

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

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

## Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC [MIM 114500]) is the most frequent autosomal dominant predisposition to the development of colorectal

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

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

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

The fact that germline *MSH2* and *MLH1* mutations cannot be identified in a still-considerable proportion of the families with "classical" HNPCC may be explained by the involvement of additional HNPCC genes. *MSH6*, *PMS2*, and the more recently described *MLH3* and *EXO1* genes may represent candidate disease-causing genes for *MSH2*- and *MLH1*-negative families (Wu et al. 2001a, 2001b). However, preliminary data indicate that germline mutations in these genes are more likely to either result in atypical HNPCC phenotypes and are rare in families with "classical" HNPCC (*MSH6*

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

## Patients and Methods

### *The Henry Lynch HNPCC Cohort*

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

**Table 1**  
**Clinical Features of the 59 Families with HNPCC Studied in this Article**

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

<sup>a</sup> Ovary cancer, stomach cancer, urinary tract cancer, and small-bowel cancer.

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

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

#### Denaturing Gradient Gel Electrophoresis (DGGE)

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

#### Southern Blot Analysis

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

probes encompassing exons 1–12 and exons 11–19; *MSH6* Southern analysis was performed with *Xba*I and *Apa*I digests, followed by hybridization with two cDNA probes encompassing exons 1–4 and exons 5–10.

#### Characterization of the Founder Deletion in *MSH2*

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

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

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

#### MSI, IHC, and MAMA

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

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

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

## Results

### Point-Mutation Analysis

A total of 37 germline mutations was detected in *MSH2*, *MLH1*, and *MSH6* by DGGE analysis, including seven mutations of uncertain pathologic significance (six missense mutations and one in-frame 3-nt deletion) (table 2). In another three families, both a missense and a

**Table 2**

**Summary of Results of the Point Mutation Analysis in Our Cohort**

GENE AND MUTATION TYPE	NO. OF POINT MUTATIONS IN <sup>a</sup>		TOTAL (N = 59)
	AC-Positive Families (N = 49)	AC-Negative Families (N = 10)	
<i>MLH1</i> :			
Frameshift	8	0	8
Nonsense	2	1	3
Splice site	6	2	8
Missense	<u>3</u>	<u>1</u>	<u>4</u>
Subtotal	19	4	23
<i>MSH2</i> :			
Frameshift	4	0	4
Nonsense	3	1	4
Splice site	1	0	1
Missense	<u>1</u>	<u>1</u> <sup>b</sup>	<u>2</u>
Subtotal	9	2	11
<i>MSH6</i> :			
Frameshift	1	0	1
In-frame deletion	0	1	1
Missense	<u>1</u>	<u>0</u>	<u>1</u>
Subtotal	2	1	3
Total	30	7	37

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

<sup>a</sup> AC = Amsterdam criteria.

<sup>b</sup> A636P in *MSH2*, a pathogenic Ashkenazi Jewish founder mutation (Yuan et al. 1999; Foulkes et al. 2002).

pathogenic mutation were detected (table 3). Most point mutations (23 of 37) were detected in *MLH1*. In three independent families, an identical nonsense mutation in *MLH1* exon 12 leading to exon skipping was found (Stella et al. 2001).

