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MSH6 and *MSH3* Are Rarely Involved in Genetic Predisposition to Nonpolypotic Colon Cancer[1]

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ABSTRACT

A set of 90 nonpolypotic colon cancer families in which germ-line mutations of *MSH2* and *MLH1* had been excluded were screened for mutations in two additional DNA mismatch repair genes, *MSH6* and *MSH3*. Kindreds fulfilling and not fulfilling the Amsterdam I criteria, showing early and late onset colorectal (and other) cancers, and having microsatellite stable and unstable tumors were included. Two partly parallel approaches were used: genetic linkage analysis (19 large families) and the protein truncation test (85, mostly smaller, families). Whereas *MSH3* was not involved in any family, a large Amsterdam-positive, late-onset family showed a novel germ-line mutation in *MSH6* (deletion of CT at nucleotide 3052 in exon 4). The mutation was identified through genetic linkage (multipoint lod score 2.4) and subsequent sequencing of *MSH6*. Furthermore, the entire *MSH6* gene was sequenced exon by exon in families with frameshift mutations in the $(C)_8$ tract in tumors, previously suggested as a predictor of *MSH6* germ-line mutations; no mutations were found. We conclude that germ-line involvement of *MSH6* and *MSH3* is rare and that other genes are likely to account for a majority of *MSH2*-, *MLH1*-mutation negative families with nonpolypotic colon cancer.

INTRODUCTION

Mutations in the DNA mismatch repair genes *MSH2*[3] and *MLH1* account for a majority of families with HNPCC (1). In DNA mismatch repair, the MSH2 protein forms a heterodimer with two additional DNA mismatch repair proteins, MSH6 or MSH3, depending on whether base-base mispairs or insertion/deletion loops are to be repaired (2, 3). In the former case, MSH6 is required, whereas in the latter case, MSH3 and MSH6 have partially redundant functions. To our knowledge, no germ-line mutations in the *MSH3* gene have been identified. However, recent observations in *Msh3*−/− mice suggest that *MSH3* germ-line mutations, if they exist in humans, might be associated with late-onset HNPCC (4). A few dozens of HNPCC or HNPCC-like families with *MSH6* germ-line mutations are known (5–12).

Since the very first reports (5, 6), somewhat atypical clinical characteristics have been attributed to families with *MSH6* germ-line mutations, including an excess of endometrial cancers (7) and late onset (7, 8). A low degree of microsatellite instability (MSI-L; Ref. 9) and/or preferential involvement of mononucleotide repeats have been proposed to characterize tumors from *MSH6* mutation carriers (10, 12). On the other hand, typical Amsterdam I-positive (13) HNPCC families also may have *MSH6* mutations (7), and no endometrial cancers are necessarily present (8, 12). Furthermore, these mutations are occasionally associated with early onset of cancer (10) and a high degree of microsatellite instability (MSI-H) in tumor tissue (5–7). In family series not prescreened for *MSH2* and *MLH1* germ-line mutations, the frequency of *MSH6* germ-line mutations ranges from 0% among Amsterdam I-positive families with MSI-positive tumors (14) to 22% among "suspected" HNPCC families with MSI-L tumors (9). In series with *MSH2* and *MLH1* mutations excluded, *MSH6* mutations are reported to occur in 5–10% of families (7, 8).

Because the phenotypic features associated with *MSH6* mutations and the prevalence of these mutations are controversial, and thus far no *MSH3* germ-line mutations have been reported, we tested 90 HNPCC and HNPCC-like families for germ-line mutations in these genes. We report a low frequency of *MSH6* mutations and a complete absence of *MSH3* mutations, suggesting that other genes are likely to account for a majority of *MSH2*- and *MLH1*-mutation-negative families.

