Lynch Syndrome (Hereditary Nonpolyposis Colorectal Cancer) Diagnostics

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Background Preventive programs for individuals who have high lifetime risks of colorectal cancer may reduce disease morbidity and mortality. Thus, it is important to identify the factors that are associated with hereditary colorectal cancer and to monitor the effects of tailored surveillance. In particular, patients with Lynch syndrome, hereditary nonpolyposis colorectal cancer (HNPCC), have an increased risk to develop colorectal cancer at an early age. The syndrome is explained by germline mutations in DNA mismatch repair (MMR) genes, and there is a need for diagnostic tools to preselect patients for genetic testing to diagnose those with HNPCC.

Methods Patients (n = 112) from 285 families who were counseled between 1990 and 2005 at a clinic for patients at high risk for HNPCC were selected for screening to detect mutations in MMR genes MLH1, MSH2, MSH6, and PMS2 based on family history, microsatellite instability (MSI), and immunohistochemical analysis of MMR protein expression. Tumors were also screened for BRAF V600E mutations; patients with the mutation were considered as non-HNPCC.

Results Among the 112 patients who were selected for screening, 69 had germline MMR mutations (58 pathogenic and 11 of unknown biologic relevance). Sixteen of the 69 mutations (23%) were missense mutations. Among patients with MSI-positive tumors, pathogenic MMR mutations were found in 38 of 43 (88%) of patients in families who met Amsterdam criteria and in 13 of 22 (59%) of patients in families who did not. Among patients with MSI-negative tumors, pathogenic MMR mutations were found in 5 of 17 (29%) of families meeting Amsterdam criteria and in 1 of 30 (3%) of non-Amsterdam families with one patient younger than age 50 years. In three patients with MSI-negative tumors who had pathogenic mutations in MLH1 or MSH6, immunohistochemistry showed loss of the mutated protein.

Conclusion Our findings suggest that missense MMR gene mutations are common in HNPCC and that germline MMR mutations are also found in patients with MSI-negative tumors.

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Hereditary nonpolyposis colorectal cancer (HNPCC), also referred to as Lynch syndrome, is characterized by an autosomal dominant inheritance of early-onset colorectal cancer and an increased risk of other cancers, including cancers of the endometrium, stomach, ovary, urinary tract, hepatobiliary tract, pancreas, and small bowel (1,2). Males with HNPCC have a higher lifetime risk for colorectal cancer (standardized incidence ratio [SIR] = 83) than females (SIR = 48) (3), and surveillance with regular colonoscopy and polypectomy has been shown to reduce disease morbidity and mortality (4). HNPCC is caused by germline mutations in DNA mismatch repair (MMR) genes, and the prevalence of this syndrome among unselected colorectal cancer patients in Sweden has been estimated, based on family history, to be approximately 1% (5,6). Although seven genes have been associated with HNPCC (MSH2, MLH1, MSH6, PMS1, PMS2, MLH3, and EXO1), mutations in only three are currently considered to cause HNPCC: MLH1, MSH2, and MSH6.

It is important to correctly diagnose all HNPCC patients and to distinguish them from non-HNPCC patients so that all families

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CONTEXT AND CAVEATS

Prior knowledge

Lynch syndrome, or HNPCC, is currently diagnosed by the detection of germline mutations in MMR genes.

Study design

A new protocol to select patients for screening for germline mutations in MMR genes was tested on family members who had undergone counseling for being at high risk for HNPCC.

Contribution

Overall, mutations in MMR genes were found in more than half of the patients who were screened for mutations, including some who would not have been screened using current protocols.

Implications

More true HNPCC cases were identified using the new protocol than the previous protocol.

Study limitations

It is possible that some patients who were not selected for mutation screening also had tumors with germline mutations in MMR genes.

can be offered tailored counseling and surveillance and, when possible, genetic testing. Several attempts have been made to define clinical criteria that can be used to diagnose HNPCC. In the first studies, which aimed to define the genes involved in HNPCC, the International Collaborative Group on HNPCC defined and later updated the so-called Amsterdam criteria for the classification of HNPCC (7). The Amsterdam criteria require that families have three affected individuals over two generations—one being a first-degree relative of the other two and one younger than age 50 years. MMR gene mutations have been identified in up to 90% of families who meet the original Amsterdam criteria, whereas application of less stringent criteria results in lower sensitivity but higher specificity (8,9). Studies of young (<50 years) patients with nonfamilial colorectal cancer have shown that many have germline mutations in MMR genes, and thus, the use of early age of onset as a criterion in the selection of patients for genetic mutation screening has been emphasized as being important for HNPCC screening (10).

During the past 12 years, screening techniques for mutations in MMR genes have been evaluated and optimized. Most early studies used various exon-by-exon polymerase chain reaction (PCR)–based methods to screen subjects with a family history of cancer. Mutations were found in 40%–60% of patients of families who met the Amsterdam criteria and in a smaller percentage of patients in families who did not fulfill these criteria (11). Studies using a combination of other mutation detection methods to detect larger genomic rearrangements show higher mutation rates than those first used (12–17).