### Detection of Genomic Rearrangements

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

**Table 3****Overview of the Individual Probands and Mutations Detected in the 59 Families**

FAMILY	AC <sup>a</sup>	MUTATION IN		
		<i>MSH2</i>	<i>MLH1</i>	<i>MSH6</i>
101	+			
102	+		243delAATG	
103	+		Δ exons 1–13 and E578G	
104	+		G67R	
107	+	IVS5+3a→t		
108	+	Δ exons 1–6		
109	+		733insAACA	
110	+		IVS5–1g→c	
111	+	840delA		
112	+		A29S	
113	+		466delC	
114	+			
115	–	A636P		
116	+	N583S	IVS7–1g→t	
117	+	Q298X		
118	+		K461X	
119	+		64insA	
120	+		Δ exon 6	
121	+		IVS6–2a→g	
122	+	Δ exons 1–6 <sup>b</sup>		
123	+	Δ exons 1–6 <sup>b</sup>		
124	+	Δ exons 1–6 <sup>b</sup>		
125	+	T552P		
126	+	129delTC and S473L		
127	+		R226L (splice site)	
128	+		52insA	
129	+		V185L	
130	+	Δ exons 1–6 <sup>b</sup>		
131	+	55delC		
132	+		IVS18+1g→a	
133			K461X	
134	+		IVS6–2a→g	
135	+		K461X	
136	+	Δ exons 3–8		
137	+		733insAACA	
138	+	Q885X		
139	–		IVS13+1g→t	
140	+	inv exons 8–16		
141	–	R389X		
142	–		IVS9+1g→a	
143	–			
145	+		168insA	
146	+			
147	+			
148	+	Δ exons 1–6 <sup>b</sup>		
149	+	806insA		
150	+	Δ exon 3		
151	–			
152	+	Δ exons 1–6 <sup>b</sup>		
153	+			1087insC
154	–			1013delCTT
155	+	Δ exons 1–6 <sup>b</sup>		
157	+	R680X		
158	+	IVS3–3t→g		
177	+	Δ 5' <i>MSH2</i>		
178	–		G244D	
205	+		525delTT	
207	–			
208	+			M492V

NOTE.—Δ = deletion.

<sup>a</sup> AC = Amsterdam criteria.<sup>b</sup> Founder genomic deletion encompassing exons 1–6 of the *MSH2* gene.

**Table 4****Summary of Genomic Rearrangements in the 59 Families with HNPCC**

FAMILY	GENOMIC REARRANGEMENT IN	
	<i>MSH2</i>	<i>MLH1</i>
103		Deletion of exons 1–13
108	Deletion of exons 1–6	
120		Deletion of exon 6
122	Deletion of exons 1–6 <sup>a</sup>	
123	Deletion of exons 1–6 <sup>a</sup>	
124	Deletion of exons 1–6 <sup>a</sup>	
130	Deletion of exons 1–6 <sup>a</sup>	
136	Deletion of exons 3–8	
140	Inversion of exons 8–16	
148	Deletion of exons 1–6 <sup>a</sup>	
150	Deletion of exon 3	
152	Deletion of exons 1–6 <sup>a</sup>	
155	Deletion of exons 1–6 <sup>a</sup>	
177	Upstream deletion <sup>b</sup>	
Total	12	2

<sup>a</sup> The founder genomic deletion encompassing exons 1–6 of the *MSH2* gene.

<sup>b</sup> An ~13-kb deletion, the 3' breakpoint end of which maps 6 kb 5' of *MSH2*. The pathogenicity of this deletion could not be investigated.

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

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

**Molecular Analysis.**—In seven patients, aberrant fragments of ~14 kb were apparent by Southern analysis after digestion with *EcoRI* and hybridization with the *MSH2* exon 7 probe (fig. 1a). Also, on the basis of decreased intensity of the wild-type fragment and additional hybridizations with exon 1–6 probes (data not shown), the presence of the aberrant *EcoRI* fragment was indicative of a genomic deletion of exons 1–6 of the *MSH2* gene. Digestion by *BclI* revealed an ~13-kb aberrant fragment and confirmed the presence of an identical rearrangement in these seven families. To further characterize this recurring North American deletion previously not found among HNPCC samples of European origin (Wijnen et al. 1998a), PCR primers were designed around the approximate location of breakpoints inferred from the restriction map of the deleted chromosome. The 5' primer (R3) was based on genomic sequences ~2 kb upstream of exon 1, whereas the 3' primer (F3) was located in intron 6 of the *MSH2* gene. Long range PCR with these primers resulted in a deletion-specific PCR product of ~1,700 bp, exclusively observed in carriers of the common deletion and not in deletion-negative controls (data not shown). Sequencing

of the breakpoint revealed exactly the same nucleotide sequence in all seven families diagnosed with the exon 1–6 deletion by Southern analysis. The breakpoints are positioned within two *Alu* repeats (fig. 1B and 1C). Thus, the deletion is likely to have arisen through an *Alu*-mediated recombination. The presence of identical breakpoint sequences in all seven cases is suggestive of a founder mutation, since a frequently recurring recombination event would be likely to result in at least a few single-nucleotide differences.

