Hereditary Nonpolyposis Colorectal Cancer in 95 Families: Differences and Similarities between Mutation-Positive and Mutation-Negative Kindreds

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Hereditary nonpolyposis colorectal cancer (HNPCC) describes the condition of a disparate group of families that have in common a predisposition to colorectal cancer in the absence of a premalignant phenotype. The genetic basis of this disease has been linked to mutations in genes associated with DNA mismatch repair. A large proportion of families harbor changes in one of two genes, *hMSH2* and *hMLH1*. Approximately 35% of families in which the diagnosis is based on the Amsterdam criteria do not appear to harbor mutations in DNA-mismatch-repair genes. In this report we present data from a large series of families with HNPCC and indicate that there are subtle differences between families that harbor germline changes in *hMSH2* and families that harbor *hMLH1* mutations. Furthermore, there are differences between the mutation-positive group (*hMSH2* and *hMLH1* combined) of families and the mutation-negative group of families. The major findings identified in this study focus primarily on the extracolonic disease profile observed between the mutation-positive families and the mutation-negative families. Breast cancer was not significantly overrepresented in the *hMSH2* mutation-positive group but was overrepresented in the *hMLH1* mutation-positive group and in the mutation-negative group. Prostate cancer was not overrepresented in the mutation-positive groups but was overrepresented in the mutation-negative group. In age at diagnosis of colorectal cancer, there was no difference between the *hMSH2* mutation–positive group and the *hMLH1* mutation–positive group, but there was a significant difference between these two groups and the mutation-negative group.

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC [MIM 120435 and MIM 120436]) is an autosomal dominantly inherited disorder that is characterized primarily by the development of early-onset colorectal cancer (CRC) and that is also associated with the development of a variety of epithelial tumors that include endometrial cancer, stomach cancer, and ovarian cancer (Watson and Lynch 1993). The genetic basis of HNPCC has been linked to errors in DNA mismatch repair (Fishel et al. 1993; Leach et al. 1993; Bronner et al. 1994; Nicolaides et al. 1994), which leaves a characteristic tumor signa-

ture, of DNA microsatellite instability, that can be used as a surrogate marker for this syndrome (Ionov et al. 1993; Thibodeau et al. 1993). At least five genes have been associated with DNA mismatch repair and HNPCC; they are *hMSH2*, *hMLH1*, *hMSH6*, *hPMS1*, and *hPMS2* (for review, see Papadopoulos and Lindblom 1997). Together, *hMSH2* and *hMLH1* account for 50%–60% of all mutations in families in which diagnosis is based on the Amsterdam criteria—three relatives with CRC, one of whom must be a first-degree relative of the other two; CRC present in at least two generations; CRC diagnosed in one relative at age <50 years; and familial adenomatous polyposis (FAP) not present. Of families with HNPCC, 2%–20% of those which are defined on the basis of the Bethesda criteria (excluding the Amsterdam-criteria families) show association with mutations in these two genes (Buerstedde et al. 1995; Wijnen et al. 1998; Heinimann et al. 1999; Syngal et al. 1999). The contribution of *hPMS1* (now considered to be a pseudogene), *hPMS2*, and *hMSH6* remains undefined; however, on the basis of current evidence, *hPMS2*

and *hMSH6* appear to account for the condition in ~5% of all families with HNPCC (Liu et al. 1996; Peltomaki and Vasen 1997). The remaining 30%–40% of Amsterdam-criteria families that do not evidently harbor germline mutations in the genes described either contain cryptic alterations within their coding regions, harbor changes in promoter/enhancer regions, or have mutations in other genes, which await identification (Papadopoulos and Lindblom 1997).

The identification of families with HNPCC remains problematic, despite knowledge about the genetic basis of the disease. Several criteria have been proposed, which are aimed at identification of families with a high probability of harboring germline mutations in DNA-mismatch-repair genes. To date, the accumulated evidence indicates that by far the most accurate method of family identification is use of the Amsterdam criteria. Deviation from these criteria appears to result in a poor mutation-detection rate, even when other tumors that have been clearly defined within the spectrum of HNPCC substitute for CRC (Wijnen et al. 1998; Heinimann et al. 1999).

