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ORIGINAL REPORT

Lower Incidence of Colorectal Cancer and Later Age of Disease Onset in 27 Families With Pathogenic *MSH6* Germline Mutations Compared With Families With *MLH1* or *MSH2* Mutations: The German Hereditary Nonpolyposis Colorectal Cancer Consortium

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose
The aim of the study was the analysis of the involvement and phenotypic manifestations of *MSH6* germline mutations in families suspected of hereditary nonpolyposis colorectal cancer (HNPCC).

Patients and Methods
Patients were preselected among 706 families by microsatellite instability, immunohistochemistry, and/or exclusion of *MLH1* or *MSH2* mutations and were subjected to *MSH6* mutation analysis. Clinical and molecular data of *MSH6* mutation families were compared with data from families with *MLH1* and *MSH2* mutations.

Results
We identified 27 families with 24 different pathogenic *MSH6* germline mutations, representing 3.8% of the total of the families, and 14.7% of all families with DNA mismatch repair (MMR) gene mutations (n = 183). The median age of onset of colorectal cancer in putative mutation carriers was 10 years higher for *MSH6* (54 years; 95% CI, 51 to 56) compared with *MLH1* and *MSH2* (44 years; 95% CI, 43 to 45; log-rank test, $P = .0038$). Relative to other malignant tumors, colorectal cancer was less frequent in *MSH6* families compared with *MLH1* and *MSH2* families (Fisher's exact test, $P < .001$). In contrast, the frequency of non–HNPCC-associated tumors was increased (Fisher's exact test, $P < .001$).

Conclusion
Later age of disease onset and lower incidence of colorectal cancer may contribute to a lower proportion of identified *MSH6* mutations in families suspected of HNPCC. However, in approximately half of these families, at least one patient developed colorectal or endometrial cancer in the fourth decade of life. Therefore, a surveillance program as stringent as that for families with *MLH1* or *MSH2* mutations is recommended.

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INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC; MIM 114500) is a highly penetrant, autosomal dominant cancer-susceptibility syndrome. Affected individuals are at high risk for developing colorectal and endometrial cancer. In addition, there is an excess of cancers of ovary, stomach, small bowel, pancreas, hepatobiliary tract, brain and upper uroepithelial tract.[1] A hallmark of most of these malignancies is the contraction/expansion of simple sequence motifs,[2-4] termed microsatellite instability (MSI) or microsatellite mutator

phenotype. Disease-causing germline mutations in the mismatch repair genes *MLH1* (MIM No. 120436; GenBank accession: AH003234), *MSH2* (MIM No. 120435; GenBank accession: AH003235), *PMS2* (MIM No. 600259; GenBank accession: U13696), and *MSH6* (MIM No. 600678; GenBank accession: AH005068) have been described.[5] The majority of germline mutations in HNPCC and suspected HNPCC cases have been identified in *MLH1* and *MSH2*.[5,6]

A surveillance program should be offered to family members with pathogenic DNA mismatch repair (MMR) gene mutations or high clinical suspicion of HNPCC. People should undergo colonoscopy every 1 to 2 years beginning at age 20 to 25 years, or 10 years earlier than the youngest age of colon cancer diagnosis in the family.[7] For extracolonic manifestations, the International Collaborative Group on HNPCC (ICG-HNPCC) recommends gynecologic examinations for women, including transvaginal ultrasonography and measurement of CA-125 every 1 to 2 years beginning at age 30 to 35 years. Surveillance for other extracolonic tumors should be tailored to the spectrum of malignancies observed in the family. Therefore, if at least one family member is affected with stomach cancer or cancer of the urinary tract, gastroscopy or sonography and urine cytology every 1 to 2 years starting at age 30 to 35 should be offered, respectively (http://www.nfdht.nl/guidelines.htm). Whether prophylactic surgery should be considered at the time of first diagnosis of colorectal cancer in HNPCC patients is under debate.[8-10] As an individualized decision after counseling, prophylactic surgery represents an option to some HNPCC patients.[11]

At present, data from the HNPCC mutation database (http://www.nfdht.nl) and from the literature indicate that *MSH6* mutations may account for 10% to 15% of all HNPCC germline mutations. Studies on patients with familial non-HNPCC colorectal carcinomas[12] and on unselected patients with endometrial carcinomas[13] suggest that approximately 1% to 2% of these cancers may be caused by *MSH6* germline mutations. A predominance of instability at mononucleotide repeats in *MSH6*-deficient tumors has been reported,[14-17] which is in accordance with the recognition of base-base mispairs and insertion/deletion loops (IDLs) of single bases by the MutSα protein complex (MSH2 + MSH6).[18] However, low levels of instability at mono- and dinucleotide repeats, and a substantial fraction of tumors without any MSI have been reported by others.[19,20]