Tumor microsatellite instability (MSI), a hallmark of HNPCC and a sign of an increased mutation rate in HNPCC tumors, is also observed in approximately 15% of unselected colorectal cancers (18). Although MSI in unselected colorectal cancer is most often associated with somatic CpG island methylation of the promoter of the MMR gene MLH1, a positive MSI test in familial or early-onset colorectal cancer is highly associated with HNPCC (19,20). Immunohistochemical analysis to detect loss of expression of the MMR proteins MLH1, MSH2, MSH6, and PMS2 can identify HNPCC-associated MMR-defective tumors and potential mutations in these genes and can thus facilitate mutation analysis (21). However, the inability of immunohistochemistry to identify loss of MMR protein expression in all HNPCC-associated MMR-defective tumors motivated the continuation of MSI analysis (22,23).

Recently, somatic mutations in the BRAF oncogene have been used to distinguish HNPCC-associated tumors from sporadic tumors with MSI. BRAF mutations were first demonstrated in melanomas and other types of cancers (24). Approximately 10% of sporadic colorectal cancers have mutations in BRAF, and the mutation is almost always V600E (25,26). There is a strong association between BRAF mutation status and MSI in colorectal tumors (26). Moreover, BRAF is often mutated in association with methylation of the MLH1 promoter and is almost never mutated in HNPCC tumors (26,27).

The number of HNPCC families whose genetic background has been clarified has gradually increased over time with the improvement of diagnostic procedures. In this study, we evaluated our current protocol to diagnose HNPCC. We reanalyzed all families who were counseled for an increased risk of colorectal cancer according to this updated protocol using family history, MSI, and immunohistochemistry testing to preselect patients for mutation screening of MLH1, MSH2, MSH6, and PMS2 genes. We also tested for the BRAF V600E mutation (Fig. 1).

Subjects and Methods

Patients

Families who were referred to the Karolinska University Hospital for genetic counseling during 1990–2005 were included in the study if they fulfilled the following criteria: 1) at least one member was diagnosed with colorectal cancer before age 50 years or two or more first- or second-degree relatives were diagnosed with colorectal cancer at any age, 2) colorectal tumor tissue was available for MSI analysis, and 3) at least one member was available for mutation screening. In total, 285 families met the criteria to be included in this study, including 37 patients who had no family history of colorectal cancer and were diagnosed when younger than age 50 years. A complete family history was available from every family that was included in the study. All diagnoses were verified through medical records or death certificates.

The families were first classified as Amsterdam or as non-Amsterdam according to family history. The Amsterdam criteria require that families have three affected individuals over two generations—one being a first-degree relative of the other two and one younger than age 50 years. Because HNPCC families also have high risks for other tumor types, these criteria were modified in 1999 to include endometrial cancer and cancers of the small bowel and the upper urothelial tract; the revised criteria are referred to as the Amsterdam criteria II (7). Of the 285 families included in the study, 60 met the Amsterdam criteria. The families that did not fit the criteria (non-Amsterdam families) could be divided into two groups—one group of 119 families

