH2 IHC analysis was positive in all samples with the exception of the rectal carcinoma from IV.13, negative for MSH2.

Discussion

Genetic heterogeneity and variable phenotypic expression represent major complications in the diagnosis and clinical management of HNPCC, one of the most common inherited predispositions to multiorgan tumorigenesis. Although the hallmark of this condition is represented by colorectal lesions, a broad range of cancer types, including tumours of the endometrium, stomach, small intestine, hepatobiliary system, and ureter, are established clinical expressions of HNPCC. The vast majority of HNPCC cases are known to result from germline mutations in the DNA mismatch repair genes *MSH2* and *MLH1*.[17] In these kindreds, high penetrance and early onset of colorectal and endometrial cancer are the major clinical features. The set of diagnostic criteria, Amsterdam criteria I and II, established by the International Collaborative Group on HNPCC[18 19] well serve the purpose of selecting families with a high likelihood of carrying *MSH2* and *MLH1* mutations.[17] More recently, atypical HNPCC families, that is, not complying with the Amsterdam criteria (ACI and II) though clearly suggestive of a HNPCC-like inherited condition, have been reported to be caused by germline mutations in a third MMR gene, namely *MSH6*.[7–9] Preliminary phenotypic analysis of these families showed a reduced occurrence of colorectal cancer and a prevalence of endometrial tumours.

Here, we have presented a detailed analysis of an extended Dutch HNPCC pedigree resulting from an inactivating germline mutation of the *MSH6* gene.

The expression of the *MSH6* mutation in this family suggests a reduced penetrance and a delayed age of onset for colorectal cancer. Using the Kaplan-Meyer method, 7% of the carriers developed colorectal cancer by the age of 50 years and 32% by the age of 80 years. This is significantly reduced ($p<0.001$) compared to "classical" HNPCC caused by *MSH2* and *MLH1* (~55% by age 50 and >80% by age 80).[15] Admittedly, these calculations are based on a limited number of cases and may represent an overestimate because not all healthy eligible relatives were tested for the *MSH6* mutation. Moreover, the selection bias introduced when studying a family with such a striking clinical history may also lead to an overestimation of the penetrance. The mean age of onset of the colorectal carcinomas in this family is 55 years. This is delayed when compared with HNPCC families caused by *MSH2* (44 years) and *MLH1* (41 years) mutations.[15] This delayed age of onset may also bias the estimated penetrance. Notably, the youngest diagnosed case was at 32 years, implying that periodic screening recommendations should not differ from those established for classical HNPCC until more data are available.

In this family, endometrial cancer is the most common tumour type (six out of 13 malignant tumours) among female carriers. All the endometrial cancers were diagnosed above the age of 50 and their mean age of onset was 55 years, that is, five to 10 years later when compared with "classical" HNPCC caused by mutations in *MLH1* and *MSH2*.[7 20]

Another striking clinical phenotype in this family is the papillary transitional cell carcinoma of the ureter and renal pelvis observed in three relatives (10% of the carriers). Notably, the lifetime cumulative risk of this tumour type in *MLH1* or *MSH2* mutation carriers is only 2.6%.[21]

As previously reported by us and others,[7–9] the MSI phenotype caused by loss of *MSH6* function is reduced when compared with *MLH1* and *MSH2*, and differs in its predominance at mononucleotide runs. This is in agreement with previous studies on yeast and mouse model systems.[22 23] From this point of view, the set of markers previously recommended by NCI for MSI analysis[16] may not be suitable for *MSH6* mutation carriers and should be complemented with a set of mononucleotide markers. In six of the cases reported here (III.4, IV.4U, IV.13E, IV.15C, IV.27, and IV.32) (table 3), instability was observed at only one or two mononucleotide repeats (one of which was not included in the NCI panel) leading to an MSI-low or stable classification. In five of these cases, IHC was performed and indicated the loss of MSH6 in the tumour. Therefore, we recommend IHC analysis in MSI-L and MSS tumours from cases with a family history suggestive of HNPCC.[12]

We also show the feasibility of the IHC approach not only on colorectal tumours but also in carcinoma of the endometrium, urinary tract, and breast. The latter finding is relevant for the inclusion of breast cancer in the HNPCC tumour spectrum: breast tumour samples from IV.15 showed both MSI-high and a negative IHC staining pattern in accordance with the presence of the *MSH6* germline mutation in this person. Two additional perimenopausal breast cancer cases were found in the present study (IV.14 and IV.20). However, as no material was available from these patients, we could not establish their *MSH6* mutation carrier status.

IHC analysis was also helpful in the assessment of the likely phenocopy status relative to III.13. As a limited amount of a colorectal carcinoma was available from this dead patient, we limited our analysis to IHC and found normal MSH6 protein expression. Moreover, all her descendants tested negative for the mutation. Another dead patient from

this family, III.19, was diagnosed with astrocytoma at the age of 75. Again, no archival material was available from this patient. However, the delayed age of onset and the fact that three of his children tested negative for the mutation, do not allow us to draw any conclusion on the relationship between the presence of the brain tumour and the *MSH6* defect.

Patient IV.6 presented with a clinical phenotype more suggestive of attenuated polyposis[24] rather than HNPCC. Accordingly, MSI analysis of the colonic polyps was negative (MSS) and normal MSH6 expression was found by IHC. Failure to detect the *MSH6* mutation in this patient confirmed that the attenuated polyposis is likely to result from an unlinked genetic predisposition.

In conclusion, the clinical features associated with *MSH6* germline mutations strongly support the inclusion of endometrial cancer and transitional cell cancer of the ureter and renal pelvis in the diagnostic criteria for HNPCC in the new Amsterdam Criteria (ACII) as formulated by the ICG-HNPCC.[19] In addition, we propose to perform MSI in combination with IHC in all Amsterdam negative families with a clustering of endometrial or urinary tract cancers. The combined MSI and IHC analysis will direct subsequent mutation analysis for *MSH6*.

We thank Dr J J P M Pieters, gynaecologist, for referring the family to the Department of Clinical Genetics, Rotterdam.

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3.2.

Cancer Risk in Hereditary Nonpolyposis Colorectal Cancer Due to *MSH6* Mutations; Impact on Counselling and Surveillance

Gastroenterology 2004:127;17-25

CLINICAL–ALIMENTARY TRACT

Cancer Risk in Hereditary Nonpolyposis Colorectal Cancer Due to *MSH6* Mutations: Impact on Counseling and Surveillance

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See editorial on page 334.

Background & Aims: Hereditary nonpolyposis colorectal carcinoma (HNPCC) is caused by a mutated mismatch repair (MMR) gene. The aim of our study was to determine the cumulative risk of developing cancer in a large series of *MSH6* mutation carriers. *Methods:* Mutation analysis was performed in 20 families with a germline mutation in *MSH6*. We compared the cancer risks between *MSH6* and *MLH1/MSH2* mutation carriers. Microsatellite instability (MSI) analysis and immunohistochemistry (IHC) were performed in the available tumors. *Results:* A total of 146 *MSH6* mutation carriers were identified. In these carriers, the cumulative risk for colorectal carcinoma was 69% for men, 30% for women, and 71% for endometrial carcinoma at 70 years of age. The risk for all HNPCC-related tumors was significantly lower in *MSH6* than in *MLH1* or *MSH2* mutation carriers ($P = 0.002$). In female *MSH6* mutation carriers, the risk for colorectal cancer was significantly lower ($P = 0.0049$) and the risk for endometrial cancer significantly higher ($P = 0.02$) than in *MLH1* and *MSH2* mutation carriers. In male carriers, the risk for colorectal cancer was lower in *MSH6* mutation carriers, but the difference was not significant ($P = 0.0854$). MSI analysis in colorectal tumors had a sensitivity of 86% in predicting a MMR defect. IHC in all tumors had a sensitivity of 90% in predicting a mutation in *MSH6*. *Conclusions:* We recommend starting colonoscopic surveillance in female *MSH6* mutation carriers from age 30 years. Prophylactic hysterectomy might be considered in carriers older than 50 years. MSI and IHC analysis are sensitive tools to identify families eligible for *MSH6* mutation analysis.

Colorectal carcinoma is the second most common cause of death due to malignancy in the western world. The cause of colorectal carcinoma is multifactorial, involving both hereditary and environmental factors.[1] A family history of colorectal carcinoma is a clinically significant risk factor and may be found in up to 15% of all patients with colorectal carcinoma.[2] The most common hereditary colorectal carcinoma syndrome is hereditary nonpolyposis colorectal carcinoma (HNPCC), which accounts for 1%–6% of all cases of colorectal carcinoma.[3] HNPCC is an autosomal dominant inherited disorder characterized by the development of colorectal carcinoma, endometrial carcinoma, and various other cancers at an early age. The Amsterdam (I and II) and Bethesda criteria are clinical criteria that can be used to identify families with HNPCC.[4–6] In HNPCC, germline mutations have been found in 4 mismatch repair (MMR) genes: *MSH2*,[7] *MLH1*,[8] *PMS2*,[9] and *MSH6*.[10,11] In 50%–85% of the families fulfilling the Amsterdam criteria, a germline mutation is detected in *MLH1* or *MSH2*.[12–14] The

†Deceased.

Abbreviations used in this paper: HNPCC, hereditary nonpolyposis colorectal carcinoma; IHC, immunohistochemistry; MMR, mismatch repair; MSI, microsatellite instability.

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cumulative lifetime risk of developing any cancer is 85%–90% in carriers of a mutation in *MLH1* or *MSH2*.[15]

The hallmark of HNPCC is microsatellite instability (MSI) in tumor tissue,[16–18] which is caused by a failure of the DNA MMR.[19] MSI is reported in 85%–92% of colorectal carcinomas and in at least 75% of endometrial carcinomas associated with HNPCC, while it occurs in 10%–15% of sporadic colorectal carcinomas[16] and in 17% of sporadic endometrial carcinomas.[20–25] MSI analysis can be used as a prescreening tool to identify families eligible for mutation analysis of the MMR genes. Previous studies have shown that colorectal carcinomas and especially endometrial carcinomas in *MSH6* mutation carriers demonstrate an MSI-high phenotype less frequently using the 5 standard markers.[26–28] Another tool for selecting families for genetic testing is immunohistochemistry (IHC) analysis with monoclonal antibodies directed against the MLH1, MSH2, and MSH6 proteins.[29,30]

In 1997, Miyaki et al.[10] and Akiyama et al.[11] described 2 families with a truncating germline *MSH6* mutation. Neither of the 2 families fulfilled the Amsterdam I criteria. The family reported by Miyaki et al. was characterized by a high age at onset of cancer and a predominance of endometrial carcinoma. In 1999, Wijnen et al.[26] described 10 kindred with 9 different truncating germline *MSH6* mutations. Most of these families did not fulfill the Amsterdam (I and II) criteria and were characterized by a predominance of endometrial carcinoma and a higher age at diagnosis of cancer compared with families with an *MLH1* or *MSH2* mutation. After this publication, more *MSH6* truncating germline mutations have been reported.[27,28,31–34]

The aims of this study were to (1) evaluate the clinical phenotype of a large series of families with an *MSH6* mutation, (2) evaluate the value of MSI and IHC analysis in the identification of such families, and (3) discuss the appropriate surveillance protocol for *MSH6* mutation carriers.

Patients and Methods

Patients

A total of 20 families with a truncating germline mutation in the *MSH6* gene were included in the study. These families originated from 2 sources. The first is a group of 214 families, tested negatively for pathogenic mutations in *MLH1* or *MSH2,* collected for scientific purposes through The Netherlands Foundation for the Detection of Hereditary Tumours and departments of clinical genetics in The Netherlands and Norway. Most of the families collected by The Netherlands Foundation for the Detection of Hereditary Tumours were selected using the Amsterdam criteria. The families collected by departments of clinical genetics in The Netherlands and Norway were selected on the basis of familial clustering of colorectal carcinoma. The group consists of 71 Amsterdam I–positive and 143 Amsterdam I–negative families. Nine different truncating *MSH6* germline mutations were identified in 10 families, as reported previously by our group.[26,34] Eight of the 10 families agreed to participate in this study.

The second group is composed of 12 families recruited through the departments of clinical genetics of the University Medical Centers of Leiden, Rotterdam, and Amsterdam (VU University Medical Center), The Netherlands, and at the Institute of Medical Genetics of the University Medical Center in Rome, Italy. Most of the referred families do not fulfill the Amsterdam II criteria. The families were referred for genetic analysis because of a positive family history of (colorectal) cancer. Only families with a protein truncating germline mutation in the *MSH6* gene were included in this study.

In the total group of 20 families, 17 different truncating mutations were identified (Table 1). Only 6 families fulfill the Amsterdam II criteria (Table 1).

We collected clinical information, including the age at diagnosis of cancer, site of the tumor, and pathology reports for as many affected individuals as possible. In addition, we collected the results of colonoscopic and gynecologic screening of the high-risk unaffected relatives. Genetic counseling and testing were offered to all relevant relatives. MSI and IHC analyses were performed on all available tumors.