The risk of developing colorectal or endometrial cancer has been described as slightly higher in families with *MSH2* mutations compared with families with *MLH1* mutations, without reaching statistical significance.[21] In contrast, *MSH6* germline mutations were reported in families with a later age of tumor onset and a higher incidence of endometrial cancers when compared with families with *MSH2* germline mutations.[16] The average age of disease onset has been shown as higher in *MSH6* mutation carriers than in *MLH1* and *MSH2* mutation carriers, which might reflect a lower penetrance of *MSH6* mutations.[15,20] Detailed analyses of phenotypic manifestations associated with hereditary *MSH6* defects has been hampered by the limited number of identified germline mutations. Here we report on the molecular and clinical data of 27 families with pathogenic *MSH6* mutations demonstrating a lower incidence of colorectal cancer and later age of disease onset compared with families with *MLH1* or *MSH2* mutations.

PATIENTS AND METHODS

Patients were recruited from the German HNPCC registry established in 1999. The main goal of the registry is to support surveillance in families suspected of HNPCC. Patients are referred to the registry nationwide based on the Amsterdam I and II criteria[22] without age restriction, and the Bethesda guidelines for the identification of patients with HNPCC.[23] These criteria include (1) patients of families with at least three members affected with histologically verified colorectal, endometrial, small bowel, renal pelvis, or ureter cancer, in which one affected member is a first-degree relative of the other two and at least two generations are affected; (2) individuals with two HNPCC-associated cancers (colon, rectum, endometrium, ovary, stomach, biliary duct, small-bowel, ureter); (3) individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma in which one of the cancers was diagnosed prior to age 45 years, and the adenoma was diagnosed prior to age 40 years; (4) individuals with colorectal cancer or endometrial cancer diagnosed before age 45 years; (5) individuals with adenoma diagnosed before age 40 years; and (6) individuals with at least one relative fulfilling one of criteria 1 through 5. Familial adenomatous polyposis coli is excluded. Pedigree information is traced by clinical geneticists.

A total of 706 families had been registered by January 2003. Whenever available, tumors samples have been analyzed for MSI and MLH1 and MSH2 protein expression. From this cohort, we selected candidates for *MSH6* germline mutations using the following criteria: (1) the patients had developed tumors that were either of low or high MSI (MSI-L or MSI-H, respectively), and (2) MLH1 and MSH2 were expressed in the tumor cells. Tumor samples from selected patients were studied for MSH6 expression using immunohistochemistry techniques. In addition, patients were included in the study if no *MLH1* or *MSH2* germline mutation had been identified and no information on MSI and protein expression was available. Written informed consent was obtained from all patients investigated.

Information on MSI was obtained from the application of the National Cancer Institute/ICG-HNPCC (NCI/ICG-HNPCC) reference marker panel for the evaluation of MSI in colorectal cancer (BAT25, BAT26, D2S123, D5S346, D17S250)[24] for most tumors. If only one marker scored as instable, we applied a second marker panel (BAT40, D3S1619, D10S197, D18S58, MycL). In addition, other markers have been used as previously described.[25,26] Tumors were classified as MSI-H or MSI-L if at least 30% or less than 30% of the markers showed instability, respectively.

Immunohistochemical staining was performed on 5-μm-thick formalin-fixed, paraffin-embedded tumor sections using a

mouse monoclonal primary antibody against MSH6 (clone 44; Transduction Laboratories, Lexington, UK; 250 μg/mL, 1:50) as described previously.[17] Loss of expression in the tumor cells was considered solely when there was normal nuclear staining in adjacent non-neoplastic cells, which served as internal controls.

Mutation analysis was performed on all exons of *MSH6*, including flanking intronic regions from genomic DNA isolated from peripheral blood lymphocytes either by denaturing high-performance liquid chromatography screening and subsequent sequencing or by direct sequencing as previously described.[26,27] The functional effect of a splice donor site mutation was analyzed by reverse transcriptase-polymerase chain reaction with exonic primers after mRNA isolation from peripheral blood and reverse transcription applying the Quick-Prep *Micro* mRNA Purification Kit (Amersham Biosciences, Freiburg, Germany) and the First-Strand cDNA Synthesis Kit (Amersham Biosciences), respectively. To test whether two *MSH6* mutations occurring in one of the patients were located on the same allele, a 7-kb fragment covering both mutations was amplified, cloned with the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany), and sequenced from both ends. Sequence information on the applied primers is available on request.