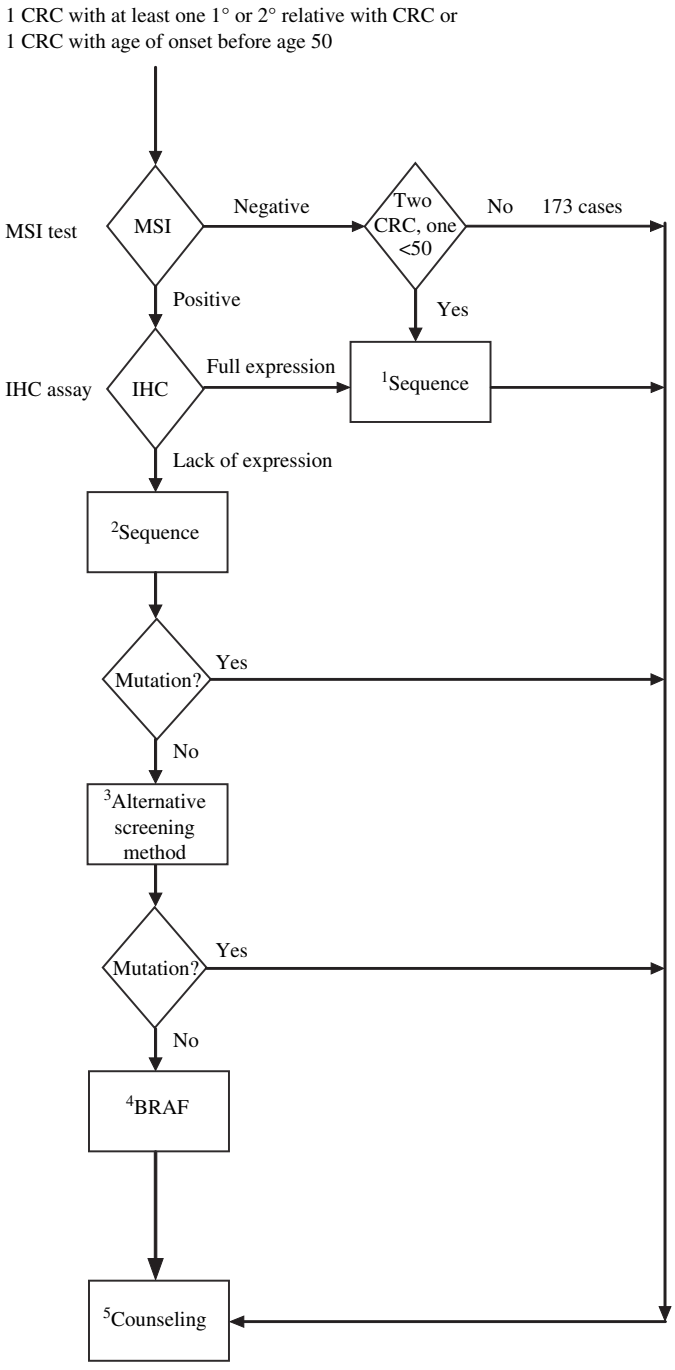

Fig. 1. Flow diagram of the hereditary nonpolyposis colorectal cancer (HNPCC) diagnostic procedure used in this study. **Top**, inclusion criteria. **1**) Microsatellite instability (MSI)–positive samples expressing all four mismatch repair (MMR) genes (MLH1, MSH2, MSH6, and PMS2) and MSI-negative samples from familial cases, among which one patient was younger than age 50 years, were sequenced for MLH1, MSH2, and MSH6. **2**) MSI-positive samples were sequenced according to which protein had low or no expression by immunohistochemistry (IHC), e.g., MLH1 if loss of MLH1 (with or without simultaneous loss of PMS2), MSH2 if loss of MSH2 (with or without simultaneous loss of MSH6), MSH6 if loss of MSH6, PMS2 if loss of PMS2 only. **3**) Alternative screening methods were quantitative multiplex polymerase chain reaction of short fluorescent fragments or multiplex ligation-dependent probe amplification. **4**) BRAF was sequenced only to verify a potential V600E mutation when no germline MMR gene mutation was found. **5**) Counseling for HNPCC if a MMR gene germline mutation was found and considered pathogenic. Disease was considered non-HNPCC if no pathogenic germline mutation was found. CRC = colorectal cancer.

each with two patients only (of which 21 had one patient younger than age 50 years) and another group of 69 families each with at least three patients affected with colorectal cancer. The second group of 69 families did not fulfill the Amsterdam criteria for the following reasons: 60 did not have any patient younger than age 50 years, 23 did not have three patients with a first-degree relationship, and 11 did not fulfill the criteria across two generations. The study was undertaken in accordance with the decisions in the Regional Ethical Review Board in Stockholm (97/205, 00/291, and 05/566).

Laboratory Diagnostic Procedures

This retrospective study used the protocol in Fig. 1 to diagnose HNPCC patients. Dissection of tumor cells from paraffin-embedded tissue for MSI and gene mutation assays were performed at the Department of Clinical Genetics (Karolinska University Hospital). The technique for MSI testing followed the international Bethesda guidelines (28) and used at least one colorectal cancer patient from each family. We preferred to test the youngest patient in the family because of the risk that an older patient might have sporadic cancer. If the preferred patient could not be used for MSI testing, if the tumor was MSI negative and HNPCC was still suspected, or for research purposes, at least one more tumor from the same patient or another family member was tested, if possible. More than one tumor was tested in a total of 35 families. In 19 families, three or more tumors were tested. A pathologist at the hospital where the specimens were archived provided the tumor tissue to be used for MSI testing. None of the MSI tests was performed using adenoma tissue. MSI testing was performed as described previously (19) or using a commercial kit (Microsatellite Instability Multiplex System Kit, Promega Corp, Madison, WI) according to the manufacturer's instructions. Briefly, genomic DNA prepared from tumor tissue was amplified by PCR in a multiplex PCR, amplifying selected microsatellite markers. Microsatellite analysis was performed using an ABI310 (Applied Biosystems, Foster City, CA) with the GeneScan 3.1 software (Applied Biosystems). MSIH (MSI high) was considered MSI positive and MSS (MS stable) and MSIL (MSI low) were considered MSI negative. The classification of MSIH, MSS, and MSIL were according to the Bethesda/National Institutes of Health (NIH) guidelines (28).

