Am. J. Hum. Genet. 61:329–335, 1997

Hereditary Nonpolyposis Colorectal Cancer Families Not Complying with the Amsterdam Criteria Show Extremely Low Frequency of Mismatch-Repair-Gene Mutations

Juul Wijnen,[1] P. Meera Khan,[1] Hans Vasen,[2] Heleen van der Klift,[1] Adri Mulder,[1] Inge van Leeuwen-Cornelisse,[1,2] Bert Bakker,[1] Monique Losekoot,[1] Pål Møller,[3] and Riccardo Fodde[1]

[1]MGC-Department of Human Genetics, Medical Genetics Center, Sylvius Laboratory, Leiden University, and [2]Foundation for the Detection of Hereditary Tumors, University Medical Center, Leiden; and [3]The Norwegian Radium Hospital, Oslo

Summary

Hereditary nonpolyposis colorectal cancer (HNPCC) is a common autosomal dominant cancer-susceptibility condition characterized by early onset colorectal cancer. Germline mutations in one of four DNA mismatch repair (MMR) genes, *hMSH2, hMLH1, hPMS1,* or *hPMS2,* are known to cause HNPCC. Although many mutations in these genes have been found in HNPCC kindreds complying with the so-called Amsterdam criteria, little is known about the involvement of these genes in families not satisfying these criteria but showing clear-cut familial clustering of colorectal cancer and other cancers. Here, we applied denaturing gradient-gel electrophoresis to screen for *hMSH2* and *hMLH1* mutations in two sets of HNPCC families, one set comprising families strictly complying with the Amsterdam criteria and another set in which at least one of the criteria was not satisfied. Interestingly, *hMSH2* and *hMLH1* mutations were found in 49% of the kindreds fully complying with the Amsterdam criteria, whereas a disease-causing mutation could be identified in only 8% of the families in which the criteria were not satisfied fully. In correspondence with these findings, 4 of 6 colorectal tumors from patients belonging to kindreds meeting the criteria showed microsatellite instability, whereas only 3 of 11 tumors from the other set of families demonstrated this instability. Although the number of tumors included in the study admittedly is small, the frequencies of mutations in the MMR genes show obvious differences between the two clinical sets of families. These results also emphasize the practical importance of the Amsterdam criteria, which provide a valid clinical subdivision between families, on the basis of their chance of carrying an *hMSH2* or an *hMLH1* mutation, and which bear important consequences for genetic testing and counseling and for the management of colorectal cancer families.

Received February 21, 1997; accepted for publication May 21, 1997.

Address for correspondence and reprints: Dr. Riccardo Fodde, MGC-Department of Human Genetics, Sylvius Laboratories, University of Leiden, Wassenaarseweg 72, P.O. Box 9503, 2300 RA Leiden, The Netherlands. E-mail: fodde@ruly46.medfac.leidenuniv.nl

0002-9297/97/6102-0011$02.00

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common genetic condition that determines susceptibility to colorectal tumorigenesis. It is characterized by an autosomal dominant pattern of inheritance, a variable age at onset, and a high degree of penetrance. Moreover, tumors in other organs—including the endometrium, stomach, small intestine, hepatobiliary system, kidney, ureter, and ovary—also are observed frequently in HNPCC patients (Lynch et al. 1993; Watson and Lynch 1993).

Recently, HNPCC has been shown to be due to germline mutations in one of four DNA mismatch repair (MMR) genes, *hMSH2* (Fishel et al. 1993; Leach et al. 1993), *hMLH1* (Papadopoulos et al. 1994), or *hPMS1* or *hPMS2* (Nicolaides et al. 1994). The majority of mutations have been detected in *hMSH2* and *hMLH1*, whereas only three germ-line mutations have been described so far in *hPMS1* and *hPMS2* (Nicolaides et al. 1994). Inactivation of MMR genes leads to genomic instability characterized by the expansion or the contraction of short repeated DNA sequences (i.e., microsatellites) (Aaltonen et al. 1993; Ionov et al. 1993). This form of instability, designated as "microsatellite instability" ("MIN") or as "replication error" ("RER"), is thought to result in a rapid accumulation of somatic mutations in different oncogenes and tumor suppressor genes, which play crucial roles in tumor initiation and progression (Lazar et al. 1994; Markowitz et al. 1995; Parsons et al. 1995). Thus, tumor progression is believed to be accelerated in HNPCC patients, since they manifest colorectal cancer more than two decades earlier than those in the general population. The same type of genetic instability has been observed, in low but significant percentages, in different types of nonfamilial cases of tumors—including colorectal cancer (Thibodeau et al. 1993; Aaltonen et al. 1994), pancreatic and gastric cancer (Han et al. 1993), endometrial carcinomas (Burks et al. 1994; Risinger et al. 1994), breast cancer and ovarian cancer, and soft-tissue sarcomas (Wooster et al. 1994)—suggesting that the same MMR genes responsible for