Unlike FAP, in which genotype-phenotype correlations have been described, no such obvious relationship appears to be apparent in HNPCC. More-subtle differences have been described, indicating that there may be histological differences between *hMSH2* mutation–associated tumors and *hMLH1* mutation–associated tumors in HNPCC (Shashidharan et al. 1999)—and, indeed, that there may be clinical differences as well (Vasen and Wijnen 1999).

In this report we further characterize the relationship between HNPCC and the likelihood of identification of a germline mutation in either *hMSH2* or *hMLH1*, and we compare the features displayed in families that are either mutation positive or mutation negative. Furthermore, a comparison between *hMSH2* mutation–positive families and *hMLH1* mutation–positive families is made that suggests that there may be some subtle differences between these two HNPCC groups. Finally, in comparing differences between the mutation-positive groups and the mutation-negative group, we were able to establish that, in disease phenotype, the mutation-negative group resembles the *hMLH1* mutation–positive group more closely than it resembles the *hMSH2* mutation–positive group. This evidence suggests that proteins, which interact with and affect *hMLH1*, could be good candidates for genetic analysis.

Patients and Methods

Index patients from 95 families enrolled in this study signed an informed-consent document (which, prior to use, was assessed by the appropriate institutional review body) authorizing genetic testing of genes associated with HNPCC. Cascade testing was performed on family members after they too had given informed consent for testing when a germline mutation had been identified in the family. Each pedigree was classified as either Amsterdam-criteria positive or Bethesda-criteria positive. Genetic analysis was performed on a fresh blood specimen from the youngest living affected proband in each family. Disease verification in affected individuals was based on either examination of pathology reports or death certificates.

hMSH2 *and* hMLH1 *Molecular Analysis*

DNA was isolated from 20 ml of peripheral blood lymphocytes, by the salting-out method first described by Miller et al. (1988). All 35 exons constituting *hMSH2* and *hMLH1* were analyzed by denaturing gradient gel electrophoresis, as described by Wijnen et al. (1995; 1996). In brief, each exon was amplified by PCR using specific primers, which have incorporated into either one of their 5′ ends a 40-bp GC clamp. The resulting PCR product was applied to a denaturing gradient gel and was subjected to electrophoresis for ⩾15 h. Then the gels were stained with ethidium bromide (1 mg/ml) and were photographed on a UV-Trans-illuminator. All polymorphisms were subjected to DNA sequencing by a semiautomated sequencing unit (PE Biosystems model 310), to determine the precise genetic change. Sequence alterations were analyzed for the presence of pathogenic mutations (nonsense, insertion, or deletion mutations). Sequence changes with a frequency of >5% were considered to be normal variants and were described as such. Polymorphisms that occurred at a frequency of <5% were considered to represent changes that were most likely to be associated with an increased predisposition to develop early-onset HNPCC.
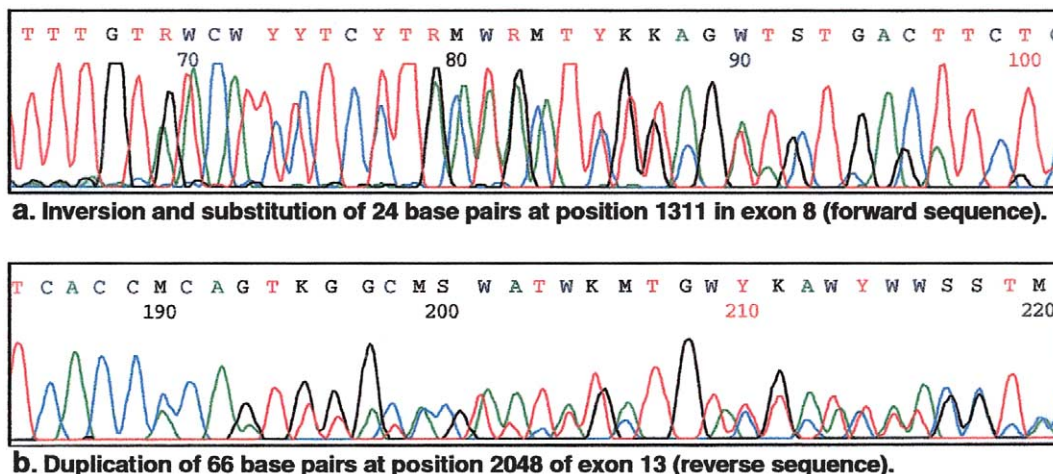
Statistical Analysis