Statistical analyses were performed applying the χ^2 test, Fisher's exact test, the Mann-Whitney *U* test, or the log-rank test wherever appropriate. *P* values below .05 were considered significant. Bonferoni adjustment was applied when multiple testing was performed. SPSS Release 10.0.7 (SPSS Inc, Chicago, IL) was used for all statistical data analyses. The index patient with identified germline mutation and his first- and second-degree relatives were included in the statistical analyses of family characteristics and frequencies of tumor entities. In a subset of this cohort, the Kaplan-Meier method was used to analyze the age of tumor onset. This subset consisted solely of proven and obligate mutation carriers and individuals with colorectal or endometrial cancer, referred to as putative carriers. Proven noncarriers were excluded. Clinical data of 156 families with pathogenic *MLH1* or *MSH2* germline mutations, identified in the same cohort of 706 families, were used for comparative analyses.

RESULTS

We identified 24 different germline mutations in *MSH6* considered to be pathogenic in 27 families (Table 1). The mutations comprised eight nonsense mutations, seven small insertions, seven small deletions, and two genomic rearrangements. There was little redundancy, and only one mutation was found twice and three times, respectively. Mutations were distributed equally alongside the gene, with the exception of six (24%) of the 25 small mutations (not considering the two genomic rearrangements) that occurred in exon 5, representing only 6.5% of the coding region (six mutations in 266 bp coding region *v* 19 mutations in 3,817 bp; χ^2 $P < .001$).

MSI analysis of 24 available tumors from patients harboring pathogenic mutations revealed 19 (79%) MSI-H tumors, four (17%) MSI-L tumors, and one microsatellite-stable (MSS) tumor (Table 2). The patient with the MSS tumor (BN5, endometrial carcinoma) was included in the *MSH6* analysis since the tumor showed loss of MSH6 expression, and the mother was also affected by a MSI-L endometrial carcinoma at age 57 years. Notably, one colon tumor (LM2) was MSI-L without showing instability in any of the five markers of the NCI/ICG-HNPCC marker panel. Mononucleotide repeats were more often affected by instability (51 [77.3%] of 66) when compared with di- and tetranucleotide repeats (34 [36.6%] of 93; χ^2 $P < .001$). Immunohistochemical analysis revealed loss of MSH6 expression in 18 tumors, whereas three tumors expressed the protein. There was a statistical difference in the number of families fulfilling Amsterdam and Bethesda criteria between *MSH6* and *MLH1/MSH2* carriers (Fisher's exact test, $P = .017$). A lower proportion of families carrying *MSH6* mutations fulfilled the Amsterdam criteria without age restriction (48.1%) than those with *MLH1* or *MSH2* mutations (60.3%), while a higher proportion of the *MSH6* families (25.9% *v* 9.0% of *MLH1* and *MSH2* families) met Bethesda criterion 4 (one family member with colorectal or endometrial cancer before age 45 years). Four families fulfilled the Amsterdam criteria by anamnestic data but not by age restriction. The disease history of families with *MSH6* mutations is detailed in Table 1.

In addition to the 27 pathogenic mutations, five missense mutations with unknown pathogenicity were identified in *MSH6* (Table 1). One of these mutations (E619D) was located on the same allele as a 4-bp deletion affecting the splice donor site of intron 7, resulting in skipping of exon 7 from the transcript. While the 4-bp deletion was a de novo mutation, the missense mutation was inherited from the patient's father. This patient (DD4) was affected with colon cancer at age 34 years and had no history of HNPCC-associated tumors in his family. The anamnestic data of patient BN1 was similar, with colon cancer at age 31 and lack of HNPCC-associated cancers among his relatives; however, in this family, the *MSH6* mutation was inherited from the mother, who was affected with breast cancer at age 66 years.