HNPCC also may play an important role in the pathogenesis of common sporadic neoplasia.

Studies in different populations demonstrate that mutations in *hMSH2* and in *hMLH1* occur in an approximately equal proportion (~25% each) of HNPCC families (Han et al. 1995; Kolodner et al. 1995; Wijnen et al. 1995, 1996; Liu et al. 1996), with the exception of the Finnish population, in which *hMLH1* mutations were found in 83% of the HNPCC kindreds and *hMSH2* mutations in only 3% (Nyström-Lahti et al. 1996). However, this exceptionally high involvement of *hMLH1* is a reflection of the fact that two *hMLH1* mutant alleles are common in Finnish HNPCC kindreds, owing to founder effects (Nyström-Lahti et al. 1996).

To date, most of the HNPCC kindreds employed for linkage and mutation analysis satisfy the Amsterdam criteria, defined by the International Collaborative Group on HNPCC; that is, (1) at least three relatives in two successive generations, one of whom is the first-degree relative of the other two, are affected with histologically verified colorectal adenocarcinoma; (2) at least one of these relatives is diagnosed before 50 years of age; and (3) familial adenomatous polyposis is absent (either clinically or by linkage) in all of the at-risk family members (Vasen et al. 1991). Because of the extreme stringency of these criteria, it is not clear whether the same MMR genes, in particular *hMSH2* and *hMLH1,* also are responsible for HNPCC in kindreds not complying with the Amsterdam criteria but clearly showing familial clustering of colorectal cancer and other cancers.

In this study, we have analyzed the *hMSH2* and *hMLH1* genes, by use of GC-clamped denaturing gradient-gel electrophoresis (DGGE) (Myers et al. 1987; Fodde and Losekoot 1994), in 125 unrelated kindreds with clustering of colorectal cancer and other cancers, 86 of which fully comply with the Amsterdam criteria (AMS+). In the rest of the families, at least one of the criteria is not satisfied (AMS−). Tumor DNA that was available from 17 of the kindreds was investigated for MIN, by the screening of mono-, di-, tri-, and tetranucleotide repeat markers, for the examination of the involvement of DNA MMR genes in the pathogenesis of these tumors.

Patients, Material, and Methods

Patients

Of a total of 125 kindreds employed in this study, 34 have been described in previous studies (Wijnen et al. 1995, 1996). Ninety-seven of these families had been recruited from various clinical centers in the Netherlands, mainly through the Netherlands Foundation for the Detection of Hereditary Tumors. In order to exclude the presence of a founder effect, pedigrees were constructed by use of a genealogical approach for which at least three generations were investigated. No evidence of founder effects was found. Information had been collected on the type and site of the cancer, the age at diagnosis, the nature of therapeutic intervention, and the pathology and histopathology of the individual tumors, for each of the affected persons. Also, the personal data and the outcome of clinical screening, of the investigated at-risk relatives, have been documented carefully. Also, 23 Norwegian families, three Italian families, one Danish family, and a Czech family have been included. Eighty-six of these 125 families are AMS+. Of the 39 AMS− kindreds, 25 fulfill the Amsterdam criteria, with one exception, whereas in the remaining 14 kindreds more than one of the criteria were not fulfilled (table 1).

Table 1

Clinical Phenotypes of the 39 AMS− Families

Phenotype	No. of Families	No. of Mutations Found in *hMSH2*	No. of Mutations Found in *hMLH1*
All colorectal cancer patients diagnosed at >50 years of age	6	...	...
Only 2 colorectal cancer patients	11	1[a]	...
Only 1 generation affected	2	1	...
3 colorectal cancer patients not first-degree relatives	6	...	...
More than one criterion not satisfied	14	...	1[b]

[a] Germ-line mutation found in the *hMSH2* gene in an HNPCC kindred characterized by two patients with colorectal cancer and one other individual with endometrial cancer and one with ovarian cancer.