Logistic regression with a correction for clustering was used to determine whether there were differences between the groups (Royall 1986). This allows the detection of differences, with regard to the occurrence of cancer, between mutation groups. In addition to the individual records on file, the records of each family's disease-free individuals were included in the analysis, thus reflecting the total number of individuals in each family. A similar analysis was performed for each cancer type. If there were no cases of a certain type of cancer, Fisher's exact test was performed to obtain some measure of the degree of association. Data analyses were performed by means of the STATA statistical package (version 6). The typical STATA command for a logistic regression was xi: logit ycrc i.mutation [fweight = n], cluster (id), where ycrc is a binary variable indicating whether an individual has had a history of the cancer type (in this case, CRC).

Table 1

Genetic Changes Identified in the *hMLH1* and *hMSH2* Genes

Gene and Family	Exon/Intron	Position(s)	Mutation Type	Nucleotide Change	Consequence of Mutation	Reference
hMLH1:						
1	1	116	1-bp substitution	G→A	Splice site	Farrington et al. (1998)
2	1	1	1-bp substitution	A→G	Loss of translation start site	Wehner et al. (1997)
3	1	112	1-bp substitution	A→G	Arg→Ser	Present study
4	3	+2, +1, 208, 209	4-bp deletion	4-bp deletion	Loss of intron/exon junction	Present study
5	4	350	1-bp substitution	C→T	Thr→Met	Maliaka et al. (1996)
6	4	381	1-bp substitution	G→A	Loss of intron/exon junction	Present study
7	7	554	1-bp substitution	T→G	Val→Gly	Kohonen-Corish et al. (1996)
8	8	673	1-bp deletion	G deletion	Frameshift/stop	Present study
9	8	655	1-bp substitution	A→G	Ile→Val (polymorphism)	Hutter et al. (1998)
10	9	790	1-bp substitution	A→G	Splice site	Liu et al. (1995)
11	12	1164	4-bp insertion	Need info	Stop codon	Present study
12	13	+13	1-bp substitution	T→A	Cryptic splice site	Present study
13	13	1554	1-bp insertion	T insertion	Stop codon	Present study
14	13	1535	1-bp substitution	G→T	Stop codon	Present study
15	13	1460	4-bp deletion	TGAT deletion	Stop codon	Present study
16	16	1731	1-bp substitution	G→A	Splice site	Present study
17	16	1852	2-bp substitution	AA→GC	Ala→Lys	Present study
18	17	1959	1-bp substitution	G→T	Splice site	Present study
19	17	1989	1-bp substitution	G→A	Stop	Present study
20	17	1975	2-bp deletion	GA deletion	Stop codon	Present study
21	19	2253	1-bp substitution	A→G	Lys→Arg	Present study
22	19	2147	1-bp substitution	G→A	Val→Met	Wehner et al. (1997)
hMSH2:						
23	6	1009	1-bp substitution	C→T	Stop codon	Present study
24	6	965	1-bp substitution	G→A	Gly→Asp	Maliaka et al. (1996)
25	6	984	1-bp substitution	C→T	Ala→Ala (polymorphism)	
26	8	1381	Inversion and substitution	Complex[a]	Nonsense segment	Present study
27	9	1408	1-bp deletion	G deletion	Stop codon	Present study
28	9	1510	1-bp substitution	G→C	Splice site change	Present study
29	10	−12 from exon 10	1-bp substitution	A→G	Cryptic splice site	Present study
30	11	1669	1-bp substitution	T→C	Leu→Leu (polymorphism)	Wijnen et al. (1995)
31	12	1968	1-bp substitution	G→C	Stop codon	Kohonen-Corish et al. (1996)
32	13	2157	1-bp substitution	A→G	Gln→Gln (polymorphism)	
33	13	2051	66-bp duplication	Complex[b]	Reiteration	Present study
34	15	2502	7-bp deletion	Loss of TAATTTC	Stop codon	Present study

[a] See figure 1*a*.
[b] See figure 1*b*.

Figure 1   Complex DNA sequence changes identified in *hMSH2*

All other statistical analysis was performed by means of Student's *t*-test.