Among the 396 enrolled members of families with pathogenic *MSH6* mutations, 115 (29.0%) were affected by a malignant disease. This frequency was lower than in families with *MLH1* or *MSH2* mutations (37.5%; Table 3). The main tumor entity of the HNPCC syndrome, colorectal cancer, was statistically less frequent among all tumors in families with *MSH6* mutations (42.4%) compared with those with *MLH1* or *MSH2* mutations (65.5%; Table 4). Such a difference was not observed for other HNPCC-associated cancers. In contrast, the frequency of non–HNPCC-associated tumors was increased, as 46 (31.9%) of 144 primary tumors reported in *MSH6* families were not of an HNPCC-associated type, compared with 131 (15.3%) of 859 tumors in *MLH1* and *MSH2* families (Fisher's exact test, $P < .001$). These tumors included breast, lung, and prostate cancer, and leukemia (Table 5). The median age of

Table 1. *MSH6* Germline Mutations and Family Characteristics

Patient No.	Patient	Mutation			Cancer and Age (years)		Criteria and Criterium No.
		Exon	Sequence	Protein	Index Patient*	Affected Relatives	
Pathogenic mutations							
1	KB5309†	1-2	del 13.0 kb (promoter, exon 1 and 2)	No transcription	R (54), C (54)	M C (52), mU C (54)	A wa
2	DD2†	2	c.426G > A	p.W142X	R (51)	F R (54), B C (47), Ni Ly (28), mA P (60)	A
3	BN4	3	c.467C > G	p.S156X	R (41)	None	B4
4	KN1450†	4	c.651_652insT	p.K218fsX218	C (33)	M St (64), 8× mRelatives with cancer (3 × St, 1 × C)	B3
5	LM2	4	c.1190_1191delAT	p.Y397fsX399	C (47), C (47)	M E (50) + C (72), mGM C (50), 4 mA + U cancer of unknown site	A
6	RG2†	4	c.1190_1191delAT	p.Y397fsX399	C (33)	F cancer (48), pGM cancer (48)	B4
7	BO2	4	c.1422_1423insTG	p.Q475fsX481	C-Ad (39)	S C-Po (41), pGM C (40), M CeU (37), mU L	B7
8	HD2†	4	c.1632_1635delAAAA	p.E544fsX569	C (40)	mA B (60), mU Br (58), mGM C (50) + K (75)	B4
9	KT2449†	4	c.2062_2063delGT	p.V688fsX696	R (47)	F C (54), pGF C, pGA C	A
10	DU5	4	c.2194C > T	p.R732X	C (61), E (63), C (73)	B C (42) + C (47), B Ur (61), B Sk (47), F C (48)	A
11	HD1†	4	c.2614_2615insATTA	p.1872fsX881	Ly (44), C (55), E (57)	mA O (60) + E (60) + C (75), mGF St (60)	B2
12	DU4	4	c.2719_2720delGT	p.V907fsX916	Not affected	F C (53), pU C (72), pU C (73), pNi C (35), S Ut (35), M O (55), mGF St (65)	A
13	DU3	4	c.2731C > T	p.R911X	C (58), E (61), K (70)	M C (55) + St (55), S C (59), B K (31)	A wa
14	TU1	4	c.3013C > T	p.R1005X	SB (70)	B C (53), B C (57), S Gi (33) + E (33), M E	A
15	BN5	4	c.3103C > T	p.R1035X	E (38)	M E (57), mGM E (39) + C (58)	A
16	KT3922†	5	c.3202C > T	p.R1068X	R (37)	F L	B4
17	BN3	5	c.3261delC	p.P1087fsX1089	C (30)	pU Bl (47), pGF L (55)	B4
18	BO1	5	c.3261_3262insC	p.F1088fsX1092	C (37)	M C (47), D Me (22), mA C (60)	A
19	BO3	5	c.3261_3262insC	p.F1088fsX1092	C (50)	B C (45) + C (58), M R (73), S C (30), Ne R-Po (29)	A
20	DD3	5	c.3261_3262insC	p.F1088fsX1092	Not affected	M E (37) + C (57), mU St (42), mU St (70), mA St (61), mGM St (50)	A
21	RG1	5	c.3324_3325insT	p.I1109fsX1111	O (54), C (55)	S E (52), M St (52) + B (54), F C (73), mA B (66) + St (69), mA E (73)	A wa
22	KB4823	IVS 5	dup 4.9 kb (3′ end of exon 4 and exon 5)	Aberrant transcript (putative)	E (51), O (51)	S CoU	B2
23	BN1†	6	c.3513_3514delTA	p.D1171fsX1175	C (31)	F P (76), M B (66), mA B (52)	B4
24	DU2	6	c.3514_3515insA	p.R1172fsX1176	R (46), R (46), B (62), R (66), B (69)	M Ut (53), mGM Ut (56), mA Ut (58)	B2
25	DD4	7	c.3646_3646 + 3delGgta	Splice defect	C (34)	None	B4
26	LM3	9	c.3838C > T	p.Q1280X	C (58)	F C (70), pU C, pU C	A wa
27	BN2	9	c.3953_3954ins32	p.R1318fsX	R (34)	pA B (51)	B4
Missense mutations							
1	DU1	2	c.297G > T (MLH1: del exon 2 and 3)	p.K99N	C (34), C (39), C (43), E (50), E (52)	B C (38) + C (39), M E (55), mA E + C, mU C	A
2	DD4	4	c.1857A > C (MSH6: c.3646_3646 + 3delGgta)	p.E619D	C (34)	None	B4
3	BN4	4	c.2360C > T	p.A787V	R (41)	M R	B3
4	LM1	4	c.2633T > C	p.V878A	C (58)	B C (58), Ni C (32), D B (32)	A
5	HD3	5	c.3226C > T	p.R1076C	R (19), E (24)	pGM R (73)	B2