Mutation Analysis

Mutation analysis of the *MSH6* gene was performed by denaturating gradient gel electrophoresis[35] followed by sequence analysis if a variant was identified. A mutation was considered pathogenic when the nucleotide change is predicting truncation of the protein (e.g., nonsense and frameshift mutations) or when it is changing a consensus splice donor or acceptor site, confirmed by testing the mutation in splice site prediction software (Neural Network Splice Site Prediction [http://www.fruitfly.org/seq_tools/splice.html] or CBS NetGene 2 [http://www.cbs.dtu.dk/services/NetGene2]).

Statistical Analysis

Penetrance for age was calculated using the Kaplan–Meier survival analysis method with the SPSS statistical package. Only proven carriers and only cases of cancer that were confirmed by medical records and/or pathology reports were included in the analysis. If more than one tumor developed in the same organ, only the first one diagnosed was included in the analysis. For the analysis of the cumulative risk of all HNPCC-related tumors together, only the first diagnosis was included in the analysis. For the analyses of the cumulative risk of colorectal and endometrial carcinomas, all first diagnoses in the respective organs were included. The observation time was from birth until date of diagnosis of cancer, death, or the end of the study in June 2002. No individuals were lost to follow-up.

Table 1. *MSH6* Mutations and Family Characteristics

Family	Mutation	Amsterdam II criteria	Confirmed tumors with age of diagnosis in **proven carriers** and individuals with unknown mutation status
1[a,b]	1784delT, L594fsX, exon 4	−	**Py77 + Py79, Py76, C84, Py59, C55 + E55, C49 + B49, E57, E60, O50, E53, E50, C50 + O51, C32,** C74
2[a]	467C→G, S156X, exon 3	+	**C67, C45, C47**
3[a]	742C→T, R248X, exon 4	+	**C61, C58 + C59, C59, C26**
4[a]	2191C→T, Q731X, exon 4	−	**C48, C49,** C51
5	2731C→T, R911X, exon 4	−	**C56 + E56 + C57 + C70 + Py69 + Lu70, C59**
6	3103C→T, R1035X, exon 4	−	**O49 + E49,** Ut58
7[a]	467C→G, S156X exon 3	+	**C62 + Py73, C44,** O78
8	1267delT, C426fsX, exon 4	−	**E62 + C65, C63, C56, C62,** E54 + C87, C85
9[a,c]	4001G→A, R1334Q, splice donor defect	−	**C69, E57, C45 + E53 + C66, C64, E50**
10	1784delT, L594fsX, exon 4	−	**E58, E60, E53**
11[d]	2984delA, 996fsX, exon 4	−	**C48 + C67, C54,** C54
12[d]	1960–1961insGTGA, fsX, exon 4	+	**C37 + St56,** C61, C51
13[a]	3261delC, P1087fsX, exon 5	−	**B78, C54 + E56, E51 + St73, E57, E49**
14[a]	IVS7-2A→C, 3647-2A→C, splice acceptor defect	+	**E58, E50, E56,** C50, E54
15	3182delT, 1061fsX exon 5	−	**E50,** C48
16	3987–3988insGTCA, S1329fsX, exon 9	−	**E43, E50**
17	1444C→T, R482X, exon 4	+	**E53 + C78 + Bl80 + Py82, C49, E49**
18	1614–1615delTCinsG, Y538X, exon 4	−	**E65 + C81, E55, O45**
19	651–652insT, K218X, exon 4	−	**E57, C52**
20	651–652insT, K218X, exon 4	−	**C61, C41**

NOTE. Boldface indicates proven carriers.
Py, transitional cell carcinoma of the renal pyelum; C, colorectal carcinoma; E, endometrial carcinoma; B, breast carcinoma; O, ovarian carcinoma; Lu, lung cancer; St, stomach cancer; Bl, bladder carcinoma.
[a]Previously published.[26]
[b]Previously published.[34]
[c]Previously published.[26,48,49]
[d]Previously published.[50]

A Kaplan–Meier analysis was also performed in 30 families with an *MLH1* mutation and 37 families with an *MSH2* mutation, previously described by Vasen et al. in 2001,[15] in which the same detailed data were available. To evaluate whether the cancer risk differed between the 3 groups of mutation carriers, we used the Wald test criterion of the Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

MSI Analysis

MSI analysis was performed on paired tumor DNA and DNA from normal tissue using the Bethesda panel of microsatellite markers D2S123, D5S346, D17S250, BAT25, and BAT26 [19] with the additional BAT40 marker.[36] Tumors were regarded as MSI high if at least 30% of the markers showed instability, MSI low if <30% showed instability, or microsatellite stable if none of the markers showed instability.

IHC

IHC staining was performed on 4-μm sections of formalin-fixed, paraffin-embedded tissues. Slides were stained with antibodies against MLH1 (clone 14; Calbiochem, Cambridge, MA), MSH2 (clone GB12; Calbiochem), and MSH6 (clone 44; Transduction Laboratories/Becton Dickinson, Lexington, KY) in a Dako Techmate 500+ automated tissue stainer using standard protocols[36] and procedures as indicated by the manufacturer. Staining patterns of MMR proteins were evaluated using normal epithelial, stromal, or inflammatory cells or the centers of lymphoid follicles as internal controls. Stained slides were scored as either positive (showing nuclear staining in at least some tumor cells) or negative.

Results

Mutation Analysis

Mutation analysis was performed in 240 individuals (95 men and 145 women). Of the individuals tested, 55 were affected, 150 were first-degree relatives, and 35 were second-degree relatives. A mutation was identified in 119 individuals. Twenty-seven individuals were obligate carriers (13 affected and 14 not affected), based on the results of mutation analyses in their family members, and were not tested. Therefore, a total of 146 carriers were identified.

Of the 55 affected individuals who have been tested, 4 were proven not to be carriers of the *MSH6* mutation segregating in their respective families and are thus considered phenocopies. Two of these individuals developed colorectal carcinoma at 46 and 75 years of age, respectively, one woman was diagnosed with endometrial carcinoma at 45 years of age, and another women devel-

Table 2. Mean Percentage Cancer Risks at Age 30, 50, and 70 Years for Carriers of a Mutation in *MLH1, MSH2,* or *MSH6*

Gene	Age (*yr*) (95% confidence intervals)		
	30	50	70
All HNPCC-related tumours			
MLH1	4.2 (1.6-6.8)	34 (27-41)	76 (62-85)
MSH2	1.1 (0-2.3)	50 (42-57)	80 (70-86)
MSH6	0.7 (0-2.1)	22 (13-29)	73 (60-82)
Colorectal carcinoma in men			
MLH1	4.1 (0.1-7.9)	31 (19-41)	65 (39-80)
MSH2	2.0 (0-4.4)	39 (28-48)	63 (49-73)
MSH6	1.7 (0-5.0)	17 (4.4-28)	69 (42-83)
Colorectal carcinoma in women			
MLH1	4.3 (0.9-7.7)	26 (17-34)	53 (33-66)
MSH2	0	30 (18-40)	68 (43-82)
MSH6	0	10 (2.4-17)	30 (12-44)
Endometrial carcinoma			
MLH1	0	7.2 (1.4-13)	27 (14-38)
MSH2	0	23 (12-32)	40 (21-54)
MSH6	0	13 (5.3-22)	71 (50-83)

oped colorectal carcinoma at 71 years of age (and breast cancer at 50 years of age).

Statistical Analysis

The 146 proven carriers of a pathogenic *MSH6* mutation (59 men and 87 women) were included in the Kaplan–Meier analysis. Sixty-four affected carriers were identified (22 men and 42 women).

Table 2 shows the mean risks of cancer (percentages) for all HNPCC-related tumors, for colorectal carcinoma in men and women separately, and for endometrial carcinoma as well as the 95% confidence intervals for the ages of 30, 50, and 70 years for *MLH1, MSH2,* and *MSH6* carriers. The respective cumulative risk curves are shown in Figures 1–4. For all HNPCC-related tumors, the cumulative risks in *MSH6* carriers, men and women together, differed statistically significantly from the risk of *MLH1* and *MSH2* ($P = 0.002$) (Figure 1). This is because of the higher mean age at onset. However, the cumulative risks at 70 years of age were similar for the 3 genes.

In Figure 2, the age-related cumulative risk for colorectal carcinoma is shown for men only for *MLH1, MSH2,* and *MSH6*. The risks were lower in *MSH6* mutation carriers, but the difference was not significantly different ($P = 0.0854$). The mean age at diagnosis for colorectal carcinoma in male *MSH6* mutation carriers was 55 years (n = 21; range, 26–84 years) versus 43 and 44 years in *MLH1* and *MSH2* mutation carriers, respectively.

In Figure 3, the age-related cumulative risk for colorectal carcinoma is shown for women only for *MLH1, MSH2,* and *MSH6*. The age-related cumulative risk was significantly lower in *MSH6* mutation carriers ($P = 0.0049$). The mean age at diagnosis for colorectal carcinoma in female *MSH6* mutation carriers was 57 years (n = 15; range, 41–81 years) versus 43 and 44 years in *MLH1* and *MSH2* mutation carriers, respectively.

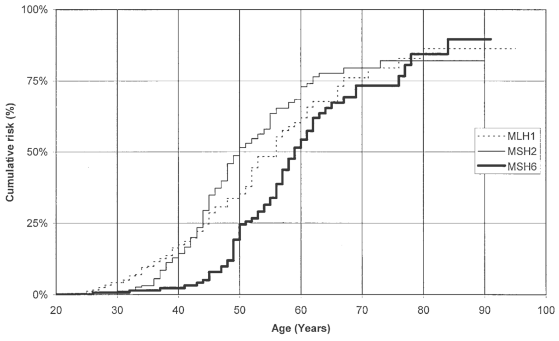

Figure 1. All HNPCC-related tumors; cumulative risks for *MLH1, MSH2,* and *MSH6* mutation carriers.

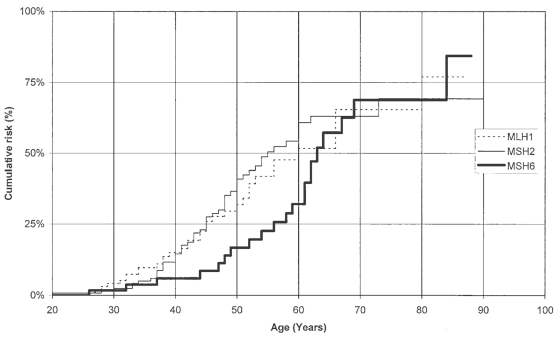

Figure 2. Colorectal carcinoma in men; cumulative risks for *MLH1, MSH2,* and *MSH6* mutation carriers.

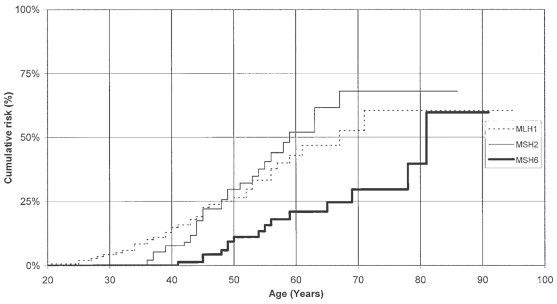

Figure 3. Colorectal carcinoma in women; cumulative risks for *MLH1, MSH2,* and *MSH6* mutation carriers.

Of the colorectal tumors in which the exact localization in the colorectum was known, 13 (39%) were located distally and 20 (61%) were located proximally (proximal to the flexura lienalis).

In Figure 4, the age-related cumulative risk for endometrial carcinoma is shown for *MLH1, MSH2,* and *MSH6*. The cumulative risk was significantly higher in *MSH6* mutation carriers ($P = 0.02$) compared with the risk in *MLH1* and *MSH2* mutation carriers. The mean age at diagnosis of endometrial carcinoma is 54 years (n = 29; range, 43–65 years) versus 48 and 49 years in *MLH1* and *MSH2* mutation carriers, respectively.

For ovarian carcinoma and transitional cell carcinoma of the upper urinary tract, cumulative risks were not calculated because the numbers were too low. The mean age at diagnosis for ovarian carcinoma was 49 years (n = 4; range, 45–51 years), and the mean age at diagnosis for transitional cell carcinoma was 72.5 years (n = 5; range, 59–82 years).

One family (family 134) was substantially more extended than the other families. To exclude the possibility that this large family biased the results, we compared the cumulative risks for the various tumors between this family and the total group. There were no substantial differences. In addition, we examined whether the degree of participation in the families influenced the results. No considerable differences in cumulative risk were found between the families with a higher and lower degree of participation. To avoid bias toward affected individuals, we performed the Kaplan–Meier analyses both with and without index patients. Because these results did not differ, we decided to include the index patients.