Immunohistochemistry for MLH1, MSH2, MSH6, and PMS2 was performed on tumors from MSI-positive patients only because our previous study (23) showed that 90% of MSI-positive tumors and none of 70 MSI-negative familial colorectal tumors lacked immunostaining pinpointing a mutated MMR gene (23). In 37 families, mutations had been identified before the introduction of immunohistochemistry analysis to the clinical protocol. Thus, tumor samples from 38 families were subjected to immunohistochemical analysis. MSI-positive samples were selected, analyzed, and evaluated as described by Halvarsson et al. (23).

Samples from the youngest colorectal cancer patient available from each Amsterdam family, MSI-positive family, and MSI-negative family with one patient younger than age 50 years were screened for mutations in MLH1, MSH2, MSH6, and PMS2. Screening was also performed in all single patients with early-onset disease and MSI-positive tumors. Exon-by-exon screening was performed using genomic DNA–based methods, such as

Table 1. Hereditary nonpolyposis colorectal cancer screening of patients from 285 families*

Criteria	MSI status	No. of patients with MMR mutation/total	MMR gene mutation type	
			Pathogenic	Unknown
Amsterdam family	Positive	43/43	38	5
	Negative	8/17	5	3
	Total	51/60	43	8
Non-Amsterdam family	Positive	10/17	9	1
	Negative	2/171†	1	1
	Total	12/188	10	2
Single patients	Positive	5/5	4	1
	Negative	0/32	0	0
	Total	5/37	4	1
All patients		68/285	57	11

* MSI = microsatellite instability; MMR = mismatch repair.

† Of these, 30 were screened for mutations because one patient in the family was younger than age 50 years.

denaturant gradient gel electrophoresis or sequencing. Screening for rearrangements used RNA- or DNA-based methods, such as protein truncation test, monoallelic mutation analysis, quantitative multiplex PCR of short fluorescent fragments, or multiplex ligation-dependent probe amplification. All nonsense mutations, splice mutations, and genomic rearrangements (deletions and insertions) were considered pathogenic. The pathogenicity of missense mutations was defined based on published data, based on the segregation of the mutation in at least three affected subjects in the family, and by comparing the frequency in 80–200 normal control subjects.

The mutation status of BRAF was assayed only to rule out HNPCC in MSI-positive patients for whom no MMR gene mutation had been identified or for whom mutations were found but considered to be of unclear biologic relevance. In brief, genomic DNA prepared from tumor tissue was amplified using PCR. The PCR product was then used for direct sequencing using the BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems) according to the manufacturer's recommendation. Sequences were analyzed using an ABI Prism 3730 Sequencer (Applied Biosystems). The chromatograms were evaluated manually by eye or with SeqScape v2.5 (Applied Biosystems). The tumor was considered V600E positive if the mutation could be detected on the chromatograms. The rationale for using BRAF V600E mutations to rule out HNPCC was based on findings that this mutation almost never occurs in HNPCC (26,27).

Results

Patients from 285 families were evaluated using a new diagnostic protocol (Fig. 1). The study included 60 families who met the Amsterdam criteria, 188 non-Amsterdam families, and 37 single patients with early-onset (younger than age 50 years at diagnosis) colorectal cancer. Patients who had an MSI-positive tumor were identified in 70% of the Amsterdam families, 9% of the non-Amsterdam families, and 13% of the early-onset patients (Table 1).

A total of 112 patients were screened for mutations in MLH1, MSH2, MSH6, and PMS2; 173 were not. Patients were screened if they were from an Amsterdam family, an MSI-positive family, or an MSI-negative family with one patient younger than age 50 years. Single patients with early-onset disease and MSI-positive tumors were also screened.

Mutations in Patients in Amsterdam Families With MSI-Positive Tumors

In total, 43 germline mutations in MLH1, MSH2, MSH6, or PMS2 were found in 42 of 43 patients in Amsterdam families with MSI-positive tumors; 38 of the mutations were considered pathogenic (88%) (Table 2). Nine missense mutations were found, as discussed below. Among families with a mixed pattern of MSI (both MSI-positive and MSI-negative tumors in the same family), MMR mutations were either not found (family 722) or did not segregate with disease within the family (families 19, 110, and 119). MMR mutations that did not segregate with disease were defined as having unknown biologic relevance.

Mutations in Patients of Non-Amsterdam Families and Early-Onset Single Patients With MSI-Positive Tumors

A germline mutation in one of the MMR genes was found in 15 of 22 MSI-positive patients in non-Amsterdam families (Table 2). Thirteen of these mutations, including two missense mutations, were considered pathogenic (59%). In two patients from different families, the same truncating PMS2 mutation was found that was considered to be of unclear biologic relevance. We considered the seven MSI-positive non-Amsterdam families with no MMR gene germline mutations as non-HNPCC families. Within these seven families, tumors of patients in five families showed lack of MLH1 protein staining and had the BRAF V600E mutation (families 237, 289, 350, 374, 409) and/or had a mixed MSI pattern (families 237, 350, 409, 301, 399). Finally, all five of the single patients with MSI-positive early-onset colorectal cancer and who were included in the non-Amsterdam group had germline mutations in one of the MMR genes (Table 2). Of those mutations, four were considered pathogenic. Thus, we found evidence for or against HNPCC in 20 of 22 patients with MSI-positive tumors in non-Amsterdam families.