Abbreviations: Ad, adenoma; B, breast; Bl, bladder; Br, brain; C, colon; CeU, cervix uteri; CoU, corpus uteri; E, endometrium; Gi, gastrointestinal tract; K, kidney; Le, leukemia; L, lung; Ly, lymphoma; Me, melanoma; O, ovary; P, pancreas; Po, polyp; R, rectum; SB, small bowel; Sk, skin; St, stomach; Ur, ureter; Ut, uterus; A, aunt; B, brother; D, daughter; F, father; GA, grand aunt; GF, grandfather; GM, grandmother; M, mother; Ne, nephew; Ni, niece; S, sister; U, uncle; p, paternal; m, maternal; A, Amsterdam I and II criteria; A wa, Amsterdam I and II criteria without restriction for age of onset; B, Bethesda guidelines for hereditary nonpolyposis colorectal cancer.

*The family member in whom the mutation has been identified.

†The mutations from these patients have been reported (references 17, 26, 29, and 35).

Table 2. Immunohistochemistry and Microsatellite Analysis on Tumors of Patients With *MSH6* Mutations

Patient No.*	Patient	Tumor and Age of Onset (years)	Immunohistochemistry			Microsatellite Analysis†				
							NCI/ICG Panel‡		All Markers Tested§	
			MLH1	MSH2	MSH6	MSI	Mono	Di	Mono	Di, Tetra
Pathogenic mutations										
1	KB5309	R (54)	+	+	−	MSI-H	2/2	2/3		3/4
2	DD2	R (51)	+	+	−	MSI-H	2/2	1/2	3/4	3/7
3	BN4	R (41)	af	af	af	MSI-H	na	1/2		3/6
4	KN1450	C (33)	+	+	−	MSI-H	2/2	1/3	7/10	1/10
5	LM2	C (47)	+	+	+	MSI-L	0/2	0/3		2/5
6	RG2	C (33)	+	+	−	MSI-H	2/2	1/3		
7	BO2	C-Ad (39)	+	+	−	MSI-H	2/2	2/2		
8	HD2	C (40)	+	+	−	MSI-H	2/2	3/3		
9	KT2449	R (47)	+	+	−	MSI-H	2/2	1/3	5/6	2/7
10	DU5	C (73)	+	+	−	MSI-H	2/2	0/3		
11	HD1	C (55)	+	+	−	MSI-H	2/2	0/3		
14	TU1	SB (70)	+	+	−	MSI-L	1/2	0/3	2/3	0/5
15	BN5	E (38)	+	+	−	MSS	0/2	0/3		
15	Mother of BN5	E (57)	+	af	af	MSI-L	2/2	0/3	2/3	0/7
16	KT3922	R (37)	+	+	−	MSI-H	2/2	1/3	5/6	3/8
17	BN3	C (30)	na	na	na	MSI-H	1/1	na	2/2	
18	BO1	C (37)	+	+	+	MSI-H	0/2	2/3		
19	Brother of BO3	C (58)	+	+	−	MSI-H	3/3	na		
20	Uncle of DD3	St (70)	+	+	−	na				
21	RG1	C (55)	+	+	+	MSI-H	2/2	3/3		
22	KB4823	E (51)	+	+	−	MSI-H	2/2	0/2		
23	BN1	C (31)	+	+	−	MSI-H	2/2	0/1		
25	DD4	C (34)	+	+	−	MSI-H	2/2	1/2		1/3
26	LM3	C (58)	+	+	−	MSI-L	1/2	0/3		
27	BN2	R (34)	na	na	na	MSI-H	0/1	0/2		2/4
Missense mutations										
1	DU1	E (52)	−	+	+	MSI-H	2/2	1/3		
3	BN4	R (41)	−	+	na	MSI-H	1/1	0/2	2/2	
4	LM1	C (58)	+	+	+	MSI-L	0/2	1/3		
5	HD3	E (24)	+	+	af	MSI-H	2/2	3/3		

Abbreviations: MSI, microsatellite instability; R, rectum; af, analysis failed; na, not analyzed; H, high-level; C, colon; -L, low-level; Ad, adenoma; SB, small bowel; E, endometrium; MSS, microsatellite stable; St, stomach.