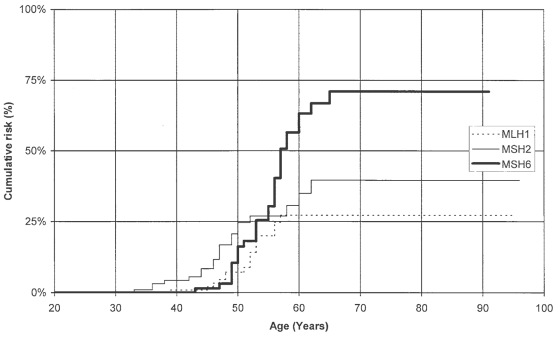

Figure 4. Endometrial carcinoma; cumulative risks for *MLH1, MSH2,* and *MSH6* mutation carriers.

Table 3. Results of MSI Analyses in Tumors of *MSH6* Mutation Carriers

Tumor	MSI high	MSI low	Microsatellite stable	Total
Colorectal carcinoma (%)	18 (86)	3 (14)	0	21
Endometrial carcinoma (%)	11 (69)	4 (25)	1 (6)	16
Transitional cell carcinoma (%)	5 (71)	2 (29)	0 (0)	7
Ovarian carcinoma	2	0	0	2
Breast carcinoma	1	0	0	1
Stomach carcinoma	0	0	1	1
Adenocarcinoma of the cervix	0	1	0	1
Total	35	9	5	49

MSI Analysis

As shown in Table 3, 49 tumors, all from mutation carriers, have been tested for MSI. Eighteen of 21 (86%) of the colorectal tumors showed an MSI-high phenotype. Two of the 3 tumors with an MSI-low phenotype would have been considered microsatellite stable if the BAT40 marker had not been tested. The third MSI-low tumor showed instability of a dinucleotide marker. If MSI-low tumors are also considered, the sensitivity for MSI analysis in colorectal tumors is 100%. Of the 16 endometrial tumors tested, 11 were MSI high (69%), 4 MSI low (25%), and one microsatellite stable (6%). Two of the MSI-high tumors and 1 of the MSI-low tumors would have been considered MSI low and microsatellite stable, respectively, if the BAT40 marker had not been tested. All MSI-low endometrial tumors showed instability of one of the mononucleotide markers. Five of the 7 (71%) transitional cell carcinomas tested showed an MSI-high phenotype. The other 2 were MSI low (29%). Two ovarian tumors were MSI high. The gastric carcinoma was microsatellite stable. The breast tumor, diagnosed in a proven carrier, showed an MSI-high phenotype. One adenocarcinoma of the cervix was MSI low. MSI in all HNPCC-related tumors together has a sensitivity of 71% and 90%, respectively, if MSI-high and both MSI-high and MSI-low tumors are considered.

IHC

As shown in Table 4, 40 tumors, all from mutation carriers, have been tested for MMR protein expres-

Table 4. Results of IHC in Tumors of *MSH6* Mutation Carriers

IHC pattern	No. of tumors (%)
MLH1+, MSH2+, MSH6−	36 (90)
MLH1+, MSH2−, MSH6−	2 (5)
MLH1−, MSH2+, MSH6−	1 (2.5)
MLH1+, MSH2+, MSH6+	1 (2.5)
Total	40

+, positive staining for protein; −, negative staining for protein.

sion by IHC: 18 colorectal tumors, 15 endometrial tumors, 4 transitional cell tumors, 1 ovarian tumor, 1 breast tumor, 1 gastric tumor, and 1 adenocarcinoma of the cervix. Thirty-six of the 40 tumors (90%) showed the expected pattern of absent staining for the MSH6 protein and retained staining for both the MLH1 and MSH2 proteins.

One of the MSI-low colorectal tumors previously mentioned showed absent MSH6 staining in IHC, indicating an MMR (*MSH6*) mutation. Another MSI-low tumor (diagnosed at age 78 years) showed positive staining for the MLH1, MSH2, and MSH6 proteins. In the same patient, bilateral transitional cell carcinoma showed an MSI-high phenotype and absent staining for the MSH6 protein. The colon tumor in this patient is likely to have been a sporadic tumor that did not develop because of defective MMR. All endometrial and transitional cell carcinomas showed negative staining for MSH6. Two colorectal tumors from different individuals showed absent staining not only for MSH6 but also for MSH2. One of these individuals also developed an endometrial carcinoma that showed negative staining for MSH6 in combination with positive staining for MLH1 and MSH2. Another colorectal tumor showed absent staining for both MLH1 and MSH6. In 98% (39 of 40) of the tested tumors, staining for the MSH6 protein was negative. In 90% (36 of 40) of the tumors, IHC specifically indicated a mutation in the *MSH6* gene by an IHC pattern with positive staining for MLH1 and MSH2 and negative staining for MSH6.

Discussion

We studied 20 families with a truncating germline *MSH6* mutation to determine the age-related cumulative risk of developing cancer and to develop a tailormade surveillance protocol. We found that the cumulative risk of all HNPCC-related tumors in *MSH6* mutation carriers was significantly lower than the risk in carriers of a truncating *MLH1* or *MSH2* mutation. In women, the cumulative risk of colorectal cancer was significantly lower ($P = 0.0049$) when compared with carriers of a mutation in *MLH1* or *MSH2,* whereas the risk of endometrial cancer was more than twice as high ($P = 0.02$). For both colorectal carcinoma (54 years) and endometrial carcinoma (55 years), the mean age at diagnosis was higher in female *MSH6* mutation carriers compared with carriers of a mutation in *MLH1* or *MSH2*. In men, the risk of colorectal carcinoma was also lower than in *MLH1* and *MSH2* mutation carriers, but the difference was not statistically significant ($P = 0.084$). The mean age at diagnosis (58.5 years) was more than 10 years higher in *MSH6* compared with *MLH1* and *MSH2* mutation carriers.

Previous studies from The Netherlands and Finland on cancer risks in carriers of an *MLH1* or *MSH2* mutation were possibly biased toward overestimation of the risk because most of the families were selected by using the Amsterdam criteria or on the basis of familial clustering of colorectal cancer.[15,27,37] However, the only population-based study (from Scotland) reported similar risks for colorectal carcinoma in men,[38] although the risk for developing colorectal carcinoma in women was lower compared with the findings in the Dutch and Finnish studies. Carayol et al.[39] discussed the fact that the current risks are probably overestimated in HNPCC because of the statistical method used and proposed a novel statistical approach. We have chosen the Kaplan–Meier analysis because all previous studies eligible for comparison with our data used the Kaplan–Meier analysis as well.[15,27,28]

The general finding of a higher age at diagnosis in *MSH6* mutation carriers when compared with carriers of a mutation in *MLH1* or *MSH2* could be explained from the functional level of the MMR proteins. MLH1 and MSH2 are involved in MMR of both single-base mismatches and insertion-deletion loops, and repair is impaired in the absence of MLH1 or MSH2. Likewise, the MSH6 protein is involved in the repair of both single-base mismatches and insertion-deletion loops. However, in the absence of MSH6, MSH3 can partially replace its repair function and such redundancy might represent a protecting factor against accumulation of DNA damage.[40–42]

A striking finding in this study is the difference in cumulative lifetime risk of colorectal carcinoma between men and women. The same trend is described in *MSH2* mutation carriers.[15,28] This cannot be explained by early death caused by endometrial carcinoma, before a colorectal carcinoma can develop, because endometrial carcinoma is not often the cause of death in these families.

The current surveillance protocol used in carriers of a mutation in one of the MMR genes is colonoscopy every

1–2 years starting at the age of 20–25 years and a yearly gynecologic examination, transvaginal ultrasound examination, and blood test for assessment of CA125 levels starting at the age of 30–35 years. If transitional cell carcinoma of the upper urinary tract or stomach cancer occurs in at least 2 individuals in a family, urine cytology yearly or gastroscopy every 1–2 years, respectively, from the age of 30–35 years is recommended.[43] In the present study, we found a mean age at diagnosis of colorectal carcinoma more than 10 years higher than found in *MLH1* and *MSH2;* the youngest age at diagnosis of colorectal cancer was 26 years in male *MSH6* carriers and 41 years in female *MSH6* carriers. We recommend the same colonoscopic surveillance protocol in male carriers of an *MSH6* mutation as recommended in *MLH1* and *MSH2* mutation carriers because the cumulative risks did not differ significantly from the risk in *MLH1* and *MSH2* carriers. However, although this might further complicate the already-complex surveillance protocol, we recommend that female carriers of an *MSH6* mutation start colonoscopy at the age of 30 years because the cumulative risk of colorectal carcinoma was significantly lower compared with carriers of a mutation in *MLH1* and *MSH2* and because the youngest age at diagnosis was 41 years.

Similar to observations in *MLH1* and *MSH2* mutation carriers, the majority (66%) of the colon carcinomas in the families we examined were located in the proximal colon. A previous study reported that 30% of the colon carcinomas associated with *MSH6* mutations were located proximally.[28] The reason for the difference between these studies is unclear.

We found that the cumulative risk of endometrial carcinoma increased sharply after the age of 50 years. It is still questionable whether surveillance of the endometrium will lead to the early detection of cancer and improvement of the prognosis.[44] Therefore, based on the substantial risk of developing this type of cancer and the overall mortality from endometrial carcinoma of approximately 14%,[45] we advocate a liberal approach toward prophylactic hysterectomy for women with a truncating *MSH6* mutation who are older than 50 years of age. For surveillance of transitional cell carcinoma, we propose starting from the age of 50 years in families in which this tumor has occurred. However, the value of urine testing for the early detection of cancer is still unknown.[46]

Because DNA analysis is expensive and time consuming, prescreening methods can be of great relevance to increasing the efficiency of genetic testing for the identification of the disease causing mutation. Two prescreening methods currently applied to identify families eligible for mutation analysis of the MMR genes are MSI analysis and IHC. MSI analysis in colorectal tumors caused by an *MSH6* mutation has been reported to show either predominance of an MSI-high phenotype[26,34] or predominance of an MSI-low phenotype.[27,28] We found an MSI-high phenotype in 86% of the *MSH6*-related colorectal carcinomas with a pattern equivalent to that found in *MLH1*- and *MSH2*-related tumors, including instability of both mononucleotide and dinucleotide markers. In the classification of MSI, we included the Bethesda panel of markers[19] as well as the BAT 40 marker because it increases the sensitivity of MSI analysis, as shown in this study and a previous study performed by our group.[36] If the MSI-low tumors are included, the sensitivity of MSI analysis is 100% in colorectal tumors. In endometrial tumors obtained from *MSH6* mutation carriers, MSI analyses have been reported to show predominantly MSI-low phenotypes with mainly instability of mononucleotide repeats.[25–28] Accordingly, in the present study, we found an MSI-low phenotype in a substantial proportion (25% [4 of 16]). An MSI-high phenotype predominated in the other types of carcinoma tested. MSI in all HNPCC-related tumors together has a sensitivity of 71% and 90%, respectively, if MSI-high and both MSI-high and MSI-low tumors are considered. IHC in both colorectal and endometrial tumors has been reported to show positive staining of the MLH1 and MSH2 proteins and absent staining for MSH6.[25,28,34,47] We found an almost 100% sensitivity in predicting an MMR defect, including a mutation in *MSH6*. In 90% of the tumors, IHC specifically predicted a germline mutation in the *MSH6* gene. Two colorectal tumors from different individuals showed absent staining not only for MSH6 but also for MSH2. A possible explanation is that in the colorectal tumor of one of these patients, both the C-8 tract in MSH6 and the A-8 tract of MSH3 were shown to be somatically instable in MSI analysis. As a result, both the MSH2–MSH6 and the MSH2–MSH3 heterodimer might be less frequently formed, which will add to loss of expression of MSH2.

In our clinic, IHC is the first step in prescreening families that fulfill the Amsterdam criteria because the yield of mutation analysis is high and IHC directly indicates which gene to test. If IHC is positive for all tested proteins, MSI analysis is performed. On the other hand, MSI analysis is the first step in prescreening families that do not fulfill the Amsterdam criteria. When an MSI-high or MSI-low phenotype, especially with instability of a mononucleotide marker, is found in an HNPCC-related tumor, IHC of the MMR proteins is the second step. In case of an MSS tumor, IHC of MSH6 is performed. Our results in this study confirm that this

approach has a high sensitivity for identifying families with an *MSH6* mutation.

In conclusion, the present study shows that female *MSH6* mutation carriers develop colorectal carcinoma at a significantly higher age than reported for *MLH1* and *MSH2* mutation carriers and that the cumulative risk is significantly lower. Based on these findings, we recommend starting colonoscopic surveillance from a higher age than recommended in *MLH1* and *MSH2* families in female *MSH6* carriers. Secondly, we found a dramatic increase in the risk of developing endometrial carcinoma after the age of 50 years in female *MSH6* mutation carriers and therefore recommend a liberal approach toward hysterectomy for women above this age. Finally, we show that both MSI analysis and IHC for the MMR proteins are very sensitive prescreening methods for identifying families eligible for mutation analysis of the *MSH6* gene.

This study underscores the distinct phenotype in *MSH6* families and provides guidelines for the identification, counseling, and management of these families.