[b] Germ-line mutation identified in the *hMLH1* gene in a patient diagnosed with colorectal cancer at 42 years of age. Unfortunately, no family history was available, since the rest of the family currently is residing in Indonesia.

DNA Isolation

Genomic DNA was isolated from whole blood, as described elsewhere (Fodde et al. 1992). DNA from formalin-fixed, paraffin-embedded colorectal adenocarcinomas was isolated as follows. Approximately 10 10-μm paraffin sections were deparaffinized with Paraclear (Earth Safe Industries) and were washed with 100% ethanol. The tissue was resuspended in a 1-ml extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 300 μg proteinase k/ml) and was incubated at 55°C for 72 h. Two additional 200-μg aliquots of proteinase k were added, with incubation intervals of 24 h. After phenol/chloroform and chloroform extraction, to remove the cellular proteins, the DNA was precipitated with 250 μl 7.5-M NH_4Ac, 20 μg glycogen, and 1 ml 100% ethanol. The precipitate was dissolved in 150 μl TE^{-4} (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA).

DNA Amplification

Amplifications of the *hMSH2* and *hMLH1* genes were performed in a 50-μl volume containing 10 mM Tris-HCl, pH 8.9, 50 mM KCl, 2.5 mM $MgCl_2$ (with the exceptions of 1.5 mM for *hMSH2* exon 1, 3.5 mM for *hMSH2* exons 2 and 4, and 5 mM for *hMLH1* exon 4), 200 μg BSA/ml, 0.01% gelatin, 0.2 mM of each dNTP, 10% glycerol, 10 pmol of each primer, and 1 unit *Taq* polymerase. The reaction was subjected to 35 PCR cycles (60 s at 94°C, 90 s at 55°C or at 58°C, and 120 s at 72°C). The primer sets were designed to encompass the entire exon, including both intron-exon boundaries, with the exceptions of *hMSH2* exons 1, 2, 4, 5, 7, 13, 14, and 15 and of *hMLH1* exons 2 and 12, for which either the nature of the intronic sequences or the limited availability of sequence information did not allow DGGE analysis of one of the two splice sites (Wijnen et al. 1995, 1996).

DGGE

For optimal DGGE conditions, DNA melting–behavior simulations were performed with the MELT87 program, developed and kindly provided by Dr. L. Lerman (Lerman and Silverstein 1987). The position of the GC-rich sequence, whether at the 5′ end or at the 3′ end of each of the primer pairs, and the optimal ranges of denaturant used for DGGE of the individual exons were described elsewhere (Wijnen et al. 1995, 1996). The general procedure for DGGE analysis was described by Fodde et al. (1992).

Sequence Analysis

DNA fragments that displayed an abnormal DGGE pattern were analyzed by solid-phase sequencing. PCR products were purified with Easyprep and the PCR-Product Prep Kit (Pharmacia), by following of the manufacturer's instructions. Then, strand separation of the PCR product was obtained by use of streptavidin-coated magnetic beads M280 (Dynal). Sequencing reactions were performed in accordance with the procedures described by Sanger et al. (1977), by use of fluorescein-isothiocyanate–labeled Universal M13 primer (Autoread Kit; Pharmacia), and were run on the automated laser fluorescent–DNA sequencing apparatus (A.L.F.; Pharmacia), in 6% polyacrylamide and 7.0 M urea, at 1,500 V, 44 mA, and 40 W, at 45°C (laser power 4mW) for 6 h.

MIN

Genomic instability was investigated in paired normal DNA (from blood lymphocytes) and tumor DNA (from colorectal adenocarcinomas), from 24 patients from 17 HNPCC families, by screening for repeat-number variations at the poly-A repeat BAT40, at seven dinucleotide repeats (CA repeats D1S102, D2S123, D3S1265, D7S440, D14S51, D19S210, and D22S257), at two trinucleotide repeats (FABP2 and DRPLA), and at two tetranucleotide repeats (D4S243 and D4S169). The repeat markers were amplified from both normal-DNA and tumor-DNA samples and were resolved by polyacrylamide-gel electrophoresis, in accordance with conventional methodologies (Aaltonen et al. 1993). The instability of a given marker was defined by the appearance of additional alleles in the tumor DNA, when compared with the corresponding normal DNA. Tumors were considered to exhibit genomic instability, or to be RER positive (RER+), whenever three or more of the above repeat markers showed novel bands in the tumor DNA that were not present in the matched normal DNA.