MATERIALS AND METHODS

Patients and Samples. This study was based on 90 families, 23 of which met the Amsterdam criteria I for HNPCC (13), whereas the remaining ("HNPCC-like") families fulfilled one of four criteria specified for Amsterdam I-negative families in Table 1. The families were from the hereditary cancer registries of Sweden (62 families), Finland (26 families), and Denmark (2 families). *MSH2* and *MLH1* mutations had been excluded in all families using DGGE or direct automated exon-specific sequencing. Linkage study comprised 19 families that were expected to be informative enough on the basis of simulation analysis. We had DNA samples from an average of five affected members/family; five or more samples were available from families 156, 224, 436, 173, 24, 219, 2113, and 2145 (Table 2), and fewer than five samples were available from the remaining families. Additionally, samples from up to 13 unaffected members/family were included in the linkage analysis. All families from which RNA samples were available ($n = 85$) were subjected to PTT (14 families were included in both linkage and PTT analysis). In addition to blood samples, specimens of fresh-frozen or archival tumor tissue were collected for microsatellite instability analysis, which was performed according to the Bethesda guidelines (15) or, in some instances, using the mononucleotide repeat markers *BAT25* and *BAT26* (16) only. All samples were obtained after informed consent according to institutional guidelines.

Linkage Analysis. Linkage to *MSH2*, *MLH1*, *MSH*6, and *MSH3* was studied using microsatellite markers flanking these loci (6–10 markers spanning 20 cM on both sides)[4]. The closest marker to *MSH2/MSH6* was *D2S123* (2.7 cM proximal to *MSH2/MSH6*), and additional markers were obtained from an integrated map (17). The closest marker to *MLH1* was *D3S1611*, located in intron 12 of this gene (18). *MSH3* was positioned through the fact that it has a common promoter with the *dihydrofolate reductase* (*DHFR*) gene (19),

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[3] The abbreviations used are: *MSH*, mut S homolog; *MLH*, mut L homolog; HNPCC, hereditary nonpolyposis colon cancer; DGGE, denaturing gradient gel electrophoresis; MSI, microsatellite instability (-H, high; -L, low); PTT, protein truncation test.

[4] Internet addresses: http://www.ncbi.nlm.nih.gov/genemap/ and ftp://ftp.genethon.fr.

Table 1 *Characteristics of families included in the PTT and/or linkage study*

	Number of families	
	PTT (n = 71)	Linkage (n = 19[a])
Amsterdam I status		
Positive	12	11
Negative	59	8
1 relative with colon cancer <35 years	9	0
2 relatives with colon cancer	27	1
≥3 relatives with colon cancer	18	7
Endometrial carcinomas only	5	0
MSI status		
Positive	10	7
Negative[b]	40	11
Not known	11	1
Average age at onset		
<50 years	16	9
≥50 years	52	10
Not known	3	0
Occurrence of endometrial cancers		
Yes	19	8
No	52	11

[a] Includes 14 families also studied by PTT.
[b] Includes MSI-L.

whose location was indicated in GeneMap'98 (closest marker: *D5S424*; 5 cM proximal to *DHFR*). Linkage was analyzed as multipoint calculations with the programs Genehunter (20, 21) and SIMPLE (22); the latter provided a powerful method in large families because it does not require splitting of such pedigrees. We used the following parameters: (*a*) autosomal dominant mode of inheritance; (*b*) gene frequency 0.001; (*c*) and frequency of phenocopies 0.03. Four age-dependent liability classes were applied, as described (23). Furthermore, patients with colorectal adenoma as a single tumor were treated in two alternative ways: considering them either to have an unknown status (strict criteria) or to be affected (relaxed criteria).

PTT. The *MSH6* and *MSH3* cDNAs were divided into three overlapping fragments and studied by the protein truncation test as described by Percesepe *et al.* (24). The sizes of observed aberrant protein fragments were estimated against molecular weight standards, and the respective areas of cDNA were sequenced.

Direct Mutation Analysis of *MSH6* and *MSH3*. *MSH6* was sequenced from genomic DNA, with published primers covering each exon and the intron/exon borders (Ref. 9; with modifications). For the amplification and sequencing of the *MSH3* exons, primers were designed taking advantage of the sequence information available for the flanking introns (25). Primer sequences and reaction conditions are available from the authors upon request. Frameshift mutations at the *MSH6*-$(C)_8$ repeat were evaluated by a radioactive method using published primers and conditions (24, 26).