*Number refers to the mutation number in Table 1.

†Data indicate markers showing instability/markers tested.

‡The National Cancer Institute/International Collaborative Group on hereditary nonpolyposis colorectal cancer (NCI/ICG-HNPCC) reference marker panel for the evaluation of MSI in colorectal cancer comprising two mono (BAT25 and BAT26) and three dinucleotide repeat markers (D2S123, D5S346, D17S250).[19]

§Only given when other/additional markers were used.

onset of colorectal cancer in putative mutation carriers was 10 years higher for *MSH6* (54 years; 95% CI, 51 to 56 years) compared with *MLH1* and *MSH2* (44 years; 95% CI, 43 to 45 years). The median age of onset of any tumor was 8 years higher for *MSH6* (51 years; 95% CI, 48 to 54 years) compared with *MLH1* and *MSH2* (43 years; 95% CI, 42 to 44 years). Accordingly, the cumulative risk by age to develop colorectal cancer or to develop any tumor was statistically

Table 3. Characteristics of Mutation-Positive Families

	MSH6	*MLH1/MSH2*	*P*
No. of families	27	156	
No. of enrolled family members*	396	1,578	
Mean age at study inclusion, years	49	45	.184 (Mann-Whitney *U* test)
No. of affected family members	115	591	.002 (Fisher's exact test)
% of affected family members	29.0	37.5	
No. of members with one tumor	94	434	
No. of members with multiple tumors	21	157	

*The index patient of each family, and its first- and second-degree relatives were considered.

Table 4. Distribution of HNPCC- and Non–HNPCC-Associated Tumors in Mutation-Positive Families

	MSH6		*MLH1/MSH2*		
	No.	%	No.	%	*P* (Fisher's exact test)
All primary tumors*	144	—	859	—	
Colorectal cancer	61	42.4	563	65.5	< .001
Other HNPCC-associated cancers†	26	18.1	136	15.8	.540
Non–HNPCC-associated tumors	46	31.9	131	15.3	< .001
Cancer of unknown site	11	7.6	29	3.4	

Abbreviation: HNPCC, hereditary nonpolyposis colorectal cancer.
*The index patient of each family, and their first- and second-degree relatives were considered.
†Comprising endometrial, ovarian, gastric, hepatobiliary, or small-bowel cancer or transitional cell carcinoma of the renal pelvis or ureter.

lower for putative *MSH6* mutation carriers compared with putative *MLH1* and *MSH2* mutation carriers, whereas no difference was obtained for non–colorectal HNPCC-associated cancers (Figs 1A to C).

DISCUSSION

In this study, molecular and clinical characteristics of 27 families with pathogenic *MSH6* germline mutations are presented and compared with data from families with *MLH1* or *MSH2* mutations. *MSH6* mutations were found in 3.8% of all HNPCC-suspected families (27 of 706 families), representing 14.7% of all 183 pathogenic germline mutations identified in the three MMR genes (*MLH1*, *MSH2*, and *MSH6*) in this cohort. These frequencies were somewhat higher, although not statistically different, when compared with the 2.4% in HNPCC-suspected families (two mutations in 84 families) reported by Peterlongo et al[28] and to the 12% of all MMR gene mutations (10 of 83 mutations) reported by Wijnen et al,[16] respectively. The age of disease onset in members of families with *MSH6* mutations was increased compared with those of families with *MLH1* and *MSH2* mutations. The median and the

Table 5. Frequencies of Tumor Entities in Mutation-Positive Families

	MSH6		*MLH1/MSH2*	
	No.	%	No.	%
All primary tumors*	144	—	859	—
Colorectal cancer	61	42.4	563	65.5
Endometrial cancer†	9	6.3	43	5.0
Ovarian cancer†	4	2.8	12	1.4
Stomach cancer†	10	6.9	37	4.3
Cancer of the renal pelvis and ureter†	0	0	13	1.5
Breast cancer	8	5.6	17	2.0
Lung cancer	7	4.9	5	0.6
Prostate cancer	4	2.8	6	0.7
Leukemia	4	2.8	4	0.5
Kidney cancer	3	2.1	8	0.9
Bladder cancer	1	0.7	10	1.2
Others and unknown site	33	22.9	141	16.4

*The index patient of each family, and their first- and second-degree relatives were considered.
†Hereditary nonpolyposis colorectal cancer–associated cancers.