Results and Discussion

The general strategy for the detection of mutations responsible for HNPCC was amplification of each of the 16 *hMSH2* exons and the 19 *hMLH1* exons, for one affected individual per family, and the analysis of the products by GC-clamped DGGE. Exons exhibiting altered migration patterns were sequenced, to determine the molecular nature of the observed variation. When sequence variants were detected, the investigations were extended to the rest of the family, to verify segregation of the nucleotide change with the disease phenotype.

Following the above-described strategy, we extended the previously reported analysis of *hMSH2* and *hMLH1* in 34 AMS+ HNPCC kindreds (Wijnen et al 1995, 1996) to an additional 91 families, 52 of which comply with the same clinical criteria. A total of 37 different mutations have been identified in 45 unrelated kindreds, of which 19 have mutations in *hMSH2* and 26 in *hMLH1* (table 2). The mutations in *hMSH2* are dispersed along the coding region of the gene, with the exceptions of exons 1 and 16, in which, to date, no mutations have been found. Moreover, 3 mutations, in exons 5, 8, and 12, were observed more frequently (table 2). The *hMLH1* mutations also were scattered throughout the entire coding region of the gene, with the exception of exon 12, in which no mutation was found. Interestingly, a 3′ mutation-cluster region, spanning exons 15–16, accounts for 10 (38%) of the 26 *hMLH1*-mutation kindreds described in this study (table 2). Five mutations, 3 in *hMSH2* and 2 in *hMLH1,* were observed in >1 HNPCC kindred (table 2). One of these mutations, the in-frame deletion of a lysine residue, in exon 16 of *hMLH1,* represents the most frequently observed mutation in this study, having been found in 4 kindreds. Genealogical and haplotype studies failed to find any relationship between 3 of these kindreds, ruling out the possibility of a founder effect.

Of particular interest is the remarkable difference observed in the involvement of *hMSH2* and *hMLH1,* between the HNPCC kindreds fully complying with and

