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Molecular and Clinical Characteristics of *MSH6* Variants: An Analysis of 25 Index Carriers of a Germline Variant

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The *MSH6* gene is one of the mismatch-repair genes involved in hereditary nonpolyposis colorectal cancer (HNPCC). Three hundred sixteen individuals who were known or suspected to have HNPCC were analyzed for *MSH6* germline mutations. For 25 index patients and 8 relatives with *MSH6* variants, molecular and clinical features are described. For analysis of microsatellite instability (MSI), the five consensus markers were used. Immunohistochemical analysis of the MLH1, MSH2, and MSH6 proteins was performed. Five truncating *MSH6* mutations, of which one was detected seven times, were found in 12 index patients, and 10 *MSH6* variants with unknown pathogenicity were found in 13 index patients. Fourteen (54%) of 26 colorectal cancers (CRCs) and endometrial cancers showed no, or only weak, MSI. Twelve of 18 tumors of truncating-mutation carriers and 3 of 17 tumors of missense-mutation carriers showed loss of MSH6 staining. Six of the families that we studied fulfilled the original Amsterdam criteria; most families with *MSH6,* however, were only suspected to have HNPCC. In families that did not fulfill the revised Amsterdam criteria, the prevalence of *MSH6* variants is about the same as the prevalence of those in *MLH1/MSH2*. Endometrial cancer and/or atypical hyperplasia were diagnosed in 8 of 12 female carriers of *MSH6* truncating mutations. Most CRCs were localized distally in the colon. Although, molecularly, missense variants are labeled as doubtfully pathogenic, clinical data disclose a great resemblance between missense-variant carriers and truncating-mutation carriers. We conclude that, in all patients suspected to have HNPCC, *MSH6*-mutation analysis should be considered. Neither MSI nor immunohistochemistry should be a definitive selection criterion for *MSH6*-mutation analysis.

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC [MIM 114400; MIM 114500]) is an autosomal dominant disorder, characterized by the early onset of gastrointestinal and urogenital cancers—in particular, colorectal cancer (CRC) and endometrial cancer. Germline mutations in four mismatch-repair (MMR) genes have thus far been associated with HNPCC: *MLH1, MSH2, MSH6,* and *PMS2*. Most of these mutations have been detected in *MLH1* and *MSH2*. The loss of MMR function—particularly, when due to *MLH1* and *MSH2* mutations—leads to somatic DNA replication errors in repetitive sequences, known as "microsatellites" (Aaltonen et al. 1993). Microsatellite instability (MSI), therefore, is the hallmark of the majority of cancers associated with HNPCC. In *MLH1*- and *MSH2*-mutation carriers, MSI has been found in >90% of CRCs and in >75% of endometrial cancers (Aaltonen et al. 1993; Peltomäki et al. 1993; Kowalski et al. 1997).

In 1995, Drummond et al. (1995), Palombo et al. (1995), and Papadopoulos et al. (1995) reported the identification of a gene that is now referred to as "*MSH6*" (MIM 600678). Whereas *MSH2* in combination with *MSH6* is involved in repair of single-nucleotide mismatches, *MSH2* in combination with *MSH3* is mainly involved in the repair of small insertions and deletions (Drummond et al. 1995; Palombo et al. 1995; Acharya et al. 1996; Marsischky et al. 1996). The first reports of human *MSH6* germline mutations were by Akiyama et al. (1997) and Miyaki et al. (1997). They described *MSH6* germline mutations in members of two families that did not fulfill the Amsterdam (I) criteria for HNPCC (Vasen et al. 1991). When looking for *MSH6* mutations in families fulfilling those criteria (i.e., typical families with HNPCC), most investigators did not find any such mutations (Papadopoulos et al. 1995; Liu et al. 1996; Akiyama et al. 1997; Kolodner et al. 1999; Wang et al. 1999). However, Wijnen et al. (1999)

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and Wu et al. (1999) reported the occurrence of *MSH6* germline mutations in a small, though significant, fraction of families that fulfilled the revised Amsterdam criteria (Vasen et al. 1999). An explanation for the lower frequency of *MSH6* mutations in such families with HNPCC may be that the loss of *MSH6* function causes only a partial MMR defect, so that the penetrance of the genetic predisposition may be lower than it is in the case of mutations in either *MLH1* or *MSH2*. Furthermore, Wu et al. (1999) showed that most of the patients who carried an *MSH6* mutation had tumors displaying an MSI-low (MSI-L) phenotype. Until now, however, MMR-gene–mutation analysis has been performed predominantly in patients with MSI-high (MSI-H) tumors. This may also explain the small numbers of *MSH6* mutations found.

Most of the *MSH6* germline mutations that have thus far been described occur in families that are only suspected to have HNPCC (Kolodner et al. 1999; Shin et al. 1999; Verma et al. 1999; Wijnen et al. 1999; Wu et al. 1999; Planck et al. 1999). In addition, current data suggest that the age at onset of CRC and of endometrial cancer is higher in *MSH6*-germline-mutation carriers than in the families with *MLH1* or *MSH2* germline mutations and that these kindreds display a high frequency of endometrial cancer or atypical endometrial hyperplasia (Miyaki et al. 1997; Wijnen et al. 1999; Wagner et al. 2001).

The aim of the present study is to describe the molecular and clinical findings in all carriers of germline *MSH6* variants whom we have detected up to January 2001, to further define the molecular and clinical implications of *MSH6* germline variants.