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3.3.

Genetic testing in hereditary non-polyposis colorectal cancer families with a *MSH2*, *MLH1* or *MSH6* mutation

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LETTER TO JMG

Genetic testing in hereditary non-polyposis colorectal cancer families with a *MSH2*, *MLH1*, or *MSH6* mutation

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About 5% of colorectal cancers are associated with the autosomal dominantly inherited cancer susceptibility syndrome hereditary non-polyposis colorectal cancer (HNPCC).[1 2] HNPCC is characterised by a high risk of developing colorectal cancer and endometrial cancer at a young age (cumulative lifetime risk 80-90% and 30-40%, respectively), and by an increased risk of developing various other tumour types, such as ovarian, uroepithelial, small intestine, biliary tract, stomach, brain, and skin cancers.[2–5] Germline mutations in one of three mismatch repair genes (*MSH2*, *MLH1*, and *MSH6*) were found to be responsible for a majority of HNPCC families.[6–9]

Knowledge of the causative mutation in a particular HNPCC family enables the identification of at risk family members by genetic testing. Clearly, the absence or presence of a mutation is of considerable medical and psychological significance. Subjects not carrying the mutation are relieved from a continuous anxiety and can be dismissed from medical surveillance, saving them trouble and reducing health care costs.[10] Importantly, subjects with the mutation can benefit from a medical surveillance programme. For HNPCC, colonoscopy has been shown to be a potent tool for the detection and treatment of premalignant adenomas or early colorectal carcinomas in at risk subjects, reducing the risk of developing colorectal cancer and decreasing the overall mortality by about 65%.[11 12] The possibility of early detection of colorectal cancer by stool analysis using the genetic markers TP53, BAT26, and K-RAS raises expectations for the development of less invasive surveillance procedures.[13] Furthermore, intervention trials with non-steroidal anti-inflammatory drugs (NSAID) in subjects at risk for developing colorectal cancer are in progress.[14 15]

So far, studies on the use of genetic testing in HNPCC families have used families or subjects who had been registered for research purposes.[10 16 17] It is conceivable, however, that these research families represent a selected group of HNPCC families where decision making processes are different from those in families in a clinical setting. Here, we report the use of genetic testing in 18 clinically ascertained HNPCC families with a known mutation in *MSH2*, *MLH1*, or *MSH6*.

Key points

- In a clinical setting, considerable interest was observed for genetic testing in HNPCC families with a known germline mutation.
- Testing was used more frequently by subjects with a higher pre-test genetic risk for the mutation, by women, and by subjects with children.
- Genetic testing has earned a place in the standard medical care for subjects at risk for HNPCC.

Table 1 Mutations in the *MLH1*, *MSH2*, and *MSH6* genes in the HNPCC families studied

Gene	Nature of the mutation (nucleotide change)	Families (n=18)
MSH2	Genomic deletion exon 3	1
	Splice acceptor site intron 9 (IVS9_2A>G)	1
	Frame shift mutation exon 2 (229_230delAG)	1
	Nonsense mutation exon 13 (2038C>T)	1
	Genomic deletion exon 1	1
	Nonsense mutation exon 8 (1285C>T)	2
	Frameshift mutation exon 2 (1705_1706delGA)	1
	Frameshift mutation exon 14 (2347delC)	1
MLH1	Splice donor site intron 8 (IVS8+1delG)	1
	In frame deletion exon 16 (1852_1854delAAG)	5
	Splice donor site exon 16 (1896G>A)	1
	Splice acceptor site intron 9 (IVS9-1G>C)	1
MSH6	Frameshift mutation exon 4 (1784delT)	1

MATERIAL AND METHODS

Patients

Eligible HNPCC families have been referred to the Department of Clinical Genetics of the Erasmus University Medical Centre Rotterdam for oncogenetic counselling by general practitioners and medical specialists since 1992. DNA analysis of the *MSH2*, *MLH1*, and *MSH6* genes was performed at the Department of Human and Clinical Genetics, Leiden University Medical Centre, as described previously.[18–20] Families were included in the study when a mutation in either of these three mismatch repair genes was identified before 2000. Subjects of these families were included when they were aged 18 years and over at the time of molecular diagnosis in the family and when they had a pre-test genetic risk for carrying the mutation of 100%, 50%, or 25% (see results).

Procedure

In general, the initial search for the causative mutation had been performed on blood DNA of the youngest colorectal cancer patient in the family (the index subject). Identified mutations were confirmed in all relatives affected with an HNPCC related tumour from which DNA samples were available. The initial counsellee and index subjects were asked to inform all the adult first and second degree relatives of patients with an HNPCC related tumour about the genetic predisposition to cancer in their family. Written information to distribute among their family members was made available to them. This information included facts on the inheritance of the cancer susceptibility in their family, the

Table 2 Characteristics of the families studied

Gene	Families (n=18)	Mean number CRC/family	Mean age CRC (range)	Mean number EC/family	Mean age EC (range)
MSH2	9	4.8	43.8 y (23–75)	1	46.4 y (30–54)
MLH1	8	5.4	43.7 y (27–72)	0.5	46.3 y (41–82)
MSH6	1	7	60.4 y (32–84)	5	55 y (50–60)

CRC, colorectal cancer. EC, endometrial cancer.

possibility of genetic testing, the risks of developing cancer, and the options for intervention. Relatives opting for genetic testing received one or more individual pre-test counselling sessions according to the recommendations of the American Society of Clinical Oncology,[21] including the discussion of medical, genetic, and psychosocial aspects of genetic testing. Psychological support was offered to all subjects throughout the testing procedure. Disclosure of the test results followed within 6-12 weeks after blood sampling. Mutation carriers were referred to local specialists for follow up and surveillance. In The Netherlands, this surveillance comprises colonoscopy and gynaecological examination every one to two years. In this country, prophylactic colectomy is currently not offered to unaffected mutation carriers.

Data collection and statistical analysis

All data were collected from medical records. Descriptive statistics were used to establish test rates of genetic testing. The influence of pre-test genetic risk, gender, parenthood, and age on the use of genetic testing was first assessed by univariate analysis. Subjects were categorised into subjects younger than 50 years and subjects 50 years and older. The simultaneous influence of gender, parenthood, and age was also assessed by multivariate logistic regression analysis in the 50% risk subjects. Pre-test genetic risk had been excluded from the multivariate analysis, as about half of the data on parenthood and age were missing for the 25% risk subjects.

To assess the time dependent rate of genetic testing, Kaplan-Meier survival probabilities were calculated for 50% risk subjects with a first degree relative with an HNPCC related tumour. By doing so, we avoided including the time 25% risk subjects had to wait for the genetic test result of their parent.

RESULTS

A cohort of 18 consecutive HNPCC families was selected that had a known mutation in *MSH2* (n=9), *MLH1* (n=8), or *MSH6* (n=1) (table 1). All families were of European origin. Five apparently unrelated families had an identical *MLH1* mutation and two apparently unrelated families had an identical *MSH2* mutation (table 1). At the time of clinical ascertainment, 15 of the 18 families fulfilled the Amsterdam II criteria.[22] Of the other three families, two families presented with a single patient with colorectal cancer under the age of 40 years and one family had three patients with endometrial cancer and one patient with ovarian cancer, all diagnosed over the age of 50 years.[23] Specific details of the number of colorectal and endometrial cancers per family and ages of onset are listed in table 2.

The 18 selected families consisted of 523 living subjects with a 100% (n=60), 50% (n=308), or 25% (n=155) pre-test genetic risk of carrying the family specific mutation (table 3). For practical reasons, the subjects diagnosed with an HNPCC related tumour (n=56) and obligate carriers (n=4) were designated as having a 100% pre-test genetic risk. The subjects with a 50% risk had a first degree relative with an HNPCC related tumour (n=267) or a first degree relative who was a mutation carrier (n=41). The subjects with a 25% risk had a living unaffected parent with a 50% risk (n=64) or a parent with a 50% risk who had died without evidence of an HNPCC related tumour (n=91).

Genetic testing was used by 260 of 523 (50%) eligible subjects (table 3). A mutation was detected in 133 (51%) subjects, of whom 83 were unaffected. Of the subjects with a pre-test genetic risk of 100%, 50%, and 25% for carrying the mutation, 87%, 57%, and 21% respectively used genetic testing ($p<0.0001$ for 100% *v* 50%; $p<0.0001$ for 50% *v* 25%) (tables 3 and 4). Of the 25% risk subjects who had an unaffected 50% risk parent who did not opt for testing, only three of 64 (5%) subjects used genetic testing. In contrast, of the 25% risk subjects with a dead unaffected 50% risk parent, 30 of 91 (33%) subjects used genetic testing (table 4). The test rate among the 308 50% risk subjects was 62% in women versus 51% in men, suggesting a small but significant preference for women to use genetic testing ($p=0.041$, table 4). The test rate in 50% risk subjects with children was 70% versus 45% for those without children, indicating parenthood as a stronger positive predictor towards testing ($p<0.001$, table 4). The age of the subjects did not influence test rates (table 4). Multivariate analysis of the data was consistent with the univariate analysis, again indicating gender and parenthood as significant parameters for genetic test usage (table 4).The mean time of follow up after identification of the mutation in the family was 42 months (range 12-74 months). Forty-one percent of 50% risk subjects decided for genetic testing within one year. At a

Table 3 Genetic testing and outcome in 100%, 50%, and 25% risk carriers in the 18 HNPCC families

			25%			
Pre-test risk	100%	50%	Parent alive	Parent dead	Total	Total
Number	60	308	64	91	155	523
Tested	52 (87%)	175 (57%)	3 (5%)	30 (33%)	33 (21%)	260 (50%)
Mutation carrier	50 (96%)	80 (46%)	0	3 (10%)	3 (9%)	133 (51%)

Table 4 Genetic test use in relation to gender, parenthood, age, and pre-test genetic risk

	Total	Tested (%)	Univariate	Multivariate
Gender*				
Females	156	97 (62)	p=0.041	p=0.045
Males	152	78 (51)		
Children*				
Yes	182	127 (70)	p<0.001	p=0.013
No	107	48 (45)		
Age*				
<50 y	191	104 (55)	p=0.24	p=0.39
>50 y	117	72 (62)		
Pre-test risk				
50%	308	175 (57)	p<0.0001	-
25%	155	33 (21)		
25% risk carriers				
Parent alive	64	3 (5)	p<0.0001	-
Parent dead	91	30 (33)		

*In subjects with a pre-test genetic risk of 50%.

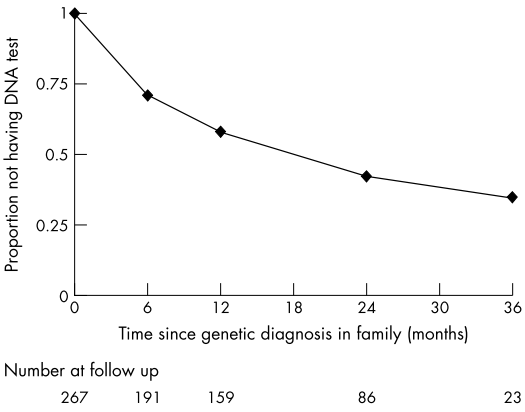

Figure 1 Time dependent rate of genetic testing in subjects with a 50% pre-test genetic risk. The proportion of 50% risk carriers not having the genetic test is shown at 0, 6, 12, 24, and 36 months after the identification of the familial mutation. Only 50% risk carriers with a first degree relative with an HNPCC related tumour are included.

follow up at two years and three years after molecular diagnosis, 58% and 65% respectively of the 50% risk subjects were tested (fig 1).

DISCUSSION

To our knowledge, the present study is the first to evaluate the use of genetic testing for HNPCC in a clinical setting. Importantly, we determined the use of genetic testing in complete pedigrees, including all affected family members and their unaffected first and second degree relatives. This was done in 18 HNPCC families with an identified pathogenic mutation in *MSH2*, *MLH1*, or *MSH6*. These families were quite extensive, with an average of 29 study subjects per family. The use of genetic testing by 50% risk carriers in our families was 57%. This test rate was lower than the 75% test rate in a Finnish cohort of 446 subjects at similar risk for an HNPCC germline mutation.[16] The Finnish subjects, however, had consented to registration and participation in research, which is likely to be positively correlated with interest in genetic testing. Also, the Finnish population is known for its positive attitude towards genetic testing.[24] In a Northern American study only 90 out of 208 subjects (43%) from four extended HNPCC research families were tested.[17] As the pre-test genetic risks of the study subjects were not specified, we cannot compare their data with ours.