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

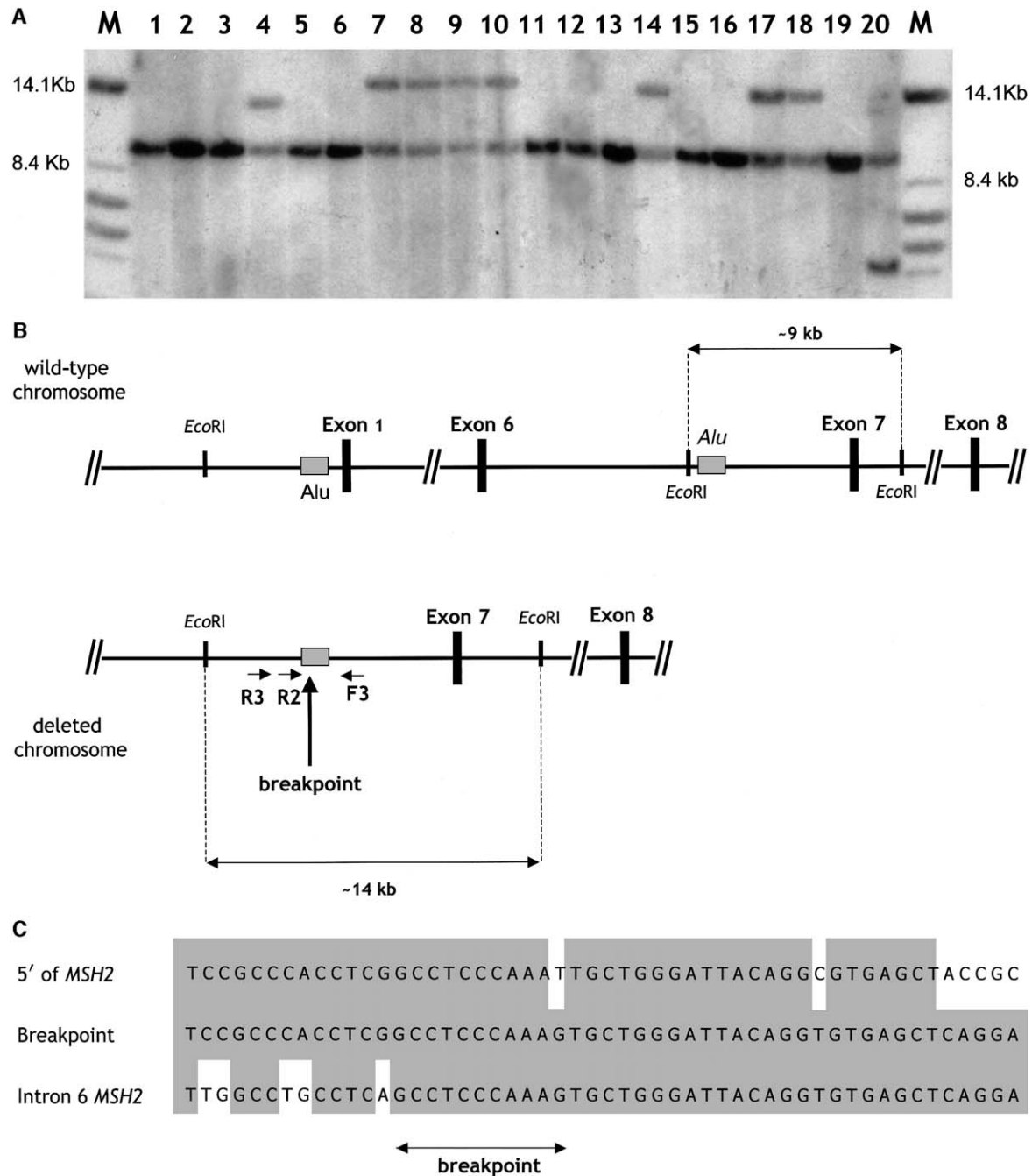
**Haplotype Analysis.**—To confirm the common genetic origin of the deletion of exons 1–6, haplotype analysis was performed (fig. 2). With the exception of families 124 and CG336, the haplotype of the deleted allele was reconstructed in the seven remaining families, six from the Lynch cohort and one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