Results

Mutation-Positive Families

Of the 95 families, 12 harbored a genetic change in the *hMSH2* gene, 22 harbored a change in the *hMLH1* gene, and 61 were found not to harbor any change in either gene. The precise genetic changes identified in the *hMLH1* gene and the *hMSH2* gene are shown in table 1. No particular types of genetic change were overrepresented in our mutation/polymorphism data set. The ratio of observed *hMSH2* genetic changes to *hMLH1* genetic changes was almost 1:2. A spectrum of changes was identified in the *hMLH1* gene; these included four nonsense mutations and three substitutions that resulted in stop codons, seven splice-site changes that resulted in exon skipping, six missense mutations, one loss of an ATG start site, and one 2-bp substitution that led to an Ala→Lys change. Similar changes were identified in the *hMSH2* gene, which included five nonsense mutations and one substitution, all of which resulted in stop codons, one missense mutation; three 1-bp substitutions that did not result in any amino acid change; and two complex mutations. The two complex changes identified in *hMSH2* were (*a*) an inversion of bases 1338–1361, substituting for bases 1311–1334 inclusive in exon 8, and (*b*) a large duplication of 66 bp in exon 13, at position 2048, both of which are predicted to result in a segment of nonfunctional peptide within the hMSH2 protein. The sequence of these two changes is shown in figure 1. In neither family were there any special features that could be related to the uniqueness of either mutation.

The polymorphisms identified in this study were checked against The International Collaborative Group on Hereditary NonPolyposis Colorectal Cancer mutation database, to determine whether they could be assigned causative status. Of the seven missense changes in *hMLH1* (see table 1), four had been identified in previous reports, with three having been reported as being causative (Kohonen-Corish et al. 1996; Maliaka et al. 1996; Wehner et al. 1997) and one having been reported as being polymorphic (Hutter et al. 1998). The three remaining changes in *hMLH1* are of unknown status. In a previous report (Maliaka et al. 1996), three of the missense mutations identified in *hMSH2* (table 1) had been shown to be polymorphisms of no apparent consequence, and one had been described as pathogenic. Since tumor tissue was not available from all patients harboring missense mutations, microsatellite-instability analysis for assignment of causation could not be performed. Nevertheless, none of the new missense mutations were detected in 50 control subjects, indicating that these changes may be associated with a change of protein function.

One family was identified as having Muir-Torre syndrome, since (*a*) one of its members presented with a

Table 2

Mutation Status of Bethesda- and Amsterdam-Criteria Families

	No. of Families			
		Mutation Positive		Proportion of Families That Are Positive
Criterion	Mutation Negative	*hMSH2*	*hMLH1*	
Bethesda	50	6	6	19.4%
Amsterdam	13	9	11	60.6%
Overall	63	15	17	33.7%

Table 3

Relative Percentage of Tumor Types in the Study Population

	No. (Percentage) in Study Population			
			Mutation Positive	
Cancer Type	Total	Mutation Negative	hMSH2	hMLH1
CRC	316 (9.86)	213 (9.80)	73 (8.92)	66 (12.15)
Endometrial/ovarian	50 (1.37)	36 (1.65)	7 (.79)	7 (1.29)
Stomach	41 (1.12)	26 (1.1)	9 (1.02)	6 (1.19)
Brain/CNS tumors	14 (.38)	11 (.5)	3 (.34)	...
Breast	55 (1.5)	44 (2.02)	2 (.22)	9 (1.66)
Renal/renal-tract	18 (.50)	9 (.410)	7 (.8)	2 (.37)
Lymphoproliferative[a]	18 (.50)	13 (.60)	2 (.23)	3 (.55)
Prostate	11 (.30)	10 (.46)	1 (.11)	...

[a] Includes all lymphoproliferative cancers except Hodgkin leukemia.

sebaceous adenoma and (*b*) there was a strong family history of CRC, which fulfilled the Amsterdam criteria. The proband harbored a silent polymorphism in exon 11 of *hMSH2* but did not appear to harbor any other change in either *hMSH2* or *hMLH1*.