RESULTS

Linkage Analysis. Possible linkage to *MSH2*, *MLH1*, *MSH6*, and *MSH3* was evaluated with flanking microsatellite markers in 19 large families, stratified according to their microsatellite instability status into MSI-negative and MSI-positive groups. Table 2 shows the multipoint lod score values at each locus. The lod score values were below −2, the formal threshold of exclusion of linkage, for both the MSI-positive and the MSI-negative group; likewise, the lod scores for the individual families were mainly negative. However, there were a few exceptions; *e.g.*, in family 173, haplotype analysis and the multipoint lod score of 0.62 with a maximum at *D3S1611* suggested linkage to *MLH1*, despite the fact that no structural alterations of *MLH1* had been detected by direct exon-specific sequencing. Whereas the involvement of *MLH1* remains to be clarified, it is possible that this family shows a "hidden" *MLH1* mutation that leads to extinct expression without any structural changes demonstrable by conventional techniques (27). Family 436 showed a multipoint lod score close to 1 for *MSH3* when strict phenotypic criteria were applied; however, no *MSH3* mutation was detected by genomic exon-specific sequencing, thus providing no further support for *MSH3* involvement.

In family 2145, linkage to the *MSH2/MSH6* region was suggested by a multipoint lod score of 2.4 (Table 2), which is close to the formal threshold of 3 for statistically significant linkage. The lod score maximum was at markers *CA5* and *CA7* located in the immediate vicinity of *MSH2* and *MSH6* (17). Because *MSH2* mutation had already been excluded by DGGE in this family, we focused on *MSH6*, and sequencing revealed a frameshift mutation in exon 4 (deletion of CT at nucleotide 3052, codon 1018) with a predicted stop codon 10 bp later (Fig. 1). The mutation is located in a region known to be important for the interaction with *MSH2* (28). This mutation was originally detected in a blood DNA sample from individual III:1 and was subsequently found to segregate with the disease phenotype in six other family members, all with colorectal and/or endometrial cancer diagnosed at 48–73 years of age (Fig. 2). In contrast, the mutation was absent in another patient, III:11, with breast cancer and intestinal neurofibroma, suggesting that these tumors arose by an unrelated mechanism. To determine the MSI status of this family, archival tumor specimens were obtained from two patients who had not been included in the linkage study, II:3 and II:7, and who had been diagnosed with colon cancer at 82 and 74 years, respectively. Evaluation of two mononucleotide (*BAT26* and *BAT25*) and three dinucleotide repeat markers (*APC*, *D2S123*, and *D17S250*) showed that both tumors were MSI-negative. Neither individual had the *MSH6* alteration in a subsequent mutation analysis, suggesting that they were phenocopies. Unfortunately, no tumor samples were available from any known mutation-carriers from this family.

PTT. Families from which RNA samples were available (n = 85) were evaluated by PTT (including 14 that were also studied for linkage as a parallel approach). No new truncating mutations were identified in either *MSH3* or *MSH6*. The frameshift mutation of family 2145 was visible as a truncated product, as expected (data not shown).

Confirmatory Experiments. Because both presently used techniques have certain limitations (notably, linkage analysis is not informative in small families and PTT is not sensitive to nontruncating mutations), three additional approaches were applied to verify that possible mutations had not been missed. First, the *MSH6* fragment ("4k"; Ref. 9), containing the presently identified frameshift mutation, was sequenced in all Finnish families because family 2145 was of Finnish origin, and founding mutations are common in this population (29). These (or other) mutations were not detected in any of 26 families studied. Second, the same cohort of families was tested for frameshift mutations in the coding *MSH6*-$(C)_8$ tract in tumors, and in five families displaying such mutations, the entire *MSH6* gene was sequenced exon by exon because similar alterations were found to predict *MSH6* germ-line mutations in a previous study (7). In our investigation, no germ-line mutations were found. Third, because the *MSH6*-$(C)_8$ tract itself has previously been implicated in germ-line mutations (5), this tract was directly sequenced in all 85 families included in the PTT analysis; no germ-line mutation was detected. In conclusion, the fact that no additional mutations were identifiable by our confirmatory experiments indicates that the low frequency for *MSH6* and *MSH3* mutations, as suggested by linkage and PTT analysis, may reflect a real biological situation in our family series, rather than possible methodological shortcomings.