**Table 5****Overview of the Results of the Complete Mutation Analysis of the *MSH2*, *MLH1*, and *MSH6* Genes in the 59 Families with HNPCC**

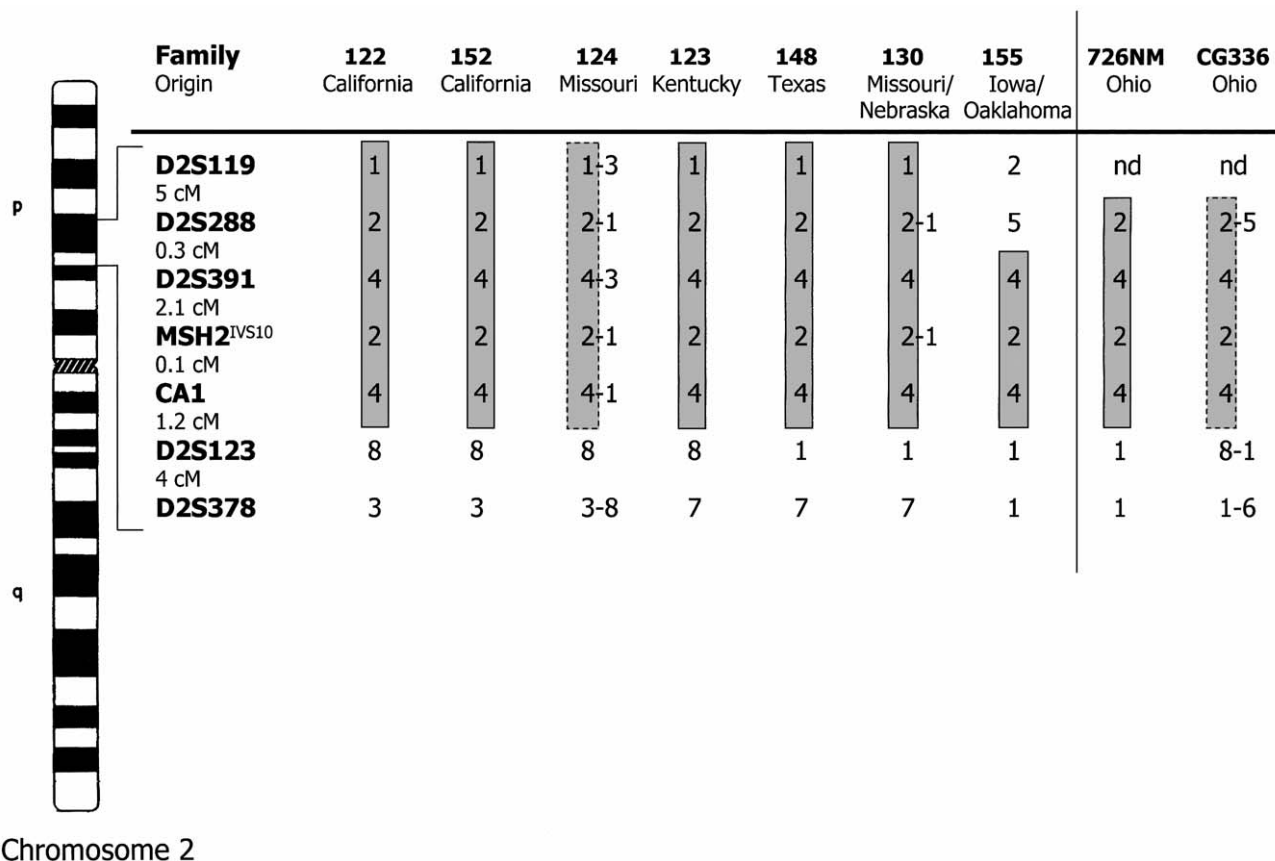
GENE AND MUTATION TYPE	NO. OF MUTATIONS IN		
	AC-Positive Families (N = 49)	AC-Negative Families (N = 10)	TOTAL (N = 59)
<b><i>MLH1</i>:</b>			
Point mutation	19	4	23
Genomic deletion	<u>2</u>	<u>0</u>	<u>2</u>
Subtotal	21	4	25 (42%)
<b><i>MSH2</i>:</b>			
Point mutation	10 <sup>a</sup>	2	12
Genomic deletion/inversion	<u>12</u>	<u>0</u>	<u>12</u>
Subtotal	22	2	24 (41%)
<b><i>MSH6</i>:</b>			
Point mutation	2	0	2
In-frame deletion	<u>0</u>	<u>1</u>	<u>1</u>
Subtotal	2	1	3 (5%)
Total	45 (92%)	7 (70%)	52 (88%)