When mutation status based on the Amsterdam criteria was compared with that based on the Bethesda criteria, differences between the two groups were observed. As expected, 60% of Amsterdam-criteria families were mutation positive, compared with 20% of Bethesda-criteria families (see table 2). There appeared to be no relationship, between the sites of mutations, that could be used to establish a genotype-phenotype correlation, for either *hMLH1* or *hMSH2*. Within each of the two groups, a comparison between the age at onset of CRC, the spectrum of extracolonic disease, and how this related to the mutation-negative group was made.

Cancer Occurrence

The overall percentage of tumors observed—in the total population, in the mutation-negative group, and in the *hMSH2* mutation–positive and *hMLH1* muta-

tion–positive groups—is shown in table 3. In the total population, the most frequently observed tumor was CRC, followed by breast cancer, endometrial cancer, and stomach cancer. Other cancers common to HNPCC were also overrepresented. The relative standardized incidence rates (SIRs), including 95% confidence intervals (95% CIs), for all cancers identified in each group are shown in table 4. When the study population was subdivided into *hMSH2* mutation–positive, *hMLH1* mutation–positive, and mutation-negative groups, similarities and differences between the three groups could be identified.

CRC

Of the 95 families, 32 fulfilled the Amsterdam criteria and 63 fulfilled the Bethesda criteria. The clinical features of each of the probands are shown in table 5. The age at diagnosis of CRC in the three groups of patients is shown in figure 2 (mutations of uncertain significance were not included). The age distributions of the *hMSH2* mutation–positive group and the *hMLH1* mutation–positive group were essentially identical, but there was an ~5-year shift in the distribution seen in the mutation-negative group. The average age (±SD) at diagnosis in *hMSH2* mutation-positive families was 45.77 (±24.35) years, and that in *hMLH1* mutation-positive families was 47.16 (±18.65) years. There was no statistical difference between the two groups (*P* = .747) when we adjusted for familial clustering. The average age at diagnosis in the mutation-negative group was 52.68 (±17.65) years, which was significantly different from that in the *hMSH2* mutation-positive group but was not statistically different from that in the *hMLH1* mutation-positive group (*P* = .031 and .135, respectively). Compared with the median age of diagnosis of CRC (peak incidence being diagnosed during the 7th and 8th decades of life), all three groups had an age at

Table 4

SIRs of Cancers Identified in All Families, as a Function of Mutational Status

	All Patients		Mutation-Positive Patients				Mutation-Negative Patients	
			hMSH2		hMLH1			
Cancer Type	No.	SIR (95% CI)	No.	SIR (95% CI)	No.	SIR (95% CI)	No.	SIR (95% CI)
CRC	361	159.54 (139.2–182.9)	73	134.24 (99.1–181.8)	66	196.76 (143.0–270.7)	213	158.61 (132.8–189.4)
Endometrial/ovarian	50	53.35 (37.0–77.0)	7	31.08 (11.7–82.9)	7	50.39 (18.9–134.3)	36	64.72 (42.0–99.7)
Stomach	41	102.2 (68.2–153.28)	9	93.35 (39.3–221.6)	6	100.89 (35.0–290.8)	26	109.20 (65.7–181.6)
Brain/CNS tumors	14	56.02 (28.0–112.0)	3	49.95 (11.2–223.2)	...	...	11	74.16 (33.9–162.0)
Breast	55	13.38 (9.4–19.0)	2	2.02 (.3–12.7)[a]	9	14.77 (6.2–35.0)	44	18.03 (12.2–26.7)
Renal/renal-tract	18	42.17 (22.9–77.7)	7	68.24 (25.7–181.8)	2	31.6 (5.1–197.6)	9	35.53 (15.0–84.3)
Lymphoproliferative[b]	18	13.93 (7.6–25.7)	2	6.44 (1.0–40.3)	3	15.66 (3.5–290.1)	13	16.95 (8.3–34.8)
Prostate	11	2.69 (1.2–5.8)	1	1.02 (.1–13.6)[a]	...	...	10	4.12 (1.8–9.4)

[a] Not significantly different from the expected value.

[b] Includes all lymphoproliferative cancers except Hodgkin leukemia.