Table 2

MMR-Gene Alterations in HNPCC Families

Gene and Family	Codon(s)	Nucleotide Change[a,b]	Nature of the Mutation[a]
hMSH2:			
NLB-376	Intron 1	aagGAG→aggGAG	a→g substitution at splice acceptor site
NLB-600	76 (exon 2)	CAGAGT→CAGT	AG deletion, frameshift; termination at codon 80
NL-10[c]	288 (exon 5)	CAG→TAG	C→T substitution, Gln→STOP
NL-39	288 (exon 5)	CAG→TAG	C→T substitution, Gln→STOP
NL-38	305 (exon 5)	GCA→ACA	G→A substitution, Ala→Thr
N-HS3	Intron 5	AGgta→AGgtt	a→t substitution at splice donor site
NL-21[c]	339–340 (exon 6)	CAAAGA→CAGA	AA deletion, frameshift; termination at codon 343
NL-7[c]	380–381 (exon 7)	GATTTA→GATTA	T deletion, frameshift; termination at codon 387
NL-23[c]	429 (exon 8)	CAG→TAG	C→T substitution, Gln→STOP
NL-220	429 (exon 8)	CAG→TAG	C→T substitution, Gln→STOP
I-219[c]	481–482 (exon 9)	TTAAG→TTAAAG	A insertion, frameshift; termination at codon 487
NLB-172	Intron 9	aagGC→aggGC	a→g substitution at splice acceptor site
NL-13[c]	532 (exon 10)	AAAGTC→AAAGGTC	G insertion, frameshift; termination at codon 535
NL-221	566 (exon 11)	AAAAAT→AAAT	AA deletion, frameshift; termination at codon 570
N-534	596 (exon 12)	CTCAATGAT→CTCGAT	AAT (Asn) in-frame deletion
N-554	596 (exon 12)	CTCAATGAT→CTCGAT	AAT (Asn) in-frame deletion
N-414	670 (exon 13)	CGA→TGA	C→T substitution, Arg→STOP
NL-203[c]	782–783 (exon 14)	ACCCAT→ACCAT	C deletion, frameshift; termination at codon 811
NL-57	834 (exon 15)	GCT→ACT	g→a substitution, Ala→Thr
hMLH1:			
NL-205	6–11 (exon 1)	Deletion of 17 nucleotides	Out-of-frame deletion, termination at codon 29
NLB-1069	6–11 (exon 1)	Deletion of 17 nucleotides	Out-of-frame deletion, termination at codon 29
NLB-100	34 (exon 1)	GAGATG→GATG	GA deletion, frameshift; termination at codon 36
N-498	62 (exon 2)	CAA→AAA	C→A substitution, Gln→Lys
N-2104	64 (exon 2)	AAT→AGT	A→G substitution, Asn→Ser
NL-20[c]	226 (exon 8)	TCGgta→TCAgta	G→A substitution at splice donor site
NL-27[c]	226 (exon 8)	TCGgta→TCGta	G or g deletion at splice donor site
NLB-296	Intron 8	TCGgtatg→TCGgtattg	t insertion at splice donor site
NL-6[c]	Intron 9	tagATC→tacATC	g→c substitution at splice acceptor site
NL-30	269 (exon 10)	TCA→TGA	C→G substitution, Ser→STOP
NL-37	295 (exon 10)	CAGgt→CGGgt	A→G substitution at splice donor site
NL-24[c]	307–308 (exon 11)	TGCACC→TGCGCACC	CG insertion, frameshift; termination at codon 367
CH-1	496–497 (exon 13)	ACCCCCCGG→ACCCCCCCGG	C insertion, frameshift; termination at codon 502
NL-25[c]	Intron 15	TCGgta→TCGata	g→a substitution at splice donor site
D-105[c]	Intron 15	tagGAG→ttgGAG	a→t substitution at splice acceptor site
NL-28[c]	593–594 (exon 16)	CAGAGAGTG→CAGAGTG	AG deletion, frameshift; termination at codon 608
NL-29[c]	616–618 (exon 16)	AAGAAGAAG→AAGAAG	AAG (Lys) in-frame deletion
I-202[c]	616–618 (exon 16)	AAGAAGAAG→AAGAAG	AAG (Lys) in-frame deletion
NL-40	616–618 (exon 16)	AAGAAGAAG→AAGAAG	AAG (Lys) in-frame deletion
NL-59	616–618 (exon 16)	AAGAAGAAG→AAGAAG	AAG (Lys) in-frame deletion
NLB-526	618 (exon 16)	AAGAAGAAG→AAGAAGGCG	AA→GC substitution, Lys→Ala
NL-4[c]	632 (exon 16)	GAGgtg→GAAgtg	G→A substitution at splice donor site
NL-204[c]	632 (exon 16)	GAGgtg→GAAgtg	G→A substitution at splice donor site
NL-56	659 (exon 17)	CGA→CCA	G→C substitution, Arg→Pro
NL-9[c]	Intron 18	AGgta→AGata	g→a substitution at splice donor site
NLB-35	Intron 18	AGgta→AGata	g→a substitution at splice donor site

[a] Uppercase letters represent exonic nucleotides, and lowercase letters represent intronic nucleotides.
[b] Nucleotides involved in the substitution/deletion events are underlined.
[c] Previously described in the studies by Wijnen et al. (1995, 1996).

those not complying with the Amsterdam criteria. Mutations in these genes could be detected in 42 (49%) of the 86 AMS+ families and in only 3 (8%) of the 39 kindreds in which one or more of the criteria were not fulfilled. This highly significant difference ($P = 9 \times 10^{-6}$) suggests that different genetic factors are responsible for tumor initiation and/or progression, in the patients belonging to these two sets of families. Similar observations were reported in Finnish HNPCC families (Nyström-Lahti et al. 1996) and in American and in German HNPCC families (Moslein et al. 1996). In Finland, Nyström-Lahti et al. (1996) found *hMSH2* and *hMLH1*