Subjects and Methods

Patient Population

Patients, who were analyzed for germline mutations in *MSH6*, were retrieved from two sources. The first source comprised the patients included in a study of the role that MMR-gene mutations played in individuals who were suspected to have HNPCC. Two hundred six patients were referred, for this study, by hospital doctors and general practitioners in the north of the Netherlands. All of the patients referred fulfilled one of the four inclusion criteria, which are given in Appendix A. Information about the family history (up to and including the third-degree relatives) was collected, and blood samples were taken from those patients who gave informed consent after written and verbal pretest counseling. Formalin-fixed paraffin-embedded tumor material was obtained and was revised. With the permission of the individuals involved, medical records of affected family members were collected, if possible, to verify the nature of reported tumors. The institutional review boards of the University Hospital Groningen and other participating hospitals approved the study. The participating patients were informed of the results of the genetic test, if they wished to be; in that case, they received verbal posttest counseling and a written summary.

The second source comprised 110 patients referred to the Department of Medical Genetics of the University Hospital Groningen because of suspicion of hereditary CRC. Half of the patients in this group fulfilled one of the criteria mentioned in Appendix A; those who did not were of more advanced age at diagnosis or were referred from other genetic departments in the absence of sufficient information about their families. In the diagnostic setting, patients with MSI-L tumors were analyzed for *MSH6* mutations, as were the patients with MSI-H tumors but without detected germline mutations in *MLH1* or *MSH2*.

Mutation Analysis

Mutation analysis of the *MSH6* gene in DNA obtained from peripheral blood lymphocytes was performed by denaturing gradient-gel electrophoresis. Variants were confirmed by direct sequencing of independently amplified PCR products, as described elsewhere (Wu et al. 1997, 1999). Sequencing was performed with an ABI PRISM 377 DNA sequencer (PE Biosystems). In almost all patients, the *MLH1* and *MSH2* genes were analyzed as well. Mutations in these genes are reported here only when occurring concurrent with an *MSH6* variant. No search for germline genomic deletions was performed. Two hundred unaffected Dutch individuals served as a control group.

MSI Analysis

MSI primers were used in the manner proposed at the international workshop on HNPCC, in Bethesda (Boland et al. 1998). These include two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, and D17S250). Another mononucleotide marker (BAT40) was also used in all patients, since MSI in *MSH6* carriers has been mainly observed at mononucleotide markers (Verma et al. 1999; Wijnen et al. 1999). DNA was extracted from formalin-fixed paraffin-embedded tumor sections. Control DNA was obtained either from normal tissue from paraffin-embedded sections or from peripheral blood of the same patients. PCR products were analyzed in 6% denaturing polyacrylamide gels on an LKB. A.L.F. DNA sequencer (Pharmacia). For data analysis, the DNA fragment analyzer (Pharmacia) was used. Tumors were classified as MSI-H when two or more of the five consensus markers showed MSI and as MSI-L when zero or one of the markers showed MSI. Since a limited number of markers

were analyzed, the classification "MSI stable" will not be used.

Immunohistochemistry

For MSH6, MLH1, and MSH2 immunohistochemistry, formalin-fixed paraffin-embedded tissue sections were collected from the proved carriers of *MSH6* variants. Three-micrometer sections of deparaffinized tumor tissue were mounted on slides with APES coating (3-aminopropyl triethoxysilan; Sigma-Aldrich). Antigen retrieval was performed by autoclave heating. The slides were incubated with mouse monoclonal antibodies to MSH6 (1:200) (clone 44; BD Transduction Laboratories), to MLH1 (1:500) (clone G168-728; PharMingen), or to MSH2 (1:100) (Ab-2; Calbiochem). The slides were successively treated with rabbit–anti-mouse peroxidase and with goat–anti-rabbit peroxidase. The chromogen was diaminobenzidine, and counterstaining was performed with hematoxylin. Protein expression in normal tissue next to the tumor served as internal positive control. The sections were scored as either negative (i.e., in the absence of detectable nuclear staining of tumor cells) or positive for MSH6, MLH1, and MSH2 expression. Scoring of the tumor staining was performed without knowledge of the MSI or of mutation status.

Results

Patient Population

A total of 316 apparently unrelated patients were analyzed for *MSH6* mutations. Of these 316 patients, 261 (106 men and 155 women) met the inclusion criteria, which are given in Appendix A. When dividing these patients according to the four criteria, 187 patients, of which 149 had CRC and 38 had endometrial cancer diagnosed at the age of <50 years, fulfilled criterion 1. Twelve patients either had an HNPCC-related tumor and a first-degree relative with CRC or endometrial cancer, one of which was diagnosed at the age of <50 years, or had CRC or endometrial cancer and a first-degree relative with an HNPCC-related tumor, one of which was diagnosed at the age of <50 years; 54 patients had multiple HNPCC-related tumors; and 8 patients had a colorectal adenoma.

Mutation Analysis

In 23 of the 261 patients, *MSH6* germline variants were found. These include five variants that have been reported elsewhere (Wu et al. 1999). Twenty-seven of the 261 patients belonged to families with HNPCC that was considered to be established, by fulfillment of the original Amsterdam criteria (Vasen et al. 1991), but without germline mutations in *MLH1* and *MSH2*. Four of these 27 patients had an *MSH6* variant. An additional 55 patients analyzed in the diagnostic setting did not fulfill the inclusion criteria. In two of these patients, an *MSH6* germline variant was found, bringing the total number of index carriers of an *MSH6* germline variant to 25.