We found that the magnitude of pre-test genetic risk for carrying the mutation was strongly correlated with test rates (87%, 57%, and 21% for 100%, 50%, and 25% risk subjects, table 3). This phenomenon has also been observed in other inherited diseases.[25–27] Interestingly, 25% risk subjects with an unaffected 50% risk parent who was alive rarely opted for genetic testing (5%), whereas subjects with the same pre-test genetic risk but with a dead unaffected 50% risk parent used genetic testing in one-third of the cases (tables 3 and 4). This may be because the identification of the mutation in a child designates the parent as an obligate carrier and children from a living unaffected 50% risk subject may therefore be more likely to refrain from genetic testing in order not to overrule their parents' preference for "not knowing". Also, children may share strategies to cope with genetic risks with their parents. The slightly higher genetic test rates in women than men (62% *v* 51%) was also seen in another late onset inherited disease.[26] In HNPCC, however, the additional risk for endometrial cancer in female mutation carriers probably also influences the use of genetic testing. The significantly higher test rates among subjects with children compared to subjects without children (70% *v* 45%) has also been observed in families with inherited breast and ovarian cancer (HBOC).[25] These findings emphasise that knowledge of the cancer risks for offspring is a major reason for subjects to opt for genetic testing. The high test rate in the affected 100% risk subjects also seems to reflect this phenomenon, since their participation is pivotal especially for the identification and conformation of the pathogenic mutation in the family, but generally has few medical implications for themselves.

A significant proportion of subjects was tested more than one year after the identification of the family specific mutation (65% of the 50% risk carriers at a follow up of three years) (fig 1). Interestingly, at our institute, the time period for deciding for genetic testing was strikingly longer for subjects at risk for HNPCC than for subjects at risk for HBOC (50% at 18 months versus 9 months).[25] The differences in time needed for decision making may perhaps be related to the one to two years interval for colonoscopy in HNPCC families versus the six months interval for breast surveillance in HBOC families.

Genetic testing rates depend on the natural history of the disease concerned, the success of treatment, the efficacy and acceptability of surveillance and prevention, and the costs involved.[28] The use of genetic testing in 50% risk subjects varies from about 20% in Huntington's disease (no interventions available, but relief of uncertainty and relevance for, for example, reproductive choices)[26 29] to ~55% in hereditary breast and ovarian cancer (*BRCA1* and *BRCA2* genes, surveillance and prophylactic mastectomy, prophylactic oophorectomy, and chemoprevention available for female carriers),[26 30] to ~90% in familial hypercholesterolaemia (lipid lowering treatment available).[31] The 57% HNPCC genetic testing rate in 50% risk carriers reported here is similar to that of women with the same genetic risk for carrying a *BRCA1* or *BRCA2* mutation. This suggests that the total load of factors that influence the choice to opt for genetic testing in HNPCC equals that in HBOC. The efficacy of regular colonoscopy in HNPCC mutation carriers is more favourable compared to regular mammography in young women with a *BRCA1* or *BRCA2* mutation.[11 32] This limited efficacy of mammography causes some women with a *BRCA1* or *BRCA2* mutation to opt for prophylactic mastectomy.[25 30] Though colonoscopy is not as invasive as the prophylactic mastectomy in HBOC, the burden and violation of physical integrity of this surveillance may be an underestimated factor. Also, the additional risks for other HNPCC related cancers, like brain tumours, for which no

options for surveillance and prevention are available, can cause subjects to refrain from testing. Other reasons not to opt for testing might be fear of financial and social discrimination and inability to cope with a positive test result. Notably, in The Netherlands, cancer predisposition is no reason for exclusion by the health insurance companies, nor for denial of access to employment. It was shown that most tested subjects are able to cope with genetic testing for cancer predisposition in the short term, particularly also for HNPCC.[16 33–36] More data on the long term psychosocial impact of genetic testing for HNPCC as well as data on the reasons and psychosocial impact of refraining from genetic testing are needed.

From the data reported here, however, it can be concluded that there is considerable interest in genetic testing in subjects from HNPCC families with a known mutation. Also, in view of the reduction in morbidity and mortality upon surveillance of identified mutation carriers, genetic testing has earned a place in the standard medical care for people at risk for HNPCC.

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3.4.

Long term follow-up of HNPCC gene mutation carriers; compliance with screening and satisfaction with counselling and screening procedures

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Long term follow-up of HNPCC gene mutation carriers: Compliance with screening and satisfaction with counseling and screening procedures

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HNPCC, colonoscopy, screening, satisfaction, mutation carriers, counseling, follow-up

Abstract

Hereditary Non Polyposis Colorectal Cancer (HNPCC) is a hereditary predisposition to colorectal and endometrial cancer, caused by mutations of the mismatch repair (MMR) genes *MSH2*, *MLH1* and *MSH6*. Regular colonoscopy reduces the incidence of colorectal cancer in mutation carriers dramatically. The aim of this study was to evaluate the use of colonoscopy by proven HNPCC mutation carriers. We also evaluated the satisfaction with the counseling and screening procedures at the long term. A questionnaire survey was performed among 94 proven MMR gene mutation carriers. Data were analyzed using univariate and multivariate analysis. The average time of follow-up was 3,5 years (range 0.5-8.5 years). The response rate was 74%. The proportion of unaffected mutation carriers under colonoscopic screening increased from 31% to 88% upon genetic testing, and for gynecological screening from 17% to 69%. However, more than half of the responders experienced colonoscopy as unpleasant or painful. About 97% felt well informed during counseling, and 88% felt sufficiently supported. Ten percent of the responders reported a high cancer worry, that was significantly (p=0.007) associated with a high perceived cancer risk. Six responders (9%) regretted being tested. Remarkably, of 4 of these 6 a close relative died recently of cancer. Problems with obtaining a disability or life insurance or mortgage were experienced by 4 out 10 healthy carriers opting for these services. In conclusion, genetic testing for HNPCC considerably improves compliance for screening, which will result in a reduction of HNPCC related cancer morbidity and mortality in mutation carriers. Most HNPCC gene mutation carriers cope well with their cancer susceptibility on the long term.

Introduction

Hereditary Non-Polyposis Colorectal Cancer (HNPCC, OMIM #114500) is an autosomal dominantly inherited predisposition to colorectal and endometrial cancer. It is caused by germline mutations of mismatch repair (MMR) genes, particularly *MSH2*, *MLH1* and *MSH6* [1-4]. MMR gene mutation carriers have cumulative lifetime risks of colorectal and endometrial cancer of 70-90% and 30-40%, respectively [5-8]. Also cancers of the stomach, ovaries, small bowel, urinary tract, skin and brain occur in mutation carriers, but the cumulative lifetime risks of each of these tumors do not exceed 15% [5-8].

The identification of MMR gene mutations in HNPCC enabled genetic testing within families with HNPCC. In the context of a known mutation in the family,

identification of individuals with or without the mutation is possible by DNA testing. Non-mutation carriers will be relieved from anxiety and can be dismissed from regular screening programs, while individuals with the mutation may benefit from these procedures. Colonoscopy was shown a potent tool for the detection and treatment of premalignant adenomas or early colorectal carcinomas in individuals at risk of HNPCC. Järvinen et al. [9] reported a decrease of overall mortality of about 65% by regular colonoscopy within this group. However, colonoscopy is an invasive screening technique with a clear burden and some risk [10]. The efficacy of screening for extra-colonic tumors in HNPCC carriers, like gynecological screening, is controversial [11, 12]. To date little is known about cancer screening practices among proven MMR gene mutation carriers. At a follow-up of 12 months Hadley et al. [13] reported a slight increase (41% to 53%) of colonoscopic screening among 17 MMR gene mutation carriers.

Here we evaluate the use of regular colonoscopy by proven MMR gene mutation carriers on the long term. In view of the significant psychosocial impact of genetic testing and related surveillance strategies, we simultaneously evaluated the long-term satisfaction with the counseling procedure and the screening program.

Patients and methods

Between November, 1994 and December, 2002, 115 MMR gene mutation carriers were identified at the Department of Clinical Genetics of Erasmus MC. Questionnaires were sent to carriers with known addresses and who were still alive on May 2003.

The counseling procedure was as follows. In the search for the causative MMR gene mutation within a family, we initially invited the relatives affected with an HNPCC related tumor for genetic testing. After identification of a pathogenic mutation, the initial counselees were asked to inform all adult first and second-degree relatives of patients with an HNPCC related tumor about the possibility of genetic testing. Written information to distribute among their family members was available to them. This information included facts on the inheritance of the cancer susceptibility in their family, the possibility of genetic testing, the risks of developing cancer, and the options for intervention. Relatives opting for genetic testing received one or more individual pre-test counseling sessions according to the recommendations of the American Society of Clinical Oncology [14]. Psychological support was offered to all subjects throughout the testing procedure. Disclosure of the test results followed within 6-12 weeks after blood sampling. Mutation carriers were referred to

local specialists for follow-up and surveillance. The advised colonic screening consisted of colonoscopy every 1-2 year from the age of 20-25 years on. The procedure was generally prepared by PEG-solution with or without fluid diet and performed under sedation. Female carriers were offered gynecological screening by vaginal ultrasound and CA125-maesurement in blood from the age of 30-35 years on. Additional screening advices for the stomach, duodenum or urinary tract were occasionally given, based on family history.

Our questionnaire addressed sociodemografic characteristics, experience with HNPCC related cancer, compliance with screening, satisfaction with the screening methods and counseling procedure, knowledge and perception of cancer risks, and discrimination by insurance companies. Sociodemographic characteristics included age, gender, marital status, highest level of education, employment and number and age of children. The use of pre- and post-test screening was asked (1=no; 2=yes), including the frequency and method of screening. Satisfaction with the screening methods and counseling procedure was rated on a 5-point scale and classified in 2 groups. Group 1, the *satisfied* group, scored 1, 2, or 3, and group 2, the *unsatisfied* group, scored 4 or 5. Since an HNPCC related colorectal cancer risk of about 80% had generally been counseled, reported colorectal cancer risks for MMR mutation carriers were scored as underestimated or overestimated if lower than 70% and higher than 90% respectively. Perceived colorectal cancer risk was rated on a 5-point scale and classified in 3 groups. Group 1, the *low* risk group, scored 1 or 2, the *intermediate* group scored 3, and the *high* risk group 4 or 5. For the evaluation of cancer worry three questions based on the "cancer worry scale" of Lerman et al. [15] were used, addressing (1) how often the mutation carrier worried about developing colorectal cancer and whether the carrier's (2) mood or (3) daily activities were impaired by these worries. Response scales varied from 1="almost never" to 4="always". Thus, the range of the total score of these three questions was 3-12. The level of worry was interpreted as *low (*when all three questions were answered by "almost never", total score =3), as *intermediate* (total score: 4-6) or as *high* (total score: 7-12). Interference of the genetic status with work and insurance were asked for (1=no; 2=yes), differentiating the type of insurance (health insurance, mortgage, life and disability insurance).

All data analyses were done with the program SPSS for Windows (version 9.0). Differences in sociodemographic characteristics between individuals affected and not-affected with an HNPCC related tumor, differences in compliance with screening before and after genetic testing and differences between reported HNPCC related risk, perceived

risk and cancer worry between groups were tested by Pearson's Chi square test, a Fisher's exact test, or a Mc Nemar Chi square test. Multiple logistic regression analysis was used to determine factors associated with reporting a wrong HNPCC related colorectal cancer risk. All *P* values were two sided. *P* values less than 0.05 were considered significant.

Results

We identified 24 families with a MMR gene germline mutation (11 of *MSH2*, 10 of *MLH1* and 3 of *MSH6*), encompassing 115 mutation carriers. Questionnaires were sent to 94 individuals (the remaining had died or had moved to unknown addresses). Of the 94 questionnaires, 70 were received back, resulting in a response rate of 74%. Of the 70 responders 24 (34%) were male and 46 (66%) were female, and 28 individuals (40%) were already diagnosed with an HNPCC related tumor at the time of testing (17 with colorectal cancer, 8 with endometrial cancer and 3 with both colorectal and endometrial cancer)(Table 1). There was no significant difference in gender and parenthood between the affected and not affected responders (Table 1). However, the responders affected with an HNPCC related tumor were significantly older than the not affected responders ($p<0.001$). The 24 non-responders did not differ significantly from the responders with respect to gender, clinical status, age and parenthood (data not shown). The average time of follow-up from the individual genetic diagnosis was 3,5 years (range 0.5-8.5 years).

Thirty-one percent of the unaffected risk carriers had regular colonoscopy before genetic testing (Table 2); 62% every 2 years, and 38% less frequent. The vast majority (79%) of individuals who had no screening prior to genetic testing was not aware of being at increased risk for colorectal cancer. After being identified as a mutation carrier, 88% of the healthy risk carriers indicated to have colonoscopic screening every 1-2 year. Five individuals refrained from colonoscopic screening so far; one because of the burdensome procedure, one because of lack of time due to a busy lifestyle, and three individuals planned to go for screening in the near future. Gynecological screening was performed in three of 18 unaffected female risk carriers (17%) that were over 35 years before the genetic test (Table 2). At the time of the questionnaire 20 of 29 female mutation carriers (69%) over 35 years of age had had gynecological screening. Noteworthy, endometrial cancer had occurred in the families of 7 of the 9 women not opting for gynecological screening. We cannot exclude that some of these women had had hysterectomy for non-malignant reasons.