Table 3

MIN in Tumors

Family	AMS Status	Mutated Gene	Results of MIN Screening, by Use of[a]			
			Mononucleotide Repeat	Dinucleotide Repeat	Trinucleotide Repeat	Tetranucleotide Repeat
NL-1	−	...	+	+	+	+
NL-22	−	...	na	+	na	na
NL-27	+	*hMLH1*	na	+	+	+
NL-30	+	*hMLH1*	na	−	−	−
NL-38	+	*hMSH2*	−	−	−	−
NL-203	+	*hMSH2*	na	+	+	−
NL-205	+	*hMLH1*	na	+	−	+
NL-206	−	...	na	−	−	−
NL-208	−	...	na	−	−	−
NL-210	−	...	na	−	−	na
NL-211	−	...	−	−	−	−
NL216	−	...	−	−	−	−
NL217	+	...	+	+	+	+
NL-218	−	...	−	−	−	−
NL-223	−	...	+	+	+	+
NLB-532	−	...	−	−	−	−
NLB-722	−	...	−	−	−	−

[a] The plus (+) sign indicates that the tumor DNA displayed additional alleles other than those observed in the paired normal DNA. The minus (−) sign indicates no difference between tumor DNA and normal DNA. "na" ("no amplification") indicates that the tumor-DNA samples did not yield any PCR product, possibly owing to the presence of impurities in the DNA preparations.

germ-line mutations in 30 (86%) of their 35 AMS+ families and in only 6 (30%) of the 20 AMS− families. However, two *hMLH1* mutations are very common among the Finnish kindreds, owing to founder effects (Nyström-Lahti et al. 1996). When a correction for founder effects is incorporated, Nyström-Lahti et al.'s (1996) data show four different mutations in 9 (44%) AMS+ families and four in 18 (22%) AMS− families. In the pooled American families and German families, Moslein et al. (1996) described comparable proportions—that is, mutations in 9 (45%) of 20 AMS+ families and in only 4 (15%) of 26 AMS− families.

Three germ-line mutations were found in HNPCC families not fulfilling the Amsterdam criteria. These families failed to meet these strict criteria because of (1) the noninclusion of endometrial and ovarian cancers, on a par with colorectal cancer, in the Amsterdam criteria, for family N-534; (2) the occurrence of affected individuals in only one generation, in family NLB-600; and (3) the unavailability of the history of the family that resides in Indonesia (family NLB-296) (tables 1 and 2).

In our study, 80 HNPCC kindreds, of which 44 are AMS+ and 36 are AMS−, revealed no mutation in either *hMSH2* or *hMLH1*. To verify the involvement of DNA MMR genes, in the tumor pathogenesis in the AMS− families, we analyzed, for MIN, the tumor DNA

Table 4

No. of Mutations, in *hMSH2* and *hMLH1*, Found in the Dutch and the European AMS+ and AMS− HNPCC Families

Family Subset	AMS+ Families				AMS− Families			
		No. (%) of Mutations				No. (%) of Mutations		
	n	*hMSH2*	*hMLH1*	Total	*n*	hMSH2	hMLH1	Total
Dutch	69	13 (19)	20 (29)	33 (48)	28	1 (4)	1 (4)	2 (7)
European[a]	17	4 (24)	5 (29)	9 (53)	11	1 (9)	...	1 (9)
Total	86	17 (20)	25 (29)	42 (49)	39	2 (5)	1 (3)	3 (8)

[a] Included 23 Norwegian families, three Italian families, one Danish family, and one Czech family.

from 11 of these kindreds. Interestingly, 8 of 11 tumors did not show RERs (RER−) (table 3), suggesting that, in this subset of AMS− HNPCC patients, DNA MMR might not represent the major cause of the disease. Tumor DNA from 4 of 6 AMS+ kindreds and from 3 of 11 AMS− kindreds exhibited MIN (RER+) (table 3). Although germ-line mutations in *hMSH2* or in *hMLH1* could be found in 3 of the 4 AMS+ kindreds with RER+ tumors, no mutations could be demonstrated in the 3 AMS− kindreds.

Among the AMS+ families, *hMSH2* mutations and *hMLH1* mutations were found in 20% and 29%, respectively (table 4). These percentages confirm our observation, in previous studies, based on a smaller number of patients (Wijnen et al. 1995, 1996). Thus, these loci account for approximately one-half of the HNPCC families analyzed here. This might represent an underestimate, since our DGGE-based mutation-detection strategy would not detect large genomic deletions, promoter mutations, or mutations in regulatory elements.