DISCUSSION

On the basis of published reports, the proportion of families not attributable to mutations in the two "major" DNA mismatch repair genes *MSH2* and *MLH1* is up to 51% among kindreds fulfilling the Amsterdam I criteria and up to 92% among kindreds not satisfying

Table 2 *Multipoint lod score values at the closest markers to MSH2/MSH6 (D2S123), MLH1 (D3S1611) and MSH3 (D5S424) calculated under the strict and relaxed criteria using programs Genehunter and SIMPLE*[a]

	D2S123			
	Lod score (strict)		Lod score (relaxed)	
Family	SIMPLE	Genehunter	SIMPLE	Genehunter
MSI-negative				
156	−0.23	−0.53	−0.88	−1.22
224	−1.80	−2.01	−1.80	−2.01
226	−0.05	−0.12	−0.66	−0.99
436	−0.58	−0.52	−1.07	−0.69
15		−0.26		−0.74
28		−0.41		−0.14
29		−0.02		−0.29
213		−0.32		−0.65
270		0.17		0.17
313		−0.36		−0.36
318		−0.73		−0.73
Total		−5.11		−7.65
MSI-positive				
173	−2.09	−0.71	−2.09	−0.71
24	−0.40	−0.65	−2.76	−0.53
171		−0.28		−0.28
211		−0.72		−0.72
219		−0.61		−0.33
2113[b]		0.33		0.33
48		−0.44		−0.44
Total		−3.08		−2.57
MSI unknown				
2145[c]	2.38	1.32	2.38	1.32

	D3S1611			
	Lod score (strict)		Lod score (relaxed)	
Family	SIMPLE	Genehunter	SIMPLE	Genehunter
MSI-negative				
156	−1.25	−0.73	−1.98	−1.12
224	−0.88	−1.77	−0.88	−1.77
226	0.01	0.14	−0.04	−0.13
436	−1.42	−0.13	−1.42	−1.29
15		0.01		−0.61
28		−0.28		−0.31
29		0.01		−0.51
213		−0.14		−0.34
270		0.05		0.05
313		0.14		0.14
318		−0.55		−0.55
Total		−3.25		−6.58
MSI-positive				
173[d]	0.62	0.61	0.62	0.61
24	−1.24	−0.581	−0.88	−0.66
171		−0.28		−0.28
211		−0.94		−0.94
219		0.26		−0.45
2113		−1.24		−1.24
48		−0.36		−0.36
Total		−3.05		−3.32
MSI unknown				
2145	−3.35	−1.41	−3.35	−1.41

Table 2. *Continued*

	D5S424			
	Lod score (strict)		Lod score (relaxed)	
Family	SIMPLE	Genehunter	SIMPLE	Genehunter
MSI-negative				
156	−1.06	−0.86	−1.62	−0.42
224	0.15	−0.37	0.15	−0.37
226[e]	0.46	0.31	−0.81	−1.02
436[f]	0.87	1.01	−0.43	0.56
15		−0.29		−0.86
28		−0.15		−0.81
29		−0.06		−0.48
213		0.04		−0.34
270		−0.02		−0.02
313		−0.28		−0.28
318		−0.79		−0.79
Total		−1.46		−4.83
MSI-positive				
173	−1.05	−1.22	−1.05	−1.23
24[g]	0.42	−0.042	−1.32	−0.58
171		−0.26		−0.26
211		−0.74		−0.74
219		−0.75		−0.83
2113		−0.67		−0.69
48		−0.04		−0.04
Total		−3.72		−4.37
MSI unknown				
2145	ND[h]	ND	ND	ND