Mutations in Patients With MSI-Negative Tumors

A germline mutation in one of the MMR genes was found in 8 of 17 MSI-negative Amsterdam families (Table 2). Five of the eight mutations were considered pathogenic (in total 29%)—one in

Table 2. Germline or somatic mutations identified during hereditary nonpolyposis colorectal cancer (HNPCC) screening

Family No.	Type*	MSI†	IHC‡	BRAF§	E-age‖	M-age‖	Gene¶	Mutation	Type	HNPCC#	Reference
					Amsterdam families with MSI-positive tumors						**
1	Amsterdam	+		–	24	29	MLH1	[del exon 1–15]	Del	Yes	This study
2	Amsterdam	+		–	34	51	MLH1	[c.131C>T]	Missense	Yes	(29)
3	Amsterdam	+		–	42	50	MLH1	[c.104T>G]	Missense	Yes	(30)
4	Amsterdam	+	–	–	32	45	MLH1	[del exon 11]	Del	Yes	(15)
5	Amsterdam	+			29	50	MSH2	[c.201del G]	Del	Yes	(31)
6	Amsterdam	+		–	44	48	MSH2	[c.892C>T]	Nonsense	Yes	(32)
7	Amsterdam	+	+	–	27	36	MLH1	[del exon 16]	Del	Yes	(33)
10	Amsterdam	+			35	46	MLH1	[c.208–2A>G]	Splice	Yes	(30)
11	Amsterdam	+	–	–	37	43	MSH2	[c.2038C>T]	Nonsense	Yes	This study
14	Amsterdam	+		–	36	45	MLH1	[del exon 16]	Del	Yes	(33)
19	Amsterdam	M	M	–	44	54	MLH1	[c.2059C>T]	Missense		This study, (34)
24	Amsterdam	+		–	24	47	MSH2	[c.1216C>T]	Nonsense	Yes	(32)
27	Amsterdam	+			27	41	MLH1	[del exon 16]	Del	Yes	(33)
30	Amsterdam	+			33	34	MSH2	[c.1097 del A]	Del	Yes	(35)
58	Amsterdam	+			31	39	MLH1	[del exon 16]	Del	Yes	(32)
63	Amsterdam	+			45	58	MLH1	[c.1769 del T]	Del	Yes	(35)
66	Amsterdam	+	–	–	41	42	MLH1	[del exon 6]	Del	Yes	(15)
69	Amsterdam	+			34	48	MLH1	[c.199G>A]	Missense	Yes	(30)
98	Amsterdam	+			32	41	MLH1	[del exon 16]	Del	Yes	(32)
110	Amsterdam	M	–		33	50	MLH1	[c.2104-10_11del GT ins A]	Intronic		This study
113	Amsterdam	+	–	–	31	48	MSH2	[c.2131C>T]	Nonsense	Yes	This study
119	Amsterdam	M	–	–	24	57	PMS2	[c.2113G>A]	Missense		(36)
124	Amsterdam	+			40	42	MSH2	[c.942+3A>T]	Nonsense	Yes	(32)
136	Amsterdam	+			38	43	MLH1	[del exon 16]	Del	Yes	This study
149	Amsterdam	+	–	–	40	45	MSH2	[del exon 8]	Del	Yes	(15)
163	Amsterdam	+	–		40	46	MLH1	[c.454–13A>G]	Splice	Yes	This study
187	Amsterdam	+			45	49	MSH2	[c.942+3A>T]	Splice	Yes	This study
198	Amsterdam	+		–	38	48	MLH1	[c.793C>T]	Missense	Yes	(32)
228	Amsterdam	+			35	42	MLH1	[c.131C>T]	Missense	Yes	This study
246	Amsterdam	+			38	59	MLH1	[c.1772_1775 del ATAG]	Del	Yes	This study
262	Amsterdam	+	–	–	31	39	MSH2	[del exon 13–15]	Del	Yes	(15)
269	Amsterdam	+		–	41	52	MLH1	[c.665 del A]	Del	Yes	This study
292	Amsterdam	+			46	51	MSH2	[c.2228 del CATT]	Del	Yes	This study
294	Amsterdam	+			34	50	MSH2	[c.1447G>T]	Nonsense	Yes	This study
302	Amsterdam	+		–	39	49	MSH2	[c.811_814 del TCTG]	Del	Yes	This study
362	Amsterdam	+	–		44	44	MLH1	[c.1225C>T]	Nonsense	Yes	This study
368	Amsterdam	+	–		35	44	MSH2	[del exon 1–6]	Del	Yes	This study
388	Amsterdam	+	–		40	51	MLH1	[del exon 16]	Del	Yes	This study
435	Amsterdam	+	–		56	57	MLH1	[c.306+3A>C]	Splice	Yes	This study
475	Amsterdam	+	–		44	44	MSH2	[c.1447_1448 del AG]	Del	Yes	This study
546	Amsterdam	+	–		39	47	MLH1	[c.1459C>T]	Nonsense	Yes	This study
722	Amsterdam	M	–		44	65					
765	Amsterdam	+	–	–	36	46	MLH1	[c.2059C>T]	Missense		This study
765							PMS2	[c.1866G>A]	Missense		This study
					Non-Amsterdam families with MSI-positive tumors						
15	Non-Amsterdam	+		–	36	51	MLH1	[c.131C>T]	Missense	Yes	(30)
16	Non-Amsterdam	+			30	36	MLH1	[c.546–2A>G]	Splice	Yes	(30)
28	Non-Amsterdam	+		–	35	39	MLH1	[c.1373T>A]	Nonsense	Yes	(32)
34	Non-Amsterdam	+		–	22	28	MLH1	[del exon 4–11]	Del	Yes	(15,31)††
88	Non-Amsterdam	+	–		41	42	MLH1	[del exon 14–15]	Del	Yes	(15,31)††
204	Non-Amsterdam	+	–		52	63	MSH2	[del exon 1–8]	Del	Yes	(15)
291	Non-Amsterdam	+		–	42	43	MLH1	[c.677+1G>T]	Splice	Yes	This study
347	Non-Amsterdam	+	–		38	44	MSH2	[del exon 1–7]	Del	Yes	(15)
400	Non-Amsterdam	+	–		45	47	MSH2	[dup ex 7–8]	Duplication	Yes	(15)
391	Non-Amsterdam	+	–	+	54	77	PMS2	[c.736 del 6 ins11]	Del		(36)
237	Non-Amsterdam	M	–	+	55	66		BRAF V600E			
289	Non-Amsterdam	+	–	+	53	64		BRAF V600E			
350	Non-Amsterdam	M		+	57	72		BRAF V600E			