We could not detect any interethnic differences, in the involvement of *hMSH2* and *hMLH1,* between HNPCC families in the Netherlands and those elsewhere in Europe (table 4). These proportions also are comparable with those observed in Japan and in North America. In Japan, 24% of the HNPCC cases have been reported to be caused by mutations in *hMLH1* (Han et al. 1995), whereas, in North America, 31% and 33% of the mutations have been found in *hMSH2* and in *hMLH1,* respectively (Liu et al. 1996). In contrast, 83% of the Finnish HNPCC families harbor mutations in *hMLH1* and only 3% have mutations in *hMSH2,* which is attributed to the founder effect (Nyström-Lahti et al. 1996).

In conclusion, we observed a highly significant difference, in the involvement of *hMSH2* and *hMLH1* gene mutations, between HNPCC kindreds complying with the Amsterdam criteria and those in which at least one of the criteria is not satisfied. This observation, consolidated by the finding that the majority of the tumors of AMS− families were RER−, suggests that two distinct tumorigenic pathways are responsible for these two categories of HNPCC kindreds. Further linkage or LOH studies will be of great importance to the identification of new loci involved in the pathogenesis of these tumors. These findings also have important implications for the management of families with colorectal cancer, since individuals belonging to AMS− families should be informed of the reduced probability of identification of the responsible mutation. Moreover, in diagnostic laboratories where the workload is potentially enormous, owing to the large number of cases with an apparent familial clustering of colorectal tumors, the Amsterdam criteria, together with the assessment of tumor MIN (RER+), presently provide a cost-effective subdivision of HNPCC families, on the basis of the probability of the identification of the responsible molecular defect in the *hMSH2* and *hMLH1* genes.

Acknowledgments

This work has been supported, in part, by the Dutch Cancer Society and the Praeventiefonds (project PRF 28-1383-1). The authors would like to thank the following clinicians for referring HNPCC families and for contributing their data on these families: Drs. F. Menko, F. Nagengast, A. Meijers-Heijboer, D. Lindhout, G. Griffioen, A. Bröcker-Vriends, A. Cats, J. Kleibeuker, L. Varesco, L. Bertario, M.-L. Bisgaard, J. Mohr, and Z. Sedlacek.

References

Aaltonen L, Peltomäki P, Leach FS, Sistonen P, Pylkkänen L, Mecklin J-P, Järvinen H, et al (1993) Clues to the pathogenesis of familial colorectal cancer. Science 260:812–816

Aaltonen L, Peltomäki P, Mecklin J-P, Järvinen H, Jass JR, Green JS, Lynch HT, et al (1994) Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. Cancer Res 54:1645–1648

Burks RT, Kessis TD, Cho KR, Hedrick L (1994) Microsatellite instability in endometrial carcinoma. Oncogene 9:1163–1166

Fishel R, Lescoe MK, Rao MRS, Copeland NG, Jenkins NA, Garber J, Kane M, et al (1993) The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell 75:1027–1038

Fodde R, Losekoot M (1994) Mutation detection by denaturing gradient gel electrophoresis (DGGE). Hum Mutat 3:83–94

Fodde R, van der Luijt R, Wijnen J, Tops C, van der Klift H, van Leeuwen-Cornelisse I, Griffioen G, et al (1992) Eight novel inactivating germ line mutations at the APC gene identified by denaturing gradient gel electrophoresis. Genomics 13:1162–1168

Han HJ, Maruyama M, Baba S, Park JG, Nakamura Y (1995) Genomic structure of human MMR gene, hMLH1, and its mutation analysis in patients with hereditary non-polyposis colorectal cancer (HNPCC). Hum Mol Genet 4:237–242

Han HJ, Yanagisawa A, Kato Y, Park JG, Nakamura Y (1993) Genetic instability in pancreatic cancer and poorly differentiated type of gastric cancer. Cancer Res 53:5087–5089

Ionov Y, Peinado MA, Malkbosyan S, Shibata D, Perucho M (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363:558–561

Kolodner RD, Hall NR, Lipford J, Kane MF, Morrison PT, Finan PJ, Burn J, et al (1995) Structure of the human MLH1 locus and analysis of a large hereditary nonpolyposis colorectal carcinoma kindred for MLH1 mutations. Cancer Res 55:242–248