The variants observed included five different truncating mutations that occurred in 12 index patients (group A; table 1). Seven patients shared the same truncating mutation in exon 4a. Haplotype analysis with four intragenic single-nucleotide polymorphisms, located in exons 2–4 and 7 of the *MSH6* gene, indicated a founder effect (data not shown). The remaining 10 variants, occurring in 13 index patients (group B), were missense variants (table 1). None of these variants was observed in the 200 control individuals. Six of these variants resulted in substitution of amino acids belonging to different polarity groups, whereas the other four variants resulted in substitution of amino acids belonging to the same polarity group (table 1). One of the missense variants was found in a patient who also carries an *MLH1* truncating mutation. This individual (patient 15) was therefore excluded from group calculations. Another 10 sequence variations of *MSH6* were found in six different exons and have been reported as polymorphisms to the International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer database. The allele frequencies varied from 49.7% to 0.3% in the Dutch population.

MSI Analysis

Various types of tumor material were available from the 25 index patients and the 8 family members, all of whom were known to carry an *MSH6* variant. Tumor material available for MSI analysis comprised 22 CRCs and 6 endometrial cancers (tables 2 and 3); 2 transitional cell cancers (1 of the renal pelvis and 1 of the bladder); 1 duodenal, 1 gastric, and 1 ovarian cancer; 3 colorectal adenomas; and 1 endometrial hyperplasia. After patient 15 was excluded, 14 (54%) of the remaining 26 CRCs and endometrial cancers were MSI-L. In group A (table 2), 5 of 13 CRCs and all 3 endometrial cancers (all of which were from founder-mutation carriers) were MSI-L. In group B (table 2), five of eight CRCs and one of two endometrial cancers were MSI-L.

Immunohistochemistry

Immunohistochemical analysis of MLH1, MSH2, and MSH6 was possible in 35 different tumors of the *MSH6*-variant carriers, including, in this analysis, patient 15 (table 1). In group A, 12 (8 CRCs, 2 endometrial cancers, 1 renal pelvis cancer, and 1 duodenal cancer) of the 18 tumors showed clear absence of MSH6 staining; in 6 tumors (3 CRCs, 1 endometrial cancer, and 2 urothelial cancers), MSH6 staining was present. In group B, 13 (8

Table 1

Molecular and Immunohistochemical Data—and MSI Results—for the Patients with *MSH6* Mutations

Patient/Family Member[a] [Sex]	Cancer[b] [Age (years)]	Mutation(s)	Polarity Data[c]	MSI[d]	Immunohistochemical[e]		
					MSH6	MLH1	MSH2
Truncating mutation (group A):							
1 [F]	EC [46]	650insT		L	No	Yes	Yes
1+ [F]	CRC [37]	650insT		L	ND	ND	ND
2 [M]	CRC-L [41]	650insT		L	No	Yes	Yes
3 [F]	CRC-L [59], EC [65]	650insT		L, L	No, yes	Yes, yes	Yes, yes
4 [F]	CRC-L [46]	650insT		L	Yes	Yes	Yes
5 [F]	EC [50], CRC-R [50], CRC-L [83]	650insT		ND, H	ND, no	ND, yes	ND, yes
5+ [F]	EC [55]	650insT		L	No	Yes	No
5++ [M]	CRC-L [37]	650insT		H	No	Yes	Yes
6 [F]	CRC-R [45], CRC-L [53], ST [62]	650insT		ND, H, ND	ND, failed, ND	ND, failed, ND	ND, failed, ND
7 [F]	CRC-L [51], DUOD [51], CAH [51]	650insT		ND, L, ND	ND, no, ND	ND, yes, ND	ND, yes, ND
8 [M]	CRC-R [38]	3263insT		H	Focal	Yes	Yes
9[f] [F]	Pelvis [63], ureter [64], BC [65], CRC-L [77]	Gln1258stop		H, ND, L, L	No, yes, yes, no	Yes, yes, yes, yes	Yes, yes, yes, yes
10 [M]	CRC-R [55], CRC-L [55]	2672delT, 2674delT		H, H	Yes, no	No, no	Yes, yes
11 [M]	CRC-R [47]	2672delT, 2674delT		H	No	Yes	No
12 [F]	OC [35], CRC-R [54]	Gln1280stop		ND, H	ND, no	ND, yes	ND, yes
Missense mutation (group B):							
13 [F]	EC [45], CAH [45]	Ser144Ile	P→NP	H, H	Yes, yes	Yes, yes	Yes, yes
13+ [F]	CRC [49]	Ser144Ile	P→NP	H	No, no IC	Yes	No
14 [M]	CRC-L [48]	Ser144Ile	P→NP	L	Yes	Yes	Yes
15[g][F]	EC [49], CRC-L [53]	Tyr850Cys	P→P	H, H	No, yes	No, no	Yes, yes
16 [M]	CRC-L [63]	Ala1021Asp	NP→AP	L	Yes	Yes	Yes
17 [M]	CRC-R [43]	His1248Asp	BP→AP	L	Yes	Yes	Yes
18 [F]	CRC-R [37], CNAH [43]	Thr1219Ile	P→NP	H, ND	Yes, yes	Yes, yes	Yes, yes
19 [M]	CRC-L [42]	Thr1100Met	P→NP	L (AD)	Yes	Yes	Yes
20 [F]	CRC-R [36]	Ile725Met	NP→NP	L	Yes	Yes	Yes
21 [F]	CRC-L [36]	Gln522Arg	P→BP	L	Yes	Yes	Yes
21+ [F]	CRC-L [40]	Gln522Arg	P→BP	H	Yes	Yes	Yes
22 [F]	BRC [44], ST [64]	Val878Ala	NP→NP	L	Failed	Failed	Failed
23 [F]	AD-R [53]	Val878Ala	NP→NP	L	ND	Yes	Yes
24 [F]	CRC-L [45]	Val878Ala	NP→NP	L (AD)	Yes (AD)	Yes	Yes
25 [F]	EC [46], OC [46]	IVS10+40ins10bp		L, H	No, ND	Yes, yes	Yes, yes

[a] Affected family members are indicated by the addition of "+" or "++" after the index patients' number.