[a] See "Materials and Methods."
[b] Shows no *MSH2* mutation by DGGE and no *MSH6* mutation by PTT or exon-specific sequencing.
[c] Shows a *MSH6* mutation.
[d] Shows no *MLH1* mutation by exon-specific sequencing.
[e] Shows no *MSH3* mutation by PTT.
[f] Shows no *MSH3* mutation by exon-specific sequencing.
[g] Shows no *MSH3* mutation by PTT.
[h] Not determined.

these criteria (14, 30–34). In our populations, these figures are (for Amsterdam-positive and -negative kindreds, respectively) 16% and 53% in Finland (32) and 50% and 82% in Sweden (35). The present study was conducted to explore further the genetic basis of the *MSH2* and *MLH1* mutation-negative families, with the emphasis on two "minor" DNA mismatch repair genes, *MSH6* and *MSH3*. Unlike many previous reports, we used no specific phenotypic criteria to select our families; instead, we included all families known to us that had screened negative for mutations in *MSH2* and *MLH1*, had at least two close relatives with nonpolypotic colon cancer, and contributed the necessary samples. As shown in Table 1, most of our families were Amsterdam I-negative, had microsatellite-stable tumors, were predominantly of late onset, and did not typically show endometrial cancers (however, five families were included that showed endometrial cancer only). In the present family series, the frequency of *MSH6* and *MSH3* germ-line mutations, respectively, was 1 of 90 (1%) and 0%, which is <1% and 0% among all Finnish and Swedish HNPCC and HNPCC-like kindreds screened for DNA mismatch repair gene mutations to date.

Our figures for *MSH6* mutations are lower than in some previous studies (see "Introduction"). Possible explanations include population-specific differences and the fact that we made no attempt to "enrich" *MSH6*-associated families by phenotypic selection. Methodological aspects could also play a role, as the techniques we used (linkage analysis and PTT) are not 100% sensitive. However, our observation that *MSH6* germ-line mutations are generally rare is compatible with a recent study (36) reporting the absence of such mutations among 41 patients with MSI-L colorectal cancer, unselected for family history. Moreover, the fact that our confirmatory experiments did not increase the mutation yield suggests that methodological flaws are not necessarily responsible. Additionally, there is a biological explanation for the disproportionate association of *MSH2* and *MLH1*, as compared with *MSH6* and *MSH3*, mutations with HNPCC, because protein products of the first two are invariably needed for DNA mismatch repair, whereas the products of the latter two are functionally redundant (see "Introduction"). In particular, *MSH6* can, at least in part, compensate for the loss of *MSH3* function,

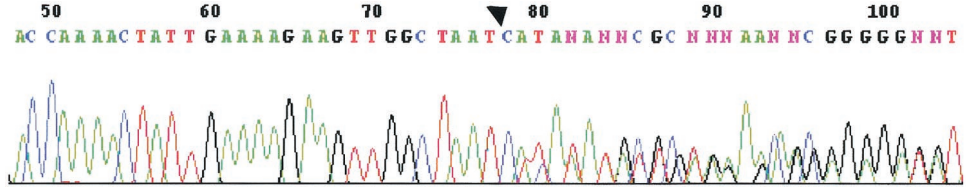

Fig. 1. Sequence tracing of *MSH6* in individual III:1 from family 2145 segregating a frameshift mutation (GCTAATC*T*CATA→GCTAATCATA) at nucleotide 3052 in exon 4, denoted by an *arrowhead*.