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

<sup>a</sup> Including the splice-site mutation detected by MAMA analysis by Yan et al. (2000).



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



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

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

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

Y.K., P.W., R.O., J.F.L., R.F., A.D., and H.T.L., unpublished data).

*Alternative Approaches to Unresolved Cases*

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

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



**Table 6****Families with Mutations of Unknown Significance and Families without Mutations**

FAMILY	AC <sup>a</sup>	MUTATION	TEST RESULT <sup>b</sup>		
			MSI	IHC	MAMA
101	+		High	Decreased expression MSH2	NP
104	+	G67R <i>MLH1</i>	NP	NP	NP
112	+	A29S <i>MLH1</i>	NP	NP	NP
114	+		NP	NP	NP
125	+	T552P <i>MSH2</i>	High	Normal expression MSH2, MLH1, MSH6	NP
129	+	V185L <i>MLH1</i>	NP	NP	NP
143	–		NP	NP	Normal expression <i>MSH2</i> , <i>MLH1</i> , <i>MSH6</i>
146	+		NP	NP	NP
147	+		NP	NP	NP
151	–		NP	NP	NP
154	–	1013delCTT <i>MSH6</i>	NP	NP	NP
177	+	D 5' <i>MSH2</i>	NP	NP	NP
178	–	G244D <i>MLH1</i>	NP	NP	NP
207	–		NP	NP	NP
208	+	M492V <i>MSH6</i>	NP	NP	NP

<sup>a</sup> AC = Amsterdam criteria.

<sup>b</sup> NP = not performed.

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

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

## Discussion

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

Apart from the more straightforward nonsense and deletion mutations that are expected to be disease-causing by nature, several missense mutations were detected in this study. One of these, the A636P mutation in *MSH2*, is a founder mutation in the Ashkenazi Jewish population and was proven to be pathogenic (Yuan et al. 1999; Foulkes et al. 2002). Two other missense mutations (G67R and V185L in *MLH1*; families 104 and 129, respectively) were previously described to affect mismatch repair (Shimodaira et al. 1998; Ellison et al. 2001). In one index patient found to carry an *MSH2* missense mutation (T552P in family 125), tumor analysis revealed an MSI-high phenotype. As described by Foulkes et al. (2002), one possible approach to the anal-

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

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

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

be detected. It is likely that genes like *MSH6* that cause an overlapping though distinct phenotype are responsible for a small subset of the families with HNPCC and for a larger proportion of the atypical kindreds.

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

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

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

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

In conclusion, improved clinical selection forms the basis of optimal mutation detection in HNPCC. Mutation analysis in “classical” HNPCC should focus on the two major MMR genes—that is, *MSH2* and *MLH1*. We underscore that, apart from point mutations, genomic rearrangements strongly contribute to the HNPCC mutation spectrum. MSI and IHC analyses will direct further mutation detection strategies in the mu-

tation-negative families with classical HNPCC and in the more atypical HNPCC cases. This approach has also led to the identification of a common North American deletion in *MSH2* accounting for ~10% of our cohort. Genealogical, molecular, and haplotype studies showed that this common deletion represents a North American founder mutation that could be traced back to the 19th century.

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## Electronic-Database Information

The URLs for data presented herein are as follows:

FamilySearch Internet, [http://www.familysearch.org/Eng/Search/frameset\\_search.asp](http://www.familysearch.org/Eng/Search/frameset_search.asp)  
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for HNPCC)  
 RepeatMasker Web Server, <http://ftp.genome.Washington.edu/cgi-bin/RepeatMasker>

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