[b] CRCs are divided among left sided (CRC-L) and right sided (CRC-R); colorectal adenomas (ADs) are divided among left sided (AD-L) and right sided (AD-R). BL = bladder cancer; BRC = breast cancer; CAH = complex atypical hyperplasia; CNAH = complex non-atypical hyperplasia; DUOD = duodenal cancer; EC = endometrial cancer; OC = ovarian cancer; ST = stomach cancer.

[c] Amino acid substitutions: P = uncharged polar; NP = nonpolar; AP = acidic charged polar; BP = basic charged polar.

[d] L = MSI-L; H = MSI-H; ND = not done.

[e] ND = not done; no IC = no internal control.

[f] This patient had multiple tumors (namely: OC, at age 49 years; sigmoid, at age 55 years; EC, at age 57 years; right renal pelvis, at age 63 years; left ureter, at age 64 years; BL, at age 65 years; basocellular, at age 75 years; ureter, at age 77 years; two rectal carcinomas, at age 77 years).

[g] This patient also had an *MLH1* truncating mutation.

Table 2

MSI Results, for CRC and Endometrial Cancer, in *MSH6*-Mutation Carriers

Patient/ Family Member[a]	Marker[b]						MSI[c]
	Bat25	Bat26	Bat40	D2S123	D5S346	D17S250	
CRC-L:							
1+	…	…	…	…	…	…	L
2	…	…	…	Yes	…	…	L
3	…	…	Yes	…	…	…	L
4	…	…	…	…	…	…	L
5	Yes	Yes	Yes	…	Yes	Yes	H
5++	Yes	Yes	ND	…	…	Yes	H
6	…	Yes	Yes	Yes	…	Yes	H
9	…	…	Yes	…	…	…	L
10	Yes	Yes	Yes	Yes	Yes	Yes	H
13+	…	ND	…	Yes	Yes?	Yes	H
14	…	Yes	…	…	…	…	L
15[1]	…	…	Yes	ND	…	Yes	H
16	…	…	…	…	…	…	L
21	…	…	…	…	…	Yes	L
21+	Yes	…	…	…	…	Yes	H
CRC-R:							
8	Yes	Yes	Yes	…	…	Yes	H
10	Yes	Yes	Yes	Yes	Yes	Yes	H
11	Yes	Yes	ND	ND	Yes	Yes	H
12	Yes	Yes	ND	Yes	…	Yes	H
17	…	…	…	…	…	ND	L
18	Yes	Yes	ND	…	Yes	Yes	H
20	ND	…	…	…	…	…	L
EC:							
1	…	…	…	…	…	…	L
3	Yes	…	Yes	…	…	…	L
5+	…	…	…	…	…	…	L
13	…	…	…	Yes	ND	Yes	H
15[d]	…	…	± Loss	Yes	Yes	Yes	H
25	…	…	…	…	…	Yes	L

[a] CRCs are divided among left sided (CRC-L) and right sided (CRC-R); EC = endometrial cancer. Patients/family members with a truncating mutation are underlined; affected family members are indicated by the addition of "+" or "++" after the index patients' number.

[b] Yes = unstable marker; ND = not done.

[c] L = MSI-L; H = MSI-H.

[d] This patient also had an *MLH1* truncating mutation.

CRCs, 1 endometrial cancer, 2 adenomas, and 2 endometrial hyperplasias) of the 16 tumors showed MSH6 staining; in 2 endometrial cancers and 1 CRC, no staining was seen (in the latter, without a positive internal control).

Clinical Data

In addition to the 25 index patients (17 female and 8 male), 8 affected family members—7 females and 1 male (these family members are indicated in tables 1 and 2, by the index patients' number appended with either "+" or "++")—of six families were also shown to be *MSH6*-variant carriers. Although the mutation status of the mother of patient 1 has not been confirmed, she must be a carrier, because she is a connecting link with patient 1+. Four of the 12 families with truncating mutations (table 4) fulfill the revised Amsterdam criteria. One of the remaining eight families is a family with late-onset CRC (i.e., in which the CRCs of the index patient and two first-degree relatives in at least two successive generations were diagnosed at the age of >50 years). Two of the 12 families with missense variants fulfill the revised Amsterdam criteria (table 4). Ten of 15 carriers of truncating mutations and 9 of 12 carriers of missense variants had at least one first-degree relative, in most cases a parent, with an HNPCC-related tumor (table 4).

The mean age at diagnosis (i.e., at the time of the first cancer diagnosis) of CRC or endometrial cancer for all *MSH6*-germline-variant carriers who have been proved was 46 years for CRC and 52 years for endometrial cancer (table 5). Of the 22 women known to be *MSH6*-variant carriers, 9 had endometrial cancer, 1 had complex atypical endometrial hyperplasia, and 1 had complex non-atypical hyperplasia. Of only the 12 female carriers of truncating mutations, 7 had endometrial cancer, and 1 had endometrial hyperplasia.