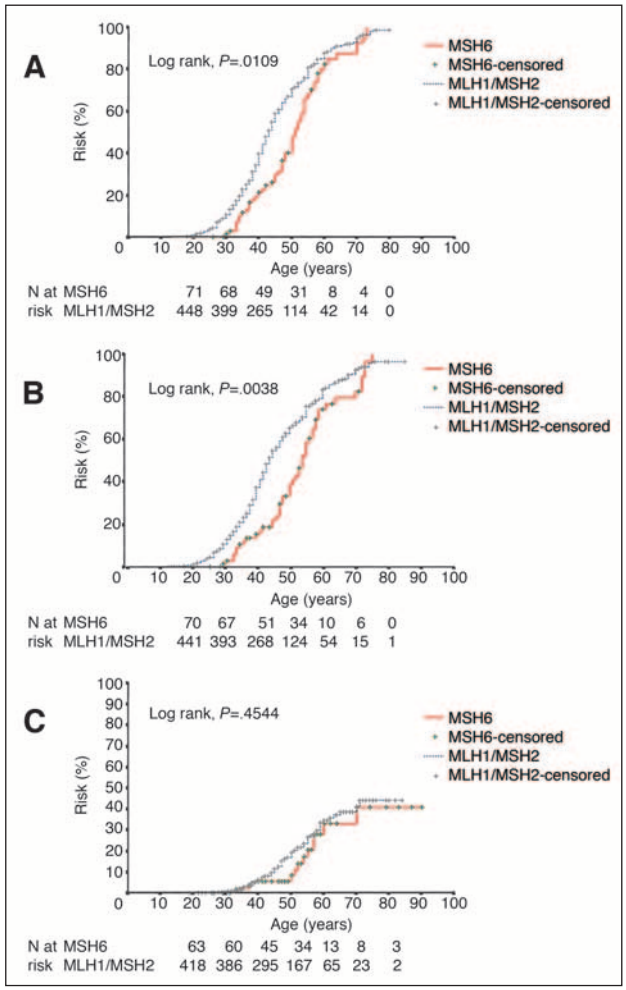

Fig 1. Cumulative risk of developing cancer in putative carriers of *MSH6* germline mutations compared with *MLH1* and *MSH2* germline mutations. (A) Any tumor; (B) colorectal cancer; (C) non-colorectal hereditary nonpolyposis colorectal cancer. N, number.

mean age of onset of colorectal cancer in putative mutation carriers (both 54 years) were similar to the mean age (55 years) reported for a large Dutch family with a *MSH6* germline mutation.[15] Whether the lower cumulative risk of disease onset by age results in a lower lifetime risk in developing cancer in *MSH6* mutation carriers cannot be concluded from our data because of the limited time of surveillance. Notably, a substantial fraction of patients with *MSH6* mutations who did not fulfill any of the Bethesda guidelines for HNPCC has been identified in several population-based approaches,[12,13,17,29] suggesting an even higher difference in the cumulative risk by age of disease onset between *MSH6* mutation carriers and *MLH1* and *MSH2* mutation carriers, if all mutations in the general population were considered. Almost two-thirds (17 of 27) of the families with *MSH6* mutations would not have been identified by application of the strict Amsterdam I and II criteria (with restriction for age of onset of at least one HNPCC-associated cancer before age 50 years). Moreover, no HNPCC-associated cancer was diagnosed before age 45 years—the limit for Bethesda criteria 3 and 4, in 10 of the families. Therefore, a relaxed age restriction in the application of clinical criteria may contribute to a more complete enrollment of families with *MSH6* mutations. Although the clinicians in North America do not currently have *MSH6* sequence analysis from all commercial laboratories providing gene-sequencing service for hereditary cancer syndromes, our data recommend the inclusion of this gene into the molecular diagnostics repertoire of HNPCC.