Fifty-seven percent of the healthy carriers experienced colonoscopy as unpleasant (Table 3), 32% as fearful, 51% as painful, 16% as shameful, and 14% as hazardous. The majority (71%) would prefer a less burdensome screening technique. About 90% of the carriers believed that screening reduced colorectal cancer risk. Ten of the 37 (27%) healthy risk carriers that underwent colonoscopic screening reported the detection of colorectal polyps. No colorectal cancers were detected during the study period.

Eighty-eight percent of the mutation carriers judged the way they were informed about the possibility of genetic testing for HNPCC as appropriate (Table 4). Also, the information about HNPCC given during counseling was judged sufficiently by 97%. However, almost one third of the mutation carriers with a previous HNPCC related cancer and half of the healthy individuals would have liked information about life-style adjustments and/or food supplements to prevent cancer. Also, updates about new developments on the field of HNPCC were appreciated on the long term.

Interestingly, 63% of the respondents affected by an HNPCC related cancer and 37.5% of the unaffected underestimated the HNPCC related cumulative lifetime colorectal cancer risk (Table 5). Reporting an incorrect HNPCC related risk was in the univariate analysis associated with age and being affected with an HNPCC related tumor, but in the multivariate analysis only with age (Table 6). About half of the respondents experienced their own colorectal cancer risk as high (Table 5).

Sixty-nine percent of the mutation carriers reported some degree of cancer worry, of whom 10% a high level (Table 7). This worry was significantly ($p=0.007$) associated with a high perceived colorectal cancer risk. Clinical status, gender, age, parenthood and reporting high or correct HNPCC related colorectal cancer risks, were not significantly associated with cancer worry. However, our study group may be too small to detect more subtle associations. Eight mutation carriers had expected more support from the genetic department (Table 4), mainly with respect to the arrangement of screening and the psychological handling of cancer risks.

Regret of genetic testing was reported by six respondents (9%). They stated that they would not choose for genetic testing for the familial MMR gene mutation with their current knowledge and experience (Table 4). Within this subgroup, four had recently lost a relative because of cancer, three had high levels of cancer worry, three expected to die of cancer despite screening, and one had insurance problems.

Insurance problems regarding disability, life insurance or mortgage, were reported by 4 out of 10 unaffected respondents, who opted for these services (Table 4). None indicated problems with getting a job or health insurance.

Discussion

We show that genetic testing largely improved colorectal and gynecological screening compliance in the studied group of proven HNPCC risk carriers (from 31% to 88% and from 17% to 69% respectively). This can be expected to lead to a considerable reduction in HNPCC related morbidity and deaths in this group.

Although, the vast majority (90%) of healthy risk carriers has faith in the efficacy and safety of the colonoscopic screening, it is certainly experienced as an invasive and burdensome technique. A main effort should be made to improve preventive options in this group of risk carriers that faces a life-long colorectal screening.

Almost two-thirds of the responders affected with an HNPCC related tumor and a third of the not affected report a lower HNPCC related cumulative lifetime colorectal cancer risk than counseled. Also, only about half of the responders perceived their own colorectal cancer risk as high. Underestimation of colorectal cancer risks by HNPCC carriers was previously described by Aktan et al. [16]. It may be due to coping strategies to deal with the personal colorectal cancer risk and to failing memory [16, 17]. Less accurate recollection of risks is also correlated with age. Since the affected responders are significantly older than the not affected, this may be an explanation for the difference in reported HNPCC related colorectal cancer risks between the responders affected and not affected with an HNPCC related tumor.

On the long term most MMR gene mutation carriers tested at our department were able to cope with having this cancer predisposition. The vast majority of proven carriers judged the information and support they received during the counseling procedure as sufficient. Noteworthy, 59% would have appreciated updates on scientific developments regarding HNPCC. High cancer worry regarding their colorectal cancer risk was indicated by 10% of the responders (3 affected and 4 not affected with an HNPCC related tumor). This is comparable to the figures presented by Aktan et al. in 83 MMR mutation carriers at one year of follow-up (8%) [16]. Cancer worry in our study was correlated significantly with a high perceived colorectal cancer risk. During counseling and follow-up an effort should be made to identify subjects with high perceived colorectal cancer risks, and

psychological support should be more actively offered. Even more so, since, in our study, underestimation of the HNPCC related colorectal cancer risk did not influence screening behavior negatively.

Regret of being tested for the familial susceptibility for HNPCC was expressed by 6 respondents (9%). Remarkably, in the families of 4 of the six responders a relative recently died of cancer. Events in the family are known to influence perceived cancer risk [17]. Also, faith in the efficacy of screening may be violated. Tailor-made psychological support may help relatives with coping problems due to newly diagnosed cancer cases or deaths in the family. Since relatives can develop HNPCC related or non-related cancer at any moment in time, it is optimal to have an ongoing follow-up of the MMR mutation positive families. This could be obtained by yearly telephone contact or information gatherings. Also, offering screening to all carriers in a multidisciplinary outpatient facility can improve long-term follow-up and satisfaction.

In the Netherlands employers or insurers are prohibited to exclude individuals with a genetic predisposition for cancer from jobs or health insurance. For life or disability insurance no questions about genetic predisposition may be asked by insurers for insurance below a certain limit; $\in$160.00 for health insurance and $\in$32.000 in the first year of a disability insurance and $\in$22.000 the following years. As a result of these regulations, the studied group experienced no problems with jobs or health insurance. However, almost half of the healthy risk carriers opting for life insurance, disability insurance or mortgage, had some kind of trouble. This represents a potential threat to the accessibility of genetic testing for cancer susceptibilities.

In conclusion, this study indicates that genetic testing for HNPCC considerably improves compliance with screening. Also, most MMR gene mutation carriers can cope with their cancer susceptibility on the long term. We identified a need for updates regarding new developments and for more support of a vulnerable minority that has coping problems at the time of genetic testing or later on. We therefore propose an ongoing access to psychological and counseling facilities for MMR gene mutation carriers, preferably in the setting of a multidisciplinary family cancer clinic.

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Table 1:
Gender, Age and Parenthood in MMR mutation carriers affected and not affected with an HNPCC-related tumor

	Affected (%) n=28	Not affected (%) n=42	Total (%) n=70	P value
Gender: Male	13/28 (46)	11/42 (26)	24/70 (34)	0.081
Female	15/28 (54)	31/42 (74)	46/70 (66)	
Age: 25-50	6/28 (21)	30/42 (71)	36/70 (51)	<0.001
>50	22/28 (79)	12/42 (29)	34/70 (49)	
Children: No	1/28 (4)	7/42 (17)	8/70 (11)	0.093
Yes	27/28 (96)	35/42 (83)	62/70 (89)	

Table 2:
Screening behaviour among MMR mutation carriers without an HNPCC-related tumor *before* and *after* genetic testing.

		Number of carriers (%)	P value
Regular colonoscopy:	*Before* genetic testing	13/42 (31)	<0.001
	After genetic testing	37/42 (88)	
Gynecological screening:	*Before* genetic testing	3/18 (17)	<0.001
	After genetic testing	20/29 (69)	

Table 3:
Satisfaction with colonoscopic screening among the studied MMR gene mutation carriers without an HNPCC-related tumor.

	Number of carriers (%)
Colonoscopy*: unpleasant	21/37 (57)
fearful	12/37 (32)
painful	19/37 (51)
shameful	6/37 (16)
Faith effectiveness of colonoscopy	38/42 (90)
Worry of complication of colonoscopy	6/42 (14)
Wish other screening method	30/42 (71)

*Scored in the 37 healthy carriers who underwent colonoscopy

Table 4:
Long term satisfaction with the counseling procedure and genetic testing in the studied MMR gene mutation carriers.

	Affected (%)	Not affected (%)	Total (%)
Appropriately invited	25/27 (93)	35/41 (85)	60/68 (88)
Sufficiently informed	26/26 (100)	40/42 (95)	66/68 (97)
Sufficiently supported	23/26 (88)	35/40 (88)	58/66 (88)
Regret of testing	2/28 (7)	4/42 (10)	6/70 (9)
Insurance problems	3/4 (75)	4/10 (40)	7/14*(50)

Not all items were scored by all 70 carriers
* only 14 carriers opted for insurance after genetic testing

Table 5:
Reported and perceived HNPCC related cumulative lifetime colorectal cancer risks by the MMR gene mutation carriers.

Reported HNPCC related colorectal cancer risk				
	Over-estimators n=4 (%)	Correct estimators n=31 (%)	Under-estimators n=32 (%)	P value
Age:				
≤50 years	0/35	24/35 (69)	11/35 (31)	<0.001
>50 years	4/32 (12)	7/32 (22)	21/32 (66)	
Affected:				
No	1/40 (2.5)	24/40 (60)	15/40 (37.5)	0.016
Yes	3/27 (11)	7/27 (26)	17/27 (63)	
Perceived colorectal cancer risk				
	High N=34 (%)	Intermediate n=13 (%)	Low n=23 (%)	P value
Age:				
≤50 years	18/36 (50)	7/36 (19)	11/36 (31)	0.91
>50 years	16/34 (47)	6/34 (18)	12/34 (35)	
Affected:				
No	19/42 (45)	9/42 (21)	14/42 (33)	0.7
Yes	15/28 (54)	4/28 (14)	9/28 (32)	

Not all respondents reported an HNPCC related colorectal cancer risk

Table 6:
Comparison of correct vs. incorrect reported HNPCC-related cumulative lifetime colorectal cancer risk in respondents affected with and without an HNPCC-related tumor and according to age.

Reported HNPCC related colorectal cancer risk								
	Correct n=31	Incorrect n=36	Univariate			Multivariate		
			OR	95% CI	P value	OR	95% CI	P value
Age:								
≤50 years	4/35	11/35	1	2.6 -	<0.001	1	1.7 -	0.004
>50 years	7/32	25/32	7.8	23.4		5.8	19.7	
Affected:								
No	24/40	16/40	1	1.5 -	0.008	1	0.6 -	0.27
Yes	7/27	20/27	4.3	12.5		2	7.0	

Not all respondents reported an HNPCC related colorectal risk

Table 7:
Long term cancer worry in the studied MMR gene mutation carriers.

Cancer worry	Low n=22 (%)	Intermediate n=31 (%)	High n=7 (%)	P value
Affected:				
No	11/42 (26)	27/42 (64)	4/42 (10)	0.47
Yes	11/28 (39)	14/28 (50)	3/28 (11)	
Gender:				
Male	8/24 (33)	13/24 (54)	3/24 (13)	0.82
Female	14/46 (30)	28/46 (61)	4/46 (9)	
Age:				
≤50yrs	10/36 (28)	23/36 (64)	3/36 (8)	0.65
>50yrs	12/34 (35)	18/34 (53)	4/34 (12)	
Children:				
No	2/8 (25)	5/8 (62.5)	1/8 (12.5)	0.91
Yes	20/62 (32)	36/62 (58)	6/62 (10)	
Reported HNPCC risk*:				
Underestimated	11/32 (34.5)	18/32 (56)	3/32 (9.5)	0.58
Correct	9/31 (29)	20/31 (64.5)	2/31 (6.5)	
Overestimated	2/4 (50)	1/4 (25)	1/4 (25)	
Perceived risk:				
Low	11/23 (48)	12/23 (52)	0	0.007
Intermediate	4/13 (31)	8/13 (61)	1/13 (8)	
High	7/34 (20.5)	21/34 (62)	6/34 (17.5)	

*Three respondents did not report an HNPCC related colorectal cancer risk

Chapter 4.

Discussion

The diagnosis of HNPCC represents a major challenge for geneticists and clinicians because of its geno- and phenotypic heterogeneity[191]. In my PhD thesis, I have attempted to define the spectrum of mutations at the main MMR genes causing HNPCC, and the establishment of genotype-phenotype correlations to allow clinical selection of families, guide their mutation analysis, and delineate surveillance protocols based on the specific genetic lesion.

In **chapter 2**, we addressed the molecular genetic analysis of HNPCC. We showed that the vast majority of 'classical' HNPCC families is due to *MLH1* or *MSH2* mutations. *MSH6* and possibly *PMS2* mutations cause a more atypical phenotype and are responsible for only a small proportion of classical families. Based on our observations, it is unlikely that other major HNPCC genes will be found in the future. Failure of mutation detection in the major MMR genes in Amsterdam criteria positive families is likely to be due to: 1. marginal fulfilment of the clinical criteria in colorectal cancer only, true mutation-negative families; 2. wrong selection of patients to be tested from otherwise mutation-positive families (e.g. phenocopies); 3. failure of mutation detection techniques in mutation-positive families.