Localization of the CRCs and MSI Status

Of the 23 known *MSH6*-variant carriers (excluding patient 15) with CRC, 14 (70%) had only a left-sided CRC, 6 (30%) had only a right-sided CRC, and 3 had both a left-sided and a right-sided CRC. Of the nine right-sided CRCs, seven tumors were available for MSI analysis, of which five, including four from truncating-mutation carriers (table 2), showed an MSI-H phenotype. Of the 16 left-sided CRCs, 14 tumors were available, of which 6, including 4 from truncating-mutation carriers and two from a missense-variant carrier, showed an MSI-H phenotype.

Discussion

In this study, 25 index patients and 8 relatives with *MSH6* germline variants are presented. The molecular and clinical data of these patients add to our understanding of the clinical implications of *MSH6* germline variants. However, new questions are raised concerning the role individual variants may play in carcinogenesis and concerning the mechanisms and the pathways that

Table 3

MSI Results for CRC and Endometrial Cancer

	No. of CRCs		No. of Endometrial Cancers		Total	
Mutation	MSI-L	MSI-H	MSI-L	MSI-H	MSI-L	MSI-H
Truncating	5	8	3	0	8	8
Missense	5	3	1	1	6	4
Total	10	11	4	1	14	12

Note.—The patient with the additional frameshift *MLH1* mutation was excluded.

Table 4

Family History of *MSH6*-Mutation Carriers

Patient	Cancer[a] [Age at Diagnosis (years)]					Family Features[b]
	Index Patient	Parents	Sibs/Children (Sex)	Second-Degree Relatives	Third-Degree Relatives (Sex)	
Truncating mutation:						
1	EC [46]	*EC* [56]	OC [35]	EC [52], lung, skin	OC [57], AD, *CRC-L* [37] (F), CRC	AC(II)
2	CRC-L [41]	BTC [82]		lung/brain [69], CA, CA, CA	CA	
3	CRC-L [59], EC [65]	CRC [74]/PRC [78]	CRC-R [55]	ST, BL, ST		Late onset
4	CRC-L [46]	CRC [41]	CRC-L [47], *AD* [49]	CRC [53], OC [50]		AC
5	EC [50], CRC-R [50], CRC-L [83], BRC [>80]	Kidney	*EC* [55], *CRC-L* [37] (M)	BRC [35]		AC(II)
6	CRC-R [45], CRC-L [53]	GI [68]	*ST* [49], *EC/CX* [50]			
7	CRC-L [51], DUOD [51], CAH [51]	Liver	CRC-L [41], LEU	Throat, CA		
8	CRC-R [38]			lung, lung, lung, lung	BRC, NHL [57], PG [51]	
9[c]	Multiple tumors [77], CRC-L [77]		BRC [49]	LEU		
10	CRC-R [55], CRC-L [55]		CRC [42]/AD, BRC [58]			
11	CRC-R [47]	CRC [51, 69, and 79]	ES [0]	CRC [73/81], lung [54], lung [56], EC [>70]	CRC [40]/EC	AC
12	OC [35], CRC-R [54]	Liver [71]				
Missense variant:						
13	EC [45]		*CRC-L* [49] (F)			
14	CRC-L [48]			CRC 78, CRC 76		
15[d]	EC [49] CRC-L [53]	CRC [72], brain	EC , *CRC-L* [32] (F)	OC, neck		AC(II)
16	CRC-L [63]		CRC [63], PRC [73]	PA [71]		
17	CRC-R [43]	BL [53]		CRC [42], lung [62], CA [70]		
18	CRC-R [37], CNAH [43]	CRC 36		CRC	CA, CA	AC
19	CRC-L [42]	CRC 41		CRC [81], CA [60]		AC
20	CRC-R [36]	CRC-R [79]/BRC [83], lung [58]	AD-L[41], AD [46]		CRC-L 52	
21	CRC-L [36]		*CRC-L* [40] (f), AD [42]	KA, RCC		
22	BRC [44], ST [64]	CRC [48]	CRC-L [49], AD-L[56]			
23	AD-R [53]	RCC [69]	AD-L [58], AD [25]	Liver, AD-L [64], CRC [60], OC [39], CRC [40]	OC [37]	
24	CRC-L [45]			ST [>50]	CRC [<50]	
25	EC/OC [46]			ST, CRC [80], BRC [>50]		

[a] Underlining indicates histologically verified, boldface italic indicates carrier, and italic indicates not a carrier. CRCs are divided among left sided (CRC-L) and right sided (CRC-R); colorectal adenomas (ADs) are divided among left sided (AD-L) and right sided (AD-R). BL = bladder cancer; BRC = breast cancer; BTC = biliary tract cancer; CA = cancer of unknown origin; CAH = complex atypical hyperplasia; CNAH = complex non-atypical hyperplasia; CX = cervical cancer; DUOD = duodenal cancer; EC = endometrial cancer; ES = esophageal cancer; GI = gastrointestinal cancer; KA = Kahler disease; LEU = leukemia; NHL = non-Hodgkin lymphoma; OC = ovarian cancer; PG = paraganglioma; PRC = prostate cancer; RCC = renal cell cancer; ST = stomach cancer.

[b] AC(II) = fulfilled (revised) Amsterdam criteria.