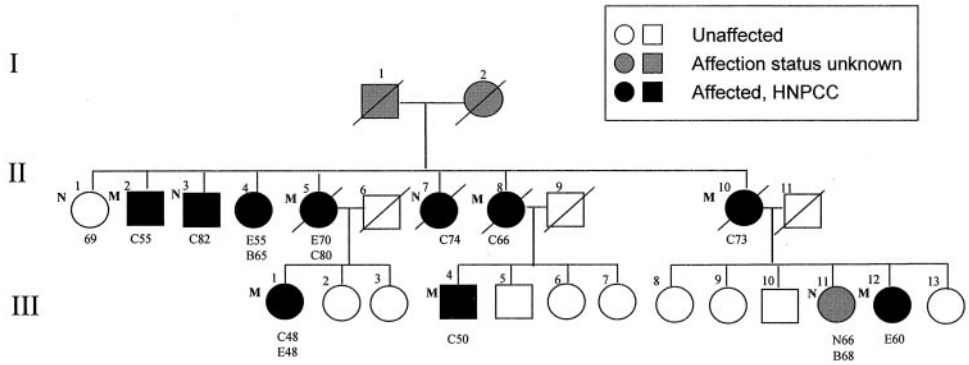

Fig. 2. Pedigree of family 2145 showing the segregation of the *MSH6* frameshift mutation (*M*, mutation carrier; *N*, noncarrier). Age at cancer diagnosis (affected individuals) or chronological age at the time of observation (unaffected individuals) is indicated *below* each symbol. The cancers are as follows: *C*, colon cancer; *E*, endometrial cancer; *B*, breast cancer; and *N*, neurofibroma. For confidentiality reasons, age and carrier status information is not shown for unaffected individuals from the youngest generation; among them, two mutation carriers were detected (51 and 71 years of age).

but not *vice versa* (4, 37), which makes it understandable why germline mutations in *MSH3* are even less frequent than those in *MSH6*. On the other hand, besides our study, *MSH3* germ-line mutations in HNPCC have really not been sought for on any large scale before, which provides an obvious additional reason for the general paucity of these mutations.

The presently detected truncating frameshift mutation in *MSH6* occurred in seven members from two generations diagnosed with colon and/or endometrial cancer at 48–73 years. Although the mean age at onset of colon cancer in mutation carriers (62 years) was more than 15 years higher than typically in HNPCC (38), it was 15 years lower than the peak incidence of colon cancer in the general population (39). The segregation data, together with the fact that mutations in the other DNA mismatch repair genes *MSH2*, *MLH1*, *MSH3*, *PMS1*, and *PMS2* had been excluded (this study and unpublished[5]), provided a strong indication that the *MSH6* mutation was responsible for cancer predisposition in this family. It is nevertheless interesting that there were three additional members, two with colon cancer and one with breast cancer, who did not have the mutation. These cases are likely to reflect a chance clustering of cancer for the following reasons. First, all were diagnosed at an advanced age (82, 74, and 68 years). Second, the two colon cancers that could be tested were MSI-negative. Third, cancers of the colorectum and breast are among the three most common cancers in the general population (39). However, epidemiological studies suggest that familial clustering of colon cancer often results from a partially penetrant inherited susceptibility (40), and therefore we cannot exclude the possibility of additional cancer-associated gene defects segregating in this family.

A final remark concerns the nature of cancer susceptibility in the remaining, quite significant number of HNPCC and HNPCC-like families with no detectable germ-line mutations in *MSH2*, *MLH1*, *MSH6*, or *MSH3*. Especially in families with microsatellite-unstable tumors, mutations in *PMS1* and *PMS2*, as previously reported in occasional HNPCC kindreds (41), as well as in *MLH3*, a newly identified human DNA mismatch repair gene (42), remain as a possibility. However, our preliminary data[5] suggest that mutations in these genes may not be very common, either. Apart from excluding the involvement of the presently known HNPCC-associated genes, the linkage approach that we used is useful to identify novel loci for HNPCC predisposition. We have recently extended our linkage study to a genome-wide search and have tentatively identified novel chromosomal regions of linkage whose further characterization is in progress.

[5] T. Liu, S. Kuismanen, P. Peltomäki, and Annika Lindblom, manuscript in preparation.

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