Lazar V, Grandjouan S, Bognel C, Couturier D, Rougier P, Bellet D, Bressac-de Paillerets B (1994) Accumulation of multiple mutations in tumor suppressor genes during colorectal tumorigenesis in HNPCC patients. Hum Mol Genet 3:2257–2260

Leach FS, Nicolaides NC, Papadopoulos N, Liu B, Jen J, Parsons R, Peltomäki P, et al (1993) Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell 75: 1215–1225

Lerman LS, Silverstein K (1987) Computational simulation of

DNA melting and its application to denaturing gradient gel electrophoresis. In: Wu R, Grossman L (eds) Recombinant DNA, part F. Vol 155 in: Methods in enzymology. Academic Press, San Diego, pp 482–501

Liu B, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, Watson P, Jass JR, et al (1996) Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. Nat Med 2:169–174

Lynch HT, Smyrk TC, Watson P, Lanspa SJ, Lynch PM, Cavalieri RJ, Boland CR (1993) Genetics, natural history, tumor spectrum and pathology of hereditary nonpolyposis colorectal cancer: an updated review. Gastroenterology 104: 1535–1549

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, et al (1995) Inactivation of the type II TGF-β receptor in colon cancer cells with microsatellite instability. Science 268:1336–1338

Moslein G, Tester DJ, Lindor NM, Honchel R, Cunningham JM, French AJ, Halling KC, et al (1996) Microsatellite instability and mutation analysis of *hMSH2* and *hMLH1* in patients with sporadic, familial and hereditary colorectal cancer. Hum Mol Genet 5:1245–1252

Myers RM, Maniatis T, Lerman LS (1987) Detection and localization of single base changes by denaturing gradient gel electrophoresis. In: Wu R, Grossman L (eds) Recombinant DNA, part F. Vol 155 in: Methods in enzymology. Academic Press, San Diego, pp 501–517

Nicolaides NC, Papadopoulos N, Liu B, Wei Y-F, Carter KC, Ruben SM, Rosen CA, et al (1994) Mutations of two PMS homologues in hereditary non-polyposis colon cancer. Nature 371:75–80

Nyström-Lahti M, Wu Y, Moisio AL, Hofstra RMW, Osinga J, Mecklin JP, Järvinen HJ (1996) DNA mismatch repair gene mutations in 55 kindreds with verified or putative hereditary non-polyposis colorectal cancer. Hum Mol Genet 5:763–769

Papadopoulos N, Nicolaides NC, Wei Y-F, Ruben SM, Carter KC, Rosen CA, Haseltine WA, et al (1994) Mutation of a *mutL* homolog in hereditary colon cancer. Science 263: 1625–1629

Parsons R, Myeroff LL, Liu B, Willson JKV, Markowitz SD, Kinzler KW, Vogelstein B (1995) Microsatellite instability and mutations of the transforming growth factor β type II receptor gene in colorectal cancer. Cancer Res 55:5548–5550

Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J (1994) Genetic instability of microsatellites in endometrial carcinoma. Cancer Res 53:5100–5103

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Thibodeau SN, Bren G, Schaid D (1993) Microsatellite instability in cancer of the proximal colon. Science 260:816–819

Vasen HFA, Mecklin J-P, Meera Khan P, Lynch HT (1991) The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). Dis Colon Rectum 34:424–425

Watson P, Lynch HT (1993) Extracolonic cancer in hereditary nonpolyposis colorectal cancer. Cancer 71:677–685

Wijnen J, Meera Khan P, Vasen H, Menko F, van der Klift H, van den Broek M, van Leeuwen-Cornelisse I, et al (1996) Majority of *hMLH1* mutations responsible for hereditary nonpolyposis colorectal cancer cluster at the exonic region 15-16. Am J Hum Genet 58:300–307

Wijnen J, Vasen H, Meera Khan P, Menko FH, van der Klift H, van Leeuwen C, van den Broek M, et al (1995) Seven new mutations in *hMSH2*, an HNPCC gene, identified by denaturing gradient-gel electrophoresis. Am J Hum Genet 56:1060–1066

Wooster R, Cleton-Jansen A-M, Collins N, Mangion J, Cornelis RS, Cooper CS, Gusterson BA, et al (1994) Instability of short tandem repeats (microsatellites) in human cancers. Nat Genet 6:152–156