[c] This patient had multiple tumors (namely: OC, at age 49 years; sigmoid, at age 55 years; EC, at age 57 years; right renal pelvis, at age 63 years; left ureter, at age 64 years; BL, at age 65 years; basocellular carcinoma, at age 75 years; ureter, at age 77 years; and two rectal carcinomas, at age 77 years).

[d] This patient also had an *MLH1* truncating mutation.

Table 5

Mean Age at Diagnosis of the First CRC or Endometrial Cancer—and Variety of Tumors—in *MSH6*–Germline-Mutation Carriers

	Founder-Mutation Carriers		Truncating-Mutation Carriers		Missense-Mutation Carriers		All Patients	
Tumor	Age [Range] (years)	No. of Patients	Age [Range] (years)	No. of Patients	Age [Range] (years)	No. of Patients	Age [Range] (years)	No. of Patients
CRC	45.8 [37–59]	8	47.3 [37–59]	13	44.7 [36–63]	11	46.1 [36–63]	24
EC	53.7 [46–65]	6	54.1 [46–65]	7	45.5 [45–46]	2	52.2 [45–65]	9

Note.—The patient with the additional frameshift *MLH1* mutation was excluded.

are involved in carcinogenesis in patients harboring different variants, as found in this study.

In 316 apparently unrelated patients, five different truncating germline mutations were detected in 12 patients. Haplotype analysis indicated a founder effect for one mutation, which was shared by seven index patients, in exon 4a (650insT). In addition to the 5 truncating mutations, another 10 different variants were found in 13 patients. Several criteria can help to make plausible that such a sequence variant has a pathogenic nature (Hofstra et al. 1997; Cotton and Scriver 1998):

First, the variant should not be found in subjects without the disease. None of the variants reported here occurred in 200 control individuals. However, as most variants were found in only 1 of the 316 patients, it cannot be concluded from this that they are not rare polymorphisms without any clinical relevance.

Second, the variant causes functional loss of the gene in a functional assay. This was previously shown for the variant Ser144Ile, by Kolodner et al. (1999), in the *Saccharomyces cerevisiae*–based functional assay, indicating that this is almost certainly a pathogenic mutation.

Third, the variant results in a substitution of amino acids belonging to different polarity groups. This was true for six of the missense variants. However, only two of these, His1248Asp and Thr1219Ile, occurred in a highly conserved region of the gene. This region shows homology with the MutS gene in *Escherichia coli* and codes for the COOH terminal region of the protein, which contains four ATP-binding–site consensus sequences. Both variants are invariable between *MSH6*, *MSH2*, and MutS (Palombo et al. 1995). For these two variants, therefore, it is likely that they have an effect on the proper function of the mutant protein and are therefore considered as probably pathogenic. Whether any of the other seven variants is pathogenic cannot be substantiated at this moment, and these should be considered as having doubtful pathogenicity.

Twenty-seven patients came from families fulfilling the original Amsterdam criteria for HNPCC (Vasen et al. 1991). In 3 of these 27 families that fulfilled the Amsterdam criteria, a probably pathogenic *MSH6* mutation was found. In addition, one missense variant of doubtful pathogenicity was identified in the proband of another family that fulfilled the Amsterdam criteria. In the diagnostic setting, 17 other families that fulfilled the Amsterdam criteria were previously found to harbor truncating mutations in either *MLH1* or *MSH2* and therefore were not analyzed for *MSH6*. Assuming that no *MSH6* germline mutations are present in these 17 families that fulfilled the Amsterdam criteria, our data indicate that probably pathogenic *MSH6* mutations occur at a lower frequency, 7% (3/44), than do *MLH1* or *MSH2* mutations in these families, thus confirming previous reports (Park et al. 1999; Wang et al. 1999). Most of the mutations that were found occurred in families that were, according to our inclusion criteria, only suspected to have HNPCC.

Our patients can be divided in three major groups: (1) young patients with CRC (including those with multiple tumors for whom CRC was diagnosed at the age of <50 years), (2) young patients with endometrial cancer (including those with multiple tumors for whom endometrial cancer was diagnosed at the age of <50 years), and (3) patients with multiple tumors. Eight (5%) of the 171 patients in the first group had a probably pathogenic *MSH6* germline variant. This was 4% (2/48) for patients in the second group and 13% (7/53) for patients in the third group. In other, very small studies, the prevalence of truncating *MSH6* mutations varied from 0% to 20% in patients whose CRC was diagnosed at the age of <50 years (Verma et al. 1999; Wang et al. 1999; Plaschke et al. 2000), whereas no *MSH6* mutations were found in 24 families with endometrial cancer (Chadwick et al. 1999) and three probably pathogenic missense *MSH6* variants were found in 19 patients with the combination of CRC and endometrial cancer (Charames et al. 2001). When comparison of the prevalence of *MSH6* mutations with that of *MLH1/MSH2* mutations is made, among patients with CRC diagnosed at the age of <50 years, the prevalence of the latter mutations varied from 4% to 11% (Wijnen et al. 1998; Montera et al. 2000; Salovaara et al. 2000; Berends et al. 2001*b*). In studies of endometrial cancer, the prevalence of truncating *MLH1* or *MSH2* mutations varied from 0% to 6% (Kobayashi et al. 1996; Kowalski et

al. 1997; Berends et al. unpublished data). Among patients with multiple HNPCC-related tumor—mainly, the combination of CRC and endometrial cancer—the percentage of *MLH1* and *MSH2* carriers was 9%–54% (Genuardi et al. 1999; Millar et al. 1999; Salovaara et al. 2000; Berends et al., unpublished data). These data show that, compared to mutations in *MLH1* and in *MSH2*, mutations in *MSH6* may contribute, to an equal extent, to the development, at an early age, of CRC and endometrial cancer and to the development of multiple tumors—in particular, CRC and endometrial cancer.