Notwithstanding that patients with pathogenic *MSH6* mutations were less frequently affected by multiple primary tumors, four of the index patients developed multiple primary colorectal cancers. These patients underwent standard oncological resections on all primary tumors, except for patient LM2, who underwent colonoscopic polypectomy of two synchronous colon carcinomas and refused the recommended subsequent standard resection. The secondary tumors were not related to ultimate mortality of these patients. Therefore, there is no indication that based on the recognition of an *MSH6* germline mutation, an extended, prophylactic surgery would have been useful in these cases.

Twenty-four of the 27 mutations were different and almost equally distributed throughout the *MSH6* gene, except for a relative accumulation in exon 5 according to its proportion of the entire coding region. Four of the six mutations in exon 5 resulted from frameshift mutations affecting the (C)8 tract. The 1-bp insertion in this tract was first reported in Korean patients[30] and is listed in the HNPCC database for an Australian patient. We have identified this mutation in an additional patient who was not included in this study due to failure to fulfill any of the criteria for patient selection (colon cancer at age 47 years). Therefore, this mutation accounts for approximately 10% of all *MSH6* germline mutations reported thus far, and constitutes, with the c.651_652insT, a founder mutation in the population of the Netherlands,[16] one of the two most common *MSH6* germline mutations. The frequent occurrence of frameshift mutations at this site might be explained by the susceptibility of homopolymeric sequences to strand slippage errors, which is also emphasized by frequent somatic mutations of this C(8) tract in MMR-deficient tumors.[31] Moreover, these findings are similar to those of *MLH1* in our cohort, where the most frequent mutation was the insertion of a cytosine residue to the (C)6 tract in exon 13 (data not shown).

The majority of tumors showed both MSI and loss of MSH6 expression, whereas few patients with identified *MSH6* mutations were included in the analysis by having developed an MSI tumor expressing MSH6 (three patients) or an MSS tumor with loss of MSH6 expression (one patient). Therefore, neither MSI analyses nor protein expression analyses were able to select all patients with *MSH6* mutations, which is similar to results reported by Wu et al[19] and Berends et al.[20] Moreover, our results do not exclude the possibility that even the combination of both methods might not identify all patients with *MSH6* mutations. The retained expression of MSH6 in some tumors may be explained by somatic mutations of the second allele impairing protein function but not expression. The MSI phenotype is, on average, less pronounced in MSH6-deficient tumors when compared with MLH1- or MSH2-deficient tumors, and mononucleotide repeat markers are more frequently affected than dinucleotide repeat markers. Therefore, the question remains as to whether the use of a larger number of long mononucleotide repeats would increase the sensitivity in the selection of patients with *MSH6* germline mutations, regardless of tumor origin.

The frequency of colorectal cancer, the main tumor type in HNPCC syndrome, was lower in *MSH6* families compared with *MLH1* and *MSH2* families, whereas non–HNPCC-associated tumors were statistically more frequent. The basis of this shift in tumor spectrum is not known. One explanation may be that the extended strand slippage errors at coding microsatellites (namely, frameshift mutations of the [A]10 tract of *TGFβRII*), which are found in 80% of colorectal cancers of the MSI-H phenotype,[32] may favor the development of colorectal cancer in individuals carrying *MLH1* and *MSH2* mutations. In contrast, the decreased frequency of frameshift mutations and similar or even increased levels of point mutations associated with MSH6-deficiency, as compared with MLH1- or MSH2-deficiency,[18,33] may explain the slower tumor development and reduced prevalence of colorectal cancer.

It is not clear why *MSH6* is less frequently affected by germline mutations when compared with *MLH1* and *MSH2,* since *MSH6* has approximately a 50% larger coding region. Our findings suggest that *MSH6* germline mutations may become less frequently evident than *MLH1* or

MSH2 mutations when the Bethesda criteria are applied because of a later age of tumor onset and a reduced frequency of colorectal cancer. Furthermore, the lower frequency of colorectal cancer along with the increased frequency of non–HNPCC-associated tumors in families with *MSH6* mutations raises the question of whether some families with tumor histories not suspected of HNPCC are associated with *MSH6* germline mutations.

The partially retained MMR capacity due to mutated *MSH6*, compared with an incapacity in mutated *MSH2*,[18,34] may be responsible for the later age of tumor onset in patients with *MSH6* germline mutations. Nonetheless, in approximately half of the families with *MSH6* germline mutations, at least one member developed colorectal or endometrial cancer in the fourth decade of life. This suggests a high variability regarding the penetrance by age of *MSH6* germline mutations, which might be based on additional genetic and/or environmental factors. Therefore, a surveillance program as stringent as that for families with *MLH1* or *MSH2* mutations is recommended.

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Appendix

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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