Colorectal cancer is a relatively common tumour type. Families may therefore fulfil the Amsterdam criteria by chance or because of additional susceptibility factors. Also, within families with a *MLH1*, *MSH2*, or *MSH6* mutation, "sporadic" colorectal cancer cases may occur. In my opinion, extensive and detailed pedigree analysis is still the cornerstone for the selection of individuals for genetic testing for HNPCC. The establishment of a detailed family history is a time consuming and painstaking exercise, but it additionally allows the selection for testing for other susceptibility factors for colorectal cancer. In classical HNPCC families mutation analysis is indicated and does not require preselection by microsatellite instability tests (MSI) or immunohistochemical (IHC) analysis, although IHC analysis can direct mutation analysis. MSI and IHC analyses of tumours are in particular helpful for selection for mutation analysis within families not or marginally fulfilling the Amsterdam criteria. For selection for MSI and IHC analysis the revised Bethesda guidelines are very useful. However, I advocate liberal inclusion for MSI and IHC analysis. Some practical guidelines for the management of HNPCC and HNPCC-like families based on our own experience and on the literature are formulated in Figure 10. IHC and/or MSI analysis as a pre-screen tool for MMR gene analysis can also be routinely offered to certain patient-groups like colorectal patients diagnosed under the age of 50 years or patients with multiple tumours. However, some form of pre-test counselling of patients is requisite, since absent staining of MSH2 and/or MSH6 are strongly indicative for the presence of a germline defect and patients should be made aware of this before testing.

Our discovery of large genomic rearrangements in *MLH1* and *MSH2* dramatically increased mutation detection rate at these major HNPCC genes. These findings underscore the need to implement molecular approaches capable to detect large genomic rearrangements. To date, many DNA-diagnostic laboratories have added PCR-based mutation techniques for large genomic deletions, e.g. MLPA[77], to their routine mutation detection techniques[35, 232, 279]. Notably, MLPA likely would have missed the 10Mb paracentric inversion of chromosome 2p we detected by Southern blot analysis in a classical HNPCC kindred, as reported in **chapter 2.3**. The identification of this inactivating *MSH2* mutation within this family was of medical benefit, as it enabled the selection of a suitable kidney donor for one of the relatives[189]. I advocate that DNA-diagnostic laboratories refine their search for atypical mutations (genomic deletion, inversions, and insertions) within families with a high chance of carrying a *MLH1*, *MSH2* and/or *MSH6* mutation which tested negative by conventional mutational analysis.

In our search for genomic rearrangements, we also identified a common North-American deletion in *MSH2*, accounting for as much as ~10% of our cohort (**chapter 2.1 and 2.2**). The combination of clinical and molecular genetic data indicated that all families carrying this *MSH2* exon 1-6 deletion descend from one ancestor who lived in Germany in the 18th century. The identification of this founder mutation enabled a conclusive genetic test in large numbers of HNPCC risk carriers in North America.

The second main diagnostic challenge of HNPCC is represented by its clinical variability. As reported in **chapter 3.1 and 3.2**, the cancer phenotype of *MSH6* mutation carriers differs from that of *MLH1* and *MSH2*. *MSH6* mutations are associated with a delayed age of onset of colorectal and endometrial cancer (55-57 years vs. 43-44 years, and 54 years vs. 48-49 years, respectively). Moreover, the cumulative lifetime risk of endometrial cancer in female mutation carriers is increased compared to *MLH1* and *MSH2* mutation carriers (71% vs. 27-40% at 70 years of age), while the risk of colorectal cancer is reduced (30% vs. 53-68% at 70 years). These observations will aid the clinical recognition of *MSH6* mutation positive families, and contribute to the development of *MSH6*-tailored cancer recommendations for screening and preventative options.

Above all, HNPCC challenges families who have to live with the disease and its threats. The availability of a genetic test for HNPCC adds to this complexity, but also offers hope for interference.

The detection of a pathogenic *MLH1*, *MSH2* or *MSH6* mutation within a family enables relatives conclusive genetic testing, allowing them to obtain certainty about their cancer risks. The use of predictive genetic testing is influenced by several factors, among which

the availability of effective and acceptable risk-reducing interventions. Former studies reported a high uptake for predictive genetic testing for HNPCC (75-81%)[12, 272]. However, we believe that these studies are likely to be biased in favour of a high uptake as only individuals who had agreed to register for research purposes were included. It is conceivable that these individuals are also willing to undertake genetic testing, and are thus not representative for HNPCC patients at large. We studied the use of genetic testing in families with a known *MLH1*, *MSH2* or *MSH6* mutation in a clinical setting (**chapter 3.3**). Fifty-seven percent of 308 individuals with an *a priori* 50% risk of carrying the germline mutation opted for genetic testing, which is approximately 20% lower than in the Finnish study. Hence, in our study a considerable portion of risk carriers refrained from genetic testing. This was confirmed by Hadley et al.[85] and Ponz de Leon et al.[235], who found a use of genetic testing of respectively 51% and 44% among 50% risk carriers. Reasons for not testing may include lack of information, fear for cancer for themselves or their children, fear for screening procedures or fear for social discrimination. In the Netherlands, knowledge of a genetic predisposition for cancer is passed among relatives. In our study, the vast majority of gene carriers appreciated the way they were informed about the possibility of genetic testing, and the information they received during counselling (88% and 97%, respectively; **chapter 3.4**). Of note, little is known about the views and feelings of relatives who refrain from counselling and testing. Both a very high and a very low cancer concern may prevent individuals from seeking genetic advice. In risk carriers for Hereditary Breast Ovarian Cancer (caused by BRCA1 or BRCA2 gene mutations), Lerman et al.[154] found that individuals who refrained from obtaining their personal genetic test result had higher depression levels than those who chose to learn their personal test result (irrespective of outcome). However, Lodder et al.[173] found no difference in anxiety and depression levels between healthy women who opted for testing for the family-specific BRCA1 or BRCA2 mutation and women who refrained from testing. The latter were higher educated, more often childless and more reluctant towards prophylactic surgery. Having children was also positively correlated with testing for HNPCC in our study, indicating that other factors than cancer fear are also important in the decision to opt for genetic testing.

Colonoscopy is experienced as a burdensome screening technique among HNPCC mutation carriers diagnosed at our department (**chapter 3.4**). The development of less invasive screening and preventative options is clearly needed. The availability of less repulsive preparation fluid would already represent a significant improvement. In the future, testing for genetic markers in stool or other non-invasive approaches such as virtual colonoscopy may become alternatives for colonoscopy[9, 50, 79, 157, 292, 293]. Nonetheless, the

above mentioned techniques share the drawback that once a polyp or a given genetic marker is detected, conventional colonoscopy has to be performed after all to allow polypectomy. In the future, significant advances in *in vivo* optical imaging based on specific molecular markers will allow the selective identification of dysplastic lesions to guide polypectomy[194]. The elucidation of the molecular mechanisms underlying tumour formation and progression will also result in the development of tailor-made pharmacological intervention. However, availability of such 'smart drugs' may not be expected in the near future. Also, the characterisation of peptides specific for MSI-positive colorectal cancer cells, that can be recognised by T cells, fuels hope for the development of a prophylactic vaccine for HNPCC carriers on the long term[250].

Finally, fear for financial and social discrimination is an important reason for not opting for genetic testing[85]. In the Netherlands, health insurance companies fund genetic counselling, genetic testing and the various screening strategies. The Dutch law prohibits discrimination of employees and exclusion from health insurance on grounds of genetic susceptibility for cancer. Since 1995 the Association of Insurance Companies (The Hague) has agreed on a moratorium which ensures that a clients' genetic susceptibility is not taken into account in case of insurances for disablement up to €32.000 in the first year and €22.000 in the following years, and life-insurances up to €160.000. As a result of this policy, no problems with health insurance or employment were reported among Dutch MMR gene mutation carriers, though 4 out of 10 healthy carriers who opted for life insurance or mortgage experienced some kind of restraint (**chapter 3.4**).

Additional research on the reasons for and psychosocial impact of refraining from genetic testing is needed. The more since, 88% of proven Dutch HNPCC carriers diagnosed at our department opted for regular colonoscopic screening after genetic testing, compared with 33% before testing (**chapter 3.4**). The significant impact of genetic testing on surveillance will lead to a considerable decrease of HNPCC-related cancer morbidity and mortality. Also, we showed that most tested individuals are able to cope with their cancer predisposition at a mean follow-up of three and a half years (**chapter 3.4**). Ten percent of the studied mutation carriers had high cancer concerns and 9% felt regret of testing. This was associated with a high-perceived colorectal cancer risk, possibly induced by recent cancer related events in the family. Years after genetic testing, some mutation carriers may thus need additional counselling and psychological support. Based on our findings and experience, I advocate that from the moment a family is identified as at risk for HNPCC, ongoing psychological support and counselling facilities should be available to the individual family members. This can easily be implemented in a multidisciplinary family cancer clinic facility.

In conclusion, future challenges for clinical geneticists and other professionals active in HNPCC diagnosis and clinical management include: further unravelling of the molecular mechanisms underlying this susceptibility, translation of this 'molecular knowledge' into preventative tools, the development of less burdensome screenings options, the delivery of optimal multidisciplinary care, and the contribution (by education and counselling) to a social climate that enables individuals at risk to make an informed and free choices about genetic testing.

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Summary

HNPCC is a hereditary cancer syndrome with an increased risk of tumours of the colorectum and endometrium. The cumulative lifetime risk of colorectal cancer is 60-90% and of endometrial cancer 30-40%. Also cancers of the stomach, small intestine, biliary system, urinary tract, ovary, brain and skin may occur among HNPCC patients though their relative risks generally do not exceed 10%. From a clinical point of view, HNPCC families are characterised by the Amsterdam criteria: two first degree relatives in two generations with colorectal, endometrial, urinary tract, or small bowel cancer, with at least one being diagnosed under the age of 50 years; FAP has to be excluded and the diagnoses must be pathologically verified. HNPCC is caused by mutations in mismatch repair (MMR) genes, involved in the repair of replication errors in DNA. Mutations in at least 4 different mismatch repair (MMR) genes are described, namely *MSH2* and *MLH1* in the majority of cases and, more rarely, *MSH6* and *PMS2*. Former studies failed to detect germline mutations in *MSH2* and *MLH1* in a still considerable proportion (36-55%) of the Amsterdam criteria positive families.

To answere the question whether unresolved HNPCC families were due to a lack of sensitivity of *MSH2* and *MLH1* mutation detection techniques, or to mutations of *MSH6* or other genes. We analysed a cohort of 59 families, selected by Prof H.T. Lynch as suspected for HNPCC, for *MSH2*, *MLH1* and *MSH6* mutations. In this cohort we detected a mutation in 88% of the families. This high mutation detection rate is partly due to the optimal clinical selection. Another reason was that the families were analysed for genomic rearrangements at the major MMR genes. In 24% of the families a genomic rearrangement was found. One of these appeared a common *MSH2* deletion accounting for ~10% of our cohort. Genealogical, molecular and haplotype studies showed that this common deletion represents a North-American founder mutation that originates from Germany and could be traced back to the 18th century. We also identified of a 10Mb paracentric inversion of chromosome 2p inactivatig the *MSH2* gene.

A small portion of the classic HNPCC families negative for *MSH2* or *MLH1* mutations is caused by *MSH6* mutations. We showed a delayed age of onset of colorectal and endometrial cancer in *MSH6* mutation carriers (55-57 years vs. 43-44 years and 54 years vs. 48-49 years respectively). Moreover, the cumulative lifetime risk of endometrial cancer in female carriers is increased when compared to *MLH1* and *MSH2* mutation carriers (71% vs. 27-40% at 70 years of age), while the risk of colorectal cancer is reduced (30% vs. 53-68% at 70 years). This is very important in view of the selection of families for

MSH6 mutation analysis and in view of the screening and preventative advices for female *MSH6* carriers, that may be modulated.
In conclusion, we showed that the vast majority of 'classical' HNPCC families is due to *MLH1* or *MSH2* mutations. *MSH6* and possibly *PMS2* mutations cause a more atypical phenotype and are responsible for only a small proportion of classical families. Based on our observations, it is unlikely that other major HNPCC genes will be found in the future.