As we reported elsewhere (Wu et al. 1999), for a smaller group of *MSH6*-mutation carriers from this study population, a high proportion of CRC and of endometrial cancers were MSI-L. This is in accordance with studies in yeast, CRC cell lines, and *MSH6* knockout mice showing that loss of *MSH6* function caused no or only weak repeat instability (Marsischky et al. 1996; Ku et al. 1999; Edelmann et al. 2000). An explanation for this is that the major function of *MSH6* is the correction of base-base mismatches and that these do not give rise to MSI. On the basis of the function of *MSH6*, it has been suggested that tumors from patients with an *MSH6* germline mutation would more often have unstable mononucleotide markers (Bhattacharyya et al. 1994; Papadopoulos et al. 1995; Verma et al. 1999; Wijnen et al. 1999). However, instability of dinucleotide markers was found just as frequently as that of mononucleotide markers, in the tumors of our patients. Substituting one of the three dinucleotide markers in the set of five consensus markers with the mononucleotide marker Bat40 would have caused a change in the designation, to MSI-H, in only one endometrial cancer (in patient 3). Somatic inactivation of other MMR genes may have been involved in causing the instability of the dinucleotide markers, and it has been reported that *MSH6* mutations in yeast and in an endometrial cancer cell line in combination with somatic frameshift mutations in other genes (*MLH1*, *MSH2*, *MSH3*) lead to an MSI-H phenotype (Marsischky et al. 1996; Risinger et al. 1996; Akiyama et al. 1997). Indeed, in our series, immunohistochemical analysis revealed the loss of MLH1 or MSH2 expression in tumors from five patients, and four of these showed an MSI-H phenotype. We did not examine the possible loss of *MSH3* function in the tumors.

The results with respect to the MSH6 staining in tumors of *MSH6*-mutation carriers are largely in accordance with the results of the mutation analysis. This is similar to the results in CRCs and in endometrial cancers of *MLH1*- and *MSH2*-mutation carriers (Leach et al. 1996; Thibodeau et al. 1996; Dietmaier et al. 1997; Ichikawa et al. 1999; Marcus et al. 1999; Berends et al. 2001*a*). However, MSH6 staining was normal in some of the tumors of truncating-mutation carriers. In two patients with double tumors, the initial CRCs had a negative MSH6 staining, whereas the second tumor (an endometrial cancer in patient 3 and a CRC in patient 10) had a normal MSH6 staining. An explanation for this discrepancy may be the absence of loss of heterozygosity, as reported by Kruse et al. (2001) in a series of sebaceous skin tumors in *MSH2*-mutation carriers. It may well also be that in the tumors mentioned above, the wild-type allele was not deleted but had a somatic mutation. This may cause the production of an enzymatically inactive but immunologically detectable protein. On the other hand, the possiblilty that the MSI-L endometrial cancer (in patient 3) and the MSI-H CRC (in patient 10) had arisen independently and still had one normally functioning *MSH6* allele cannot be excluded.

Most of the missense variants did not lead to the loss of MSH6 staining. This is not surprising in view of their relatively small effect on the protein structure. On the other hand, a missense variant may cause such a change in the conformation that the protein cannot form a stable dimer with the MSH2 protein. This causes the unstable protein to be present only in low concentration, owing to a short half-life time, and the antibody used is thus not capable of recognizing the protein properly. One could also speculate that, in addition to the wild-type allele, the variant allele is mutated again by a second hit and thus completely inactivated, causing loss of protein expression.

As mentioned above, most of the families in which we found *MSH6* germline mutations were families that were only suspected to have HNPCC. Unfortunately, we have little data on segregation and penetrance of the mutations. On the basis of the available information about family history, the founder mutation, at least, does not seem to have a low penetrance, since three of the seven families with this mutation fulfilled the (revised) Amsterdam criteria. Phenotypic analysis of these seven families with the founder mutation did not reveal a distinctive phenotype for this type of mutation. Although the sample sizes were small, previous reports suggest that the mean age at diagnosis of cancer in *MSH6*-mutation carriers is somewhat higher than it is in *MLH1*- and *MSH2*-mutation carriers (Miyaki et al. 1997; Kolodner et al. 1999; Wagner et al. 2001). In families with either *MLH1* or *MSH2*, the mean age at diagnosis of CRC is 40–45 years, and the mean age at diagnosis of endometrial cancer is 46–48 years (Vasen et al. 1993; Watson et al. 1994). Since we included predominantly patients with CRC or endometrial cancer diagnosed at the age of <50 years, we are not well able to make any reliable statement about the age at onset, in general, in *MSH6*-mutation carriers. In spite of this bias, in the *MSH6*-truncating-mutation carriers that we studied, the mean age at diagnosis of CRC is 47 years, and the mean age at diagnosis of endometrial

cancer is 54 years. This is slightly higher than in *MLH1*- and *MSH2*-mutation carriers, but, again, in general, the age at onset may well be higher in the case of an *MSH6* mutation. *MSH6* germline mutations may be associated with a high risk for endometrial cancer in female carriers. Eight (67%) of 12 proved female carriers of truncating mutations had endometrial cancer or its precursor, atypical hyperplasia. These results are comparable with those reported by Wijnen et al. (1999), who found a frequency of 73% in female carriers of *MSH6* truncating mutations. Although we have to deal with the bias of the inclusion criterion used (i.e., criterion 1; see Appendix A), the frequency of endometrial cancer in *MSH6*-mutation carriers thus may be higher than that in *MLH1*- and *MSH2*-mutation carriers, who have a lifetime risk of 40%–50% for the development of endometrial cancer.