(Table continues)

Table 2 (continued).

Family No.	Type*	MSI†	IHC‡	BRAF§	E-age‖	M-age‖	Gene¶	Mutation	Type	HNPCC#	Reference
374	Non-Amsterdam	+	–	+	55	63		BRAF V600E			
409	Non-Amsterdam	M	–	+	40	58		BRAF V600E			
301	Non-Amsterdam	M	–	–	69	75		BRAF not informative			
399	Non-Amsterdam	M	+	–	53	60		BRAF wild type			
					Single early-onset patients with MSI-positive tumors						
59	1 CRC < 50	+					MSH2	[c.1226_1227 del AG]	Del	Yes	(35)
166	1 CRC < 50	+	–	–			MSH2	[del exon 2]	Del	Yes	(15)
331	1 CRC < 50	+	–				MSH2	[del exon 1–6]	Del	Yes	(15)
480	1 CRC < 50	+	–	+			PMS2	[c.736_741 del 6 ins11]	Del		(36)
669	1 CRC < 50	+	–				MSH2	[c.1906C>T]	Missense	Yes	This study
					Amsterdam families with MSI-negative tumors						
145	Amsterdam	–			50	65	MSH6	[c.3052_3053 del CT]	Del	Yes	(37)
175	Amsterdam	–		–	50	66	MLH1	[c.1733A>G]	Missense		(38)
181	Amsterdam	–		–	40	56	MLH1	[c.1733A>G]	Missense		(38)
199	Amsterdam	–	+	–	29	53	MSH2	[c.593A>G]	Missense	Yes	(32)
241	Amsterdam	–		–	45	65	MLH1	[c.2146G>A]	Missense		This study
340	Amsterdam	–			41	53	MSH6	[c.2303 del CCT]	Del	Yes	This study
424	Amsterdam	–	–		41	45	MLH1	[c.298C>T]	Nonsense	Yes	This study
534	Amsterdam	–	–		49	52	MSH6	[c.2851_2858 del 8]	Del	Yes	This study
					Non-Amsterdam families with MSI-negative tumors						
341	Non-Amsterdam	–	–		35	43	MLH1	[c.454–1G>A]	Splice	Yes	This study
487	Non-Amsterdam	–	+	–	49	49	MSH6	[c.3674C>T]	Missense		This study

* Single early-onset patients do not meet Amsterdam criteria (7).

† MSI = microsatellite instability; + = positive; – = negative; M = more than one tumor were studied and showed different results.

‡ IHC = immunohistochemistry; + = the mutated gene expressed the protein; – = the mutated gene did not express the protein; M = results differed between tumors; missing values indicate that immunohistochemistry was not done.

§ BRAF: + = V600E; – = wild-type missing values indicate that the test was not done.

‖ E/M = early/mean; missing values for single patients younger than age 50 years.

¶ Gene names according to GenBank.

Yes indicates that the mutation was considered pathogenic and causes HNPCC. Missing values indicate unclear biologic relevance.