The detection of a familial MMR gene mutation, enables individuals at risk to obtain certainty about whether they inherited the HNPCC susceptibility or not. HNPCC is inherited in an autosomal dominant fashion. Therefore, children of MMR gene mutation carriers have 50% risk to carry the familial mutation. If tested negative, relatives can be dismissed from screening. MMR gene mutation carriers can benefit from cancer screening. Regular colonoscopic screening drasticly decreases colorectal cancer risk in HNPCC risk carriers.
We performed a pedigree study based on 18 families with a known *MLH1*, *MSH2* or *MSH6* mutation diagnosed in a clinical setting. Fifty-seven percent of the 50% risk carriers opted for genetic testing at a mean follow-up of 42 months. As for other late onset genetic predispositions, testing was used more frequently by women (women vs. men = 62% vs. 51%; p=0.045), and by subjects with children (with children vs. without children = 70% vs. 45%; p=0.013). Hence, in a clinical setting a considerable portion of the risk carriers refrains from the genetic test. Reasons for not testing may include lack of information, fear for cancer for themselves or their children, fear for screening procedures or fear for social discrimination. More data on the reasons and psychosocial impact of refraining from genetic testing are needed. The more since 88% of proven Dutch HNPCC carriers opted for regular colonoscopic screening compared to 31% before testing at our department. This leads to a considerable decrease of their colorectal cancer risk. Despite the positive impact of screening protocols, the majority of mutation carriers diagnosed at our department experienced colonoscopic screening as a burden. An important task for the professionals involved in the care for HNPCC carriers is to develop and evaluate less invasive screening and preventive options.
It has been previously shown that most tested individuals are satisfied about the counselling procedure and are able to cope with genetic testing for a cancer predisposition, including HNPCC, on the short-term (one year follow-up). At a follow-up of three and a half years, we also found that the majority of the carriers tested at our department was satisfied about the information and support during counselling. Also, the majority can cope with their cancer risks. Ten percent of the carriers experienced high

cancer worry and 9% regrets having taken the test. High cancer worry was significantly associated with a high perception of colorectal cancer risk, possibly induced by cancer related events in the family. Our data indicate that from the day suspicion of HNPCC arises in a family, relatives should have ongoing acces to counselling and psychological support. Finally, 4 out of 10 healthy carriers tested at our department who opted for life insurance, disability insurance or mortgage, experienced some kind of restraint. The latter represents a potential threat to the accessibility of genetic testing for cancer susceptibilities.

The challenge for clinical geneticists and others working in the field of hereditary cancer, is to supply optimal multidisciplinary care on the short and long term, to develop non-invasive screening and prevention options, and to contribute to a social climate necessary for individuals to make free and personal though educated choices relative to testing for genetic risk factors.

Samenvatting

HNPCC is een erfelijk kankersyndroom met een verhoogd risico op met name dikkedarm-, en baarmoederkanker. Het cumulatieve risico op dikkedarmkanker is 60-90% en op baarmoederkanker 30-40%, op de leeftijd van 70 jaar. Daarnaast komen soms andere vormen van kanker voor, namelijk maagkanker, dunnedarmkanker, urinewegtumoren, eierstokkanker, hersentumoren en huidkanker. Het risico op deze tumoren ligt meestal niet boven 10%. Om klinisch een familie met HNPCC te definiëren wordt veelal gebruik gemaakt van de *Amsterdam criteria*: drie familieleden, waarvan één eerste graads van de twee anderen, met dikkedarm-, baarmoeder-, urineweg-, of dunnedarm-kanker, bij minimaal één gediagnosticeerd onder de 50 jaar, waarbij FAP (familiaire adenomateuze polyposis) is uitgesloten en de diagnoses pathologisch bevestigd zijn.
HNPCC wordt veroorzaakt door afwijkingen (mutaties) in zogenaamde *mismatch repair* genen. De eiwitten die naar deze genen gemaakt worden, zijn betrokken bij de reparatie van fouten in het DNA die met name bij de DNA-verdubbeling (replicatie) ontstaan. Kiembaanmutaties in ten minste vier verschillende genen zijn beschreven als oorzaak van HNPCC, namelijk in *MLH1* en *MSH2* en in mindere mate in *MSH6* en *PMS2*. In eerder verrichte studies werd in 45-64% van de Amsterdam criteria positieve families een *MLH1*- of *MSH2*-mutatie gevonden. Dit betekent dat in een aanzienlijk aantal families geen moleculaire diagnose werd gesteld. Om na te gaan of dit veroorzaakt werd doordat in deze families sprake was van een mutatie in *MSH6* of een ander HNPCC-gen of door het tekort schieten van de mutatie-analyse, onderzochten wij een groep van 59 families, door Professor H.T. Lynch geselecteerd op HNPCC, op *MLH1*-, *MSH2*- en *MSH6*-mutaties. In dit cohort vonden wij een mutatie in *MLH1* of *MSH2* in 88% van de families. Daarnaast bevatten 3 families een *MSH6*-mutatie. De hoge mutatie-detectie is toe te schrijven aan de goede klinische selectie van de families. Bovendien verrichtten wij naast de conventionele mutatie-analyse ook onderzoek naar genomische herrangschikkingen. Hierbij werd in 24% van de families een genomische herrangschikking van *MLH1* of *MSH2* gevonden. Een van de genomische deleties in *MSH2* bleek een Amerikaanse *founder*-mutatie. Door middel van uitgebreid genealogisch en moleculair onderzoek kon deze mutatie teruggevoerd worden op een Duitse immigrantenfamilie, welke in het begin van de 18^{e} eeuw naar Amerika emigreerde. Ook werd een paracentrische inversie van chromosoom 2 ontdekt, die inactivatie van *MSH2* veroorzaakt.
In een klein gedeelte van de klassieke HNPCC-families wordt een mutatie in *MSH6* gevonden. Door middel van analyse van 20 *MSH6*-families, waaronder een zeer grote Nederlandse familie, toonden wij aan dat de leeftijd bij het onstaan van de darm-, en

baarmoederkanker in *MSH6*-mutatiedragers significant hoger is dan in *MLH1*- en *MSH2*-mutatiedragers (respectievelijk 55-57 jaar vs. 43-44 jaar en 54 jaar vs. 48-49 jaar). Daarnaast is het cumulatieve risico op dikkedarmkanker op de leeftijd van 70 jaar voor vrouwelijke *MSH6*-draagsters lager en het risico op baarmoederkanker hoger dan voor *MLH1*- en *MSH2*-mutatiedragers/draagsters (30% vs. 53-68% and 71% vs. 27-40%). Dit is van belang voor de selectie van families voor mutatie-analyse en voor het geven van adviezen voor controle en preventie.
Concluderend toonden wij aan dat verreweg het merendeel van de klassieke HNPCC-families het gevolg is van mutaties in *MLH1* of *MSH2*. *MSH6*- en mogelijk *PMS2*-mutaties geven een atypischer fenotype en veroorzaken maar een klein deel van de klassieke families. Bovendien lijkt het onwaarschijnlijk dat er naast de nu bekende HNPCC-genen nog een klassiek HNPCC-gen van betekenis bestaat.

Wanneer in een familie met HNPCC een ziekteveroorzakende mutatie in *MLH1*, *MSH*2 of *MSH*6 gevonden wordt, is het mogelijk familieleden te testen op de aanwezigheid van deze aanleg. HNPCC erft autosomaal dominant over. Dit betekent dat kinderen van iemand met een *MLH1*-, *MSH2*- of *MSH6*-mutatie 50% kans hebben de aanleg voor kanker te hebben geërfd. Wanneer een familielid de familiaire aanleg niet bij zich draagt, heeft hij of zij niet het familiaire risico op kanker en kan de aanleg ook niet doorgeven of doorgegeven hebben aan eventuele kinderen. Familieleden die de aanleg wel geërfd hebben, komen in aanmerking voor intensieve controle: colonoscopie iedere 1 à 2 jaar vanaf de leeftijd van 20-25 jaar, jaarlijks gynaecologische controle vanaf de leeftijd van 30-35 jaar, en controle van maag, dunnedarm en urinewegen afhankelijk van de familiegeschiedenis. Ook kunnen zij de aanleg aan hun eventuele kinderen doorgeven of gegeven hebben. Regelmatige darmcontrole brengt het risico op dikkedarmkanker bij HNPCC-mutatiedragers drastisch omlaag.
In 18 Nederlandse families met een bewezen *MLH1*-, *MSH2*- of *MSH6*- mutatie koos 57% van de personen met een *à priori* kans van 50% op de familaire aanleg voor de genetische test. Het wel of niet kiezen voor testen bleek gecorreleerd aan het risico op het hebben van de mutatie en het hebben van kinderen (wel kinderen vs. geen kinderen = 70% vs. 45%; p=0.013). Ook kozen vrouwen, net als bij het genetisch testen voor andere, later in het leven tot uiting komende aandoeningen, vaker voor testen (vrouwen vs. mannen = 62% vs. 51%; p=0.045). Hoewel dus het merendeel van de risicodragers geïnteresseerd is in genetisch testen, ziet ook een aanzienlijk deel hiervan af. Dit kan komen doordat zij niet goed zijn voorgelicht, door angst dat zij zelf of hun kinderen kanker zullen krijgen, of door angst voor de controles of voor sociale discriminatie. Het is belangrijk meer inzicht

te krijgen in de redenen waarom risicodragers afzien van testen, met name omdat van de op onze afdeling geteste dragers 88% gebruik maakt van colonoscopie, terwijl voor de genetische test 31% van hen regelmatig colonoscopie liet verrichten. Deze toename van dikkedarmcontrole brengt hun risico op het krijgen van dikkedarmkanker sterk omlaag. Ondanks dat dragers goed gebruik maken van de mogelijkheid om colonoscopie te laten verrichten, ervaart een groot deel van de dragers het onderzoek als onplezierig en zou het merendeel een andere manier van controle op prijs stellen (respectievelijk 57% en 71%). Een belangrijke taak voor de deskundigen is dan ook weggelegd voor wat betreft het ontwikkelen van minder belastende methoden van onderzoek en preventie.
Eerdere studies op het gebied van HNPCC gaven aan dat op de kortere termijn (follow-up van 1 jaar) het merendeel van de HNPCC-risicodragers tevreden is over de counselingsprocedure en goed om kan gaan met het hebben van de aanleg voor HNPCC. Op basis van vragenlijsten, ingevuld door 70 *MLH1*-, *MSH2*- en *MSH6*-mutatiedragers uit 24 Nederlandse families, blijkt dat ook op de langere termijn het merendeel van de dragers tevreden is over de tijdens de counseling ontvangen informatie en begeleiding. Bij een gemiddelde follow-up van 3,5 jaar maakt 10% van de dragers zich nog ernstig zorgen om hun kankerrisico, waarbij zes dragers (9%) zelfs spijt hebben van de genetische test. Grote kankerangst is significant gekoppeld aan het ervaren van een hoog eigen risico op dikkedarmkanker, wat mogelijk geïnduceerd wordt door het overlijden aan kanker van familieleden. Onze studie geeft aan dat vanaf het moment dat de verdenking van HNPCC in een familie rijst, de familieleden toegang moeten hebben tot counseling en psychologische ondersteuning, zowel op de korte als op de lange termijn. Tenslotte rapporteerden 4 van 10 gezonde mutatiedragers, die een levensverzekering, arbeidsongeschiktheidsverzekering of hypotheek wilden afsluiten, hierbij problemen te hebben ondervonden. Sociale discriminatie, of de angst daarvoor, kan een serieuze bedreiging vormen voor de vrije keuze van risicodragers voor het genetisch testen op een aanleg voor kanker.
De uitdaging voor deskundigen op het gebied van kankergenetica is om goede multidisciplinaire zorg te bieden, nieuwe minder invasieve controle- en preventiemogelijkheden te ontwikkelen en bij te dragen aan een sociaal klimaat dat nodig is om voor betrokken personen een vrije keus ten aanzien van testen op een genetisch risico op HNPCC te waarborgen.

List of tables and figures

Tables.

Table 1.	Proven and "candidate" HNPCC genes.	14
Table 2.	The Amsterdam criteria for the clinical diagnosis of HNPCC Families.	14
Table 3.	The Bethesda guidelines to select for MSI testing of colorectal tumours.	16
Table 4.	Selected markers for MSI analysis in colorectal cancer and criteria for the interpretation of MSI.	16
Table 5.	Cumulative lifetime risks of colorectal, endomterial, stomach, ovarian, urothelial, small bowel, biliary tract cancer and brain tumours in *MLH1*, *MSH2* and *MSH6* mutation carriers compared to the population risks.	33
Table 6.	Mean age of onset of colorectal, endometrial, stomach, ovarian, urothelial, small bowel, biliary tract cancer and brain tumours in *MLH1*, *MSH2* and *MSH6* mutation carriers.	33
Table 7.	Screening advises in HNPCC(-like) families.	38
Table 8.	Comparison of MSI and IHC analysis in 154 tumours of 119 families analysed for *MLH1*, *MSH2* and sometimes *MSH6* mutations.	51
Table 9.	Logistic regression model for selection for *MSH2*, *MLH1* mutation analysis.	51

Figures.

Figure 1.	A.S. Warthin (1866-1931), H.T. Lynch.	13
Figure 2.	The crystal structure of the MutS dimer.	17
Figure 3.	Schematic representation of the eukaryotic mismatch repair (MMR) pathways.	22
Figure 4.	Schematic representation of the Knudson model.	24
Figure 5.	The adenoma-carcinoma sequence.	26
Figure 6.	A schematic representation of the Wnt-signalling pathway.	27
Figure 7.	A schematic representation of the KRAS-signalling pathway.	28
Figure 8.	A schematic representation of the TGFβ-signalling pathway.	29
Figure 9.	A schematic representation of the p53-pathway.	30
Figure 10.	A practical flowchart for the management of individuals with multiple or early onset HNPCC-associated tumours, and of families with clustering of HNPCC-associated tumours.	50

Curriculum Vitae

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