A remarkable finding is that the majority of the CRCs in the *MSH6*-variant carriers were located distally in the colorectum. Thus far, when patients from the literature and from the population that we studied are taken together, the location of 41 CRCs of *MSH6*-variant carriers has been noted. Fourteen (34%) of these CRCs were right-sided, and 27 (66%) were left-sided; in three patients, both a left-sided and a right-sided CRC occurred (Akiyama et al. 1997; Miyaki et al. 1997; Kolodner et al. 1999; Planck et al. 1999; Shin et al. 1999; Verma et al. 1999). This is in contrast with the situation in *MLH1*- and *MSH2*-mutation carriers, in whom ~70% of the CRCs were located proximal to the splenic flexure. Such a differential preponderance of the site of CRC development in patients with different mutated genes may be a consequence of differences in the embryologic origin of the proximal and distal colon, as well as a consequence of their susceptibility to environmental carcinogens, but a ready explanation is not at hand.

As mentioned in the "Results" section, we divided the *MSH6* mutations among two groups: those with truncating mutations (i.e., group A) and those with missense variants (i.e., group B). We wondered if, in addition to the data about change of amino acids and conserved domains, other characteristics could help us to clarify the possible pathogenicity of missense variants. First, between groups A and B, the proportions (50% and 40%, respectively) of MSI-H CRCs and endometrial cancers were not significantly different. Second, clinical data may provide arguments for the pathogenicity of variants. Although we have few data about segregation of the variants, 2 of 12 carriers in group B (patient 15 excluded), compared to 4 of 12 in group A, came from families that fulfilled the Amsterdam criteria. Although the figures are small, the difference is not statistically significant. Furthermore, in 2 of the 10 families that did not fulfill the Amsterdam criteria, an affected sibling happened to be an *MSH6*-variant carrier. Most patients had a parent with an HNPCC-related tumor. No major difference, between patients in groups A and B, in the frequency of affected parents existed. On the other hand, in patients that fulfilled our inclusion criteria (Appendix A) but did not have a variant in *MSH6*, *MLH1* or *MSH2*, only 96 (42%) of 228 had a first-degree relative with an HNPCC-related tumor. Another characteristic feature of HNPCC is the early age at diagnosis. The mean age at diagnosis of CRC or of endometrial cancer for patients in group B was slightly (but not significantly) lower—not higher—than that of patients in group A. Finally, with respect to the site of the CRC, either proximal or distal, there was no significant difference between groups A and B. Taken together, the clinical data disclose a great resemblance between the patients with missense variants and the patients with a truncating mutation, thereby supporting the notion that these variants indeed may be pathogenic. Segregation analysis could help to further elucidate this issue. Also, functional assays will become important tools in the identification of both the effects and the clinical relevance of *MSH6* variants.

In summary, this study has shown that the prevalence of *MSH6* mutations is ~10% in classical families that fulfill the Amsterdam criteria. In families that are only suspected to have HNPCC, the prevalence of mutations in *MLH1* and in *MSH2* and the prevalence of *MSH6* variants is about the same. In *MSH6*-mutation carriers, a substantial proportion of tumors are MSI-L, and CRCs occur more frequently in the left colon than they do in *MLH1*- or *MSH2*-mutation carriers. Female *MSH6*-mutation carriers seem to be at a high risk for endometrial cancer. Missense variants in *MSH6* are about as common as truncating mutations, and molecular and clinical characteristics suggest that a considerable number of these missense variants are pathogenic. Tumors from *MSH6*-truncating-mutation carriers generally show no MSH6 staining, on immunohistochemical analysis; this is not the case for tumors from carriers of a missense variant.

In conclusion, one should consider performing *MSH6*-mutation analysis for all patients suspected of HNPCC. Neither MSI nor immunohistochemistry should be a definitive selection criterion for this mutation analysis.

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Appendix A

Inclusion Criteria for the Population-Based Study

Patients who fulfilled one of the following criteria were included:

1. CRC or endometrial cancer was diagnosed at the age of <50 years.
2. Patients either (*a*) had an HNPCC-related tumor (i.e., CRC; endometrial cancer; cancer of the small bowel, the stomach, the pancreas, the biliary tract, or the ovaries; or transitional cell cancer of the renal pelvis, ureter, or bladder) and a first-degree relative with CRC or endometrial cancer, one of which was diagnosed at the age of <50 years, or (*b*) had CRC or endometrial cancer and a first-degree relative with an HNPCC-related tumor, one of which was diagnosed at the age of <50 years.
3. Patients had two or more HNPCC-related tumors, irrespective of age at diagnosis.
4. Patients had a colorectal adenoma or atypical endometrial hyperplasia and had a first-degree relative with CRC or endometrial cancer, both of which were diagnosed at the age of <50 years.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

International Collaborative Group on Hereditary Non-polyposis Colorectal Cancer, http://www.nfdht.nl/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *MSH6* [MIM 600678] and HNPCC [MIM 114400; MIM 114500])

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