Table 5

Clinical Features of Probands Selected for Mutation Analysis

| Proband	Age at Diagnosis (years)	Tumor Type	Family History of Cancer?[a]	Amsterdam Criteria Positive?	Proband	Age at Diagnosis (years)	Tumor Type	Family History of Cancer?[a]	Amsterdam Criteria Positive?
1	27	CRC	Yes	Yes	58	24	CRC	Yes	Yes
2	48	CRC	Yes	No	59	48	CRC	Yes	Yes
3	65	CRC	Yes	No	60	35	CRC	Yes	No
4	46	CRC	No	No	61	54	CRC	Yes	No
5	45	CRC	Yes	Yes	62	43	CRC	Yes	No
6	55	Stomach	Yes	Yes	63	57	Adenomas	Yes	No
7	47	CRC	Yes	No	64	41	CRC	Yes	Yes
8	52	CRC	Yes	Yes	65	56	CRC	Yes	Yes
9	39	CRC	Yes	No	66	62	CRC	Yes	No
10	51	CRC	Yes	No	67	68	Adenomas	Yes	No
11	59	CRC	Yes	No	68	32	CRC	Yes	No
12	37	CRC	Yes	Yes	69	55	CRC	Yes	Yes
13	31	CRC	No	No	70	55	Stomach	Yes	Yes
14	46	CRC	Yes	No	71	29	Stomach	Yes	No
15	55	CRC	Yes	No	72	40	Endometrial cancer	Yes	Yes
16	36	CRC	Yes	Yes	73	45	Adenomas	Yes	No
17	52	CRC	Yes	No	74	45	CRC	Yes	Yes
18	41	CRC	Yes	Yes	75	57	Adenomas	Yes	No
19	48	CRC	Yes	No	76	13	CRC	Yes	No
20	26	CRC	Yes	No	77	54	Adenomas	Yes	No
21	25	CRC	Yes	No	78	40	CRC	Yes	No
22	64	CRC	Yes	Yes	79	33	CRC	Yes	No
23	58	Adenomas	Yes	No	80	36	CRC	Yes	No
24	32	CRC	Yes	Yes	81	61	CRC	Yes	No
25	47	CRC	Yes	Yes	82	65	Adenomas	Yes	No
26	17	CRC	Yes	No	83	38	CRC	No	No
27	40	Stomach	Yes	No	84	32	CRC	Yes	No
28	44	CRC	Yes	No	85	30	CRC	Yes	No
29	41	CRC	Yes	No	86	52	CRC	Yes	No
30	46	CRC	Yes	No	87	63	CRC	Yes	No
31	19	Adenomas	Yes	Yes	88	44	CRC	Yes	No
32	52	CRC	Yes	No	89	54	CRC	Yes	No
33	43	CRC	Yes	No	90	78	Ovarian cancer	Yes	No
34	37	Endometrial cancer	Yes	Yes	91	51	CRC	Yes	No
35	66	CRC	Yes	No	92	51	CRC	Yes	Yes
36	38	CRC	Yes	Yes	93	44	Stomach	Yes	No
37	48	CRC	Yes	Yes	94	50	CRC	Yes	No
38	47	Endometrial cancer	Yes	Yes	95	30	CRC	Yes	No
39	35	Adenomas	Yes	No					
40	66	CRC	Yes	No					
41	74	CRC	Yes	No					
42	49	CRC	Yes	Yes					
43	31	CRC	Yes	No					
44	44	CRC	Yes	Yes					
45	27	CRC	Yes	No					
46	51	CRC	Yes	No					
47	58	CRC	Yes	Yes					
48	56	CRC	Yes	No					
49	28	CRC	Yes	Yes					
50	45	CRC	No	No					
51	31	CRC	Yes	No					
52	25	CRC	Yes	Yes					
53	53	CRC	Yes	No					
54	69	CRC	Yes	No					
55	62	CRC	Yes	Yes					
56	45	CRC[b]	Yes	Yes					
57	50	CRC	Yes	Yes					

[a] All patients without a family history had multiple primary colon cancers; for these individuals, the age at diagnosis is that when CRC was first diagnosed.

[b] Muir-Torre syndrome.

onset that was significantly younger than would have been expected (Cancer in New South Wales: Incidence and Mortality 1997 database). When the *hMLH1* mutation–positive group was compared with the *hMSH2* mutation–positive group, there was a trend toward a lower rate of CRC in the latter group, although this trend was not statistically significant ($P = .087$).

Extracolonic Cancers

The relative frequency of other malignancies associated with HNPCC is shown in figure 3. The frequencies of the following cancer types were found not to