** In family 1, the mutation was found using the monoallelic mutation analysis technique, performed as in (14).

†† Mutation results in this study differ from the two studies because different techniques were used to detect them.

MLH1, one in MSH2, and three in MSH6. Four of the eight mutations were missense mutations (Table 2).

Mutation screening was also performed in the 30 MSI-negative non-Amsterdam families among whom one member was younger than age 50 years at diagnosis. From the 30 families screened, two mutations were found (Table 2). One was a splice mutation in MLH1; the other was a missense mutation in MSH6. To confirm MSI status, additional MSI analyses as well as immunohistochemistry analysis were performed on a subset of tumors from 38 families. Tumors from two patients in family 145 were tested for MSI and both were negative. Tumors from two patients in family 424 tested MSI negative, but one showed lack of MLH1 protein by immunohistochemistry. The MSI test was repeated once using a new section of this tumor after the immunohistochemistry result and was still negative. A tumor from a patient in family 534 was MSIL but lacked expression of MSH6 by immunohistochemistry. Two patients in family 341 had the (c.454–1G>A) MLH1 splice mutation, and both had metachronous colorectal cancers at ages 51 and 68 and 35 and 51 years, respectively. Both the tumor at age 35 years and the metachronous tumor were MSI negative. However, immunohistochemistry showed loss of MLH1 protein, and therefore, the mutation was considered to be pathogenic.

Missense Mutations

In total, we found 16 missense mutations. Missense MLH1 mutation (c.131C>T) in families 2, 15, and 228 is a Swedish founder mutation that segregated with affected status in families and was not found in normal control subjects (29,30). The MLH1 mutation (c.2059C>T) in families 19 and 765 has been observed in Polish families (34) and was not found in normal control subjects. This mutation did not segregate consistently in the families in this study and thus was considered of unclear biologic relevance. A PMS2 missense mutation (c.1866G>A), which was previously published as a polymorphism (39), was also found in family 765. The MLH1 mutation in family 198 (c.793C>T) segregated with some MSI-positive tumors and was not found in 96 control subjects. In family 198, one patient had MSI-negative colorectal cancer at 46 years of age and two metachronous tumors at 79 and 82 years of age.

However, this patient did not inherit the mutation; thus, another predisposing factor may contribute to colorectal cancer in this family. The MLH1 mutations in families 3 (c.104T>G) and 69 (c.199G>A) segregated with disease in the families and were not found in normal control subjects. The mutation in family 69 has been observed in numerous other families (40). The MSH2 missense mutation c.1906C>T in family 669 is a founder mutation in Ashkenazi Jews that is considered to cause HNPCC (41). A germline missense MSH2 mutation (c.593A>G) segregated in family 199 with three MSI-negative tumors and was not found in control subjects. We consider this MSH2 mutation to be pathogenic, although it is possible that a coexisting MLH3 mutation contributed to disease in this family (42). In family 241, a MLH1 missense mutation (c.2146G>A) segregated with disease in affected family members and was not found in normal control subjects. This mutation has been published in other HNPCC families but is still considered to be of uncertain biologic relevance (39). The same MLH1 missense variant (c.1733A>G) was found in families 175 and 181. This variant did not segregate with disease and was considered to be of unclear biologic relevance. However, this variant may serve as a low-risk/modifying allele (38). The missense MSH6 mutation (c.3674C>T), not found in control subjects, was found in family 487, in which both parent and child had MSI-negative colon tumors at age 49 years. Because segregation analysis was not possible, this missense mutation was considered as having unclear biologic relevance. The PMS2 missense mutation (c.2113G>A) in family 119 did not segregate well with disease in the family, which had a mixed pattern of MSI, and was thus considered as having an unclear biologic relevance.

Discussion

As expected, the MMR gene mutation rate was high (88%) among HNPCC patients in Amsterdam families with MSI-positive tumors. We also observed that 59% of patients in non-Amsterdam families with MSI-positive tumors had MMR gene mutations. The Bethesda criteria would have selected all mutation-positive patients to be tested for MSI or immunohistochemistry (28). If we had only screened MSI-positive patients for mutations, the 10 mutations in MSI-negative patients would have been missed. If we had used immunohistochemistry instead of MSI testing, we would have found some of the mutations in MSI-negative patients but might have missed some of the missense mutations.

We found five deleterious MMR gene mutations in tumors from MSI-negative patients (in families 145, 340, 341, 424, and 534). Three of these were in MSH6, associated with a lower degree of MSI-positive tumors (43), and two were in MLH1. MSI associated with MSH6 mutations preferentially shows mutations in mononucleotide repeats (43). The MSI test used four mononucleotide repeats, and only one patient (family 534) had a tumor that was MSIL. For three of the five MSI-negative tumors with MMR gene mutations, immunohistochemistry results showed lack of the mutated protein in all tumors, suggesting that immunohistochemistry can sometimes be more informative than MSI testing in predicting a germline predisposing mutation. The negative MSI test was confirmed in a second analysis after the immunohistochemistry result (in families 145, 341, 424, and 534). For MSI-negative tumors with MMR missense mutations, it is difficult to evaluate the risk of disease. In this study, we considered four (175, 181, 241, and 487) of five mutations to be of unknown biologic relevance.

Families among whom a full genetic investigation did not reveal a pathogenic MMR gene mutation were considered to be non-HNPCC. Because we did not continue the search for deleterious mutations in all patients using all current methods known, it is still possible that a deleterious mutation could have been undetected in patients with no mutation and even in patients with missense mutations. Mutations in at least seven genes have been associated with HNPCC: MSH2, MLH1, MSH6, PMS1, PMS2, MLH3, and EXO1. The former three gene mutations are associated with the vast majority of HNPCC (5,40). Mutations in PMS1 are no longer considered to be associated with colorectal cancer, and the consequences of germline PMS2 mutations, including the three mutations identified in our study, are still unclear (36,39,44,45). The MLH3 and the EXO1 genes have been studied in families fulfilling or not fulfilling the Amsterdam criteria, and several missense mutations of unclear biologic relevance have been identified (42,46). Because so many MSI-negative patients in our study had MMR gene mutations, it is still possible that we missed HNPCC families by not screening the 173 patients with MSI-negative tumors who were not selected by our new protocol (Fig. 1). Based on this protocol, we selected 30 families with at least one patient younger than age 50 years. This criterion resulted in mutations being found in two families, both having a pedigree that is highly suggestive of HNPCC. Thus, it might be possible to improve our protocol by defining new criteria for selecting MSI-negative patients for mutation screening. The new criteria should include the age of onset in the entire family as well as penetrance and what type of tumors should be considered. Immunohistochemistry could also be used to preselect MSI-negative patients for mutation screening.

When a germline MMR gene mutation was not detected in a family with MSI-positive tumors, we tried to obtain evidence for or against HNPCC. Five such families showed a mixed pattern of MSI. The presence of the BRAF V600E excluded HNPCC in five families. A syndrome involving a mixed pattern of MSI as well as BRAF mutations in tumors and precursor lesions has been suggested and is supported by our data (47). A new syndrome X was also recently suggested to occur in Amsterdam families with MSI-negative tumors (48). Thus, both the BRAF-associated syndrome and the syndrome X could explain some of the non-HNPCC families in our study.

For now, we consider the vast majority of the remaining 173 unscreened patients in our cohort of non-Amsterdam patients to have non-HNPCC. We hypothesize that most non-HNPCC patients are at risk for disease due to other genetic factors that act as monogenic or additive/modifying factors in a complex disease, and in the Karolinska University Hospital, we offer also non-HNPCC families tailored surveillance programs based on empiric risk figures. We also offer subjects at risk for disease who are members of families with known or suspected MMR gene mutations colonoscopy screening at 2-year intervals beginning at age 25 years. We offer all non-HNPCC risk individuals a screening colonoscopy every 3–5 years, depending on the

risk, starting at least 10 years before the earliest age of onset in the family.

Although it is critical to identify all individuals who are at increased risk for colorectal cancer to offer them surveillance programs, it is equally important to accurately exclude individuals in a specific family who are not at increased risk. Thus, it is very important to find the gene mutations that are associated with HNPCC. Different criteria to select patients for mutation screening have been outlined and evaluated. The Centre for Reviews and Dissemination (49) concluded that a mixed strategy, similar to our protocol, showed the lowest incremental cost-effectiveness ratio. A recent study (50) used a population-based strategy and performed MSI testing on 1066 unselected colorectal cancer patients, screened 208 patients' samples for mutations, and identified 23 patients with HNPCC. Our current family-based screening protocol used family history and tumor MSI within a selected cohort of 285 patients suspected of HNPCC that led to mutation screening of 112 samples and the identification of at least 57 HNPCC patients. Although a population-based mutation-screening strategy has the potential to also identify nonfamilial HNPCC patients, more patients will need to be screened, and some mutations in patients with MSI-negative tumors will be missed. The most important advantage with the family history–based selection process we used is that non-HNPCC families are also identified and can be offered preventive programs.

We present our current protocol using a family-based strategy for HNPCC detection. We estimate that this protocol detected the majority of DNA MMR gene mutations. However, the unexpected findings of several mutations also in MSI-negative patients suggest that it might be worthwhile to modify our protocol and to find new criteria to detect HNPCC in MSI-negative patients. The high number of MMR gene missense mutations in both MSI-positive and MSI-negative patients emphasizes the need for functional assays to be used in genetic counseling. For now, these mutations will have to be interpreted using clinically relevant available information in each family.

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Notes

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Mutations in this article are described according to recommendations by den Dunnen and Antonarakis (51). The following GenBank reference sequences have been used—MLH1: NM_000249, MSH2: NM_000251, MSH6: NM_000179, PMS2: NM_000535, and BRAF: NM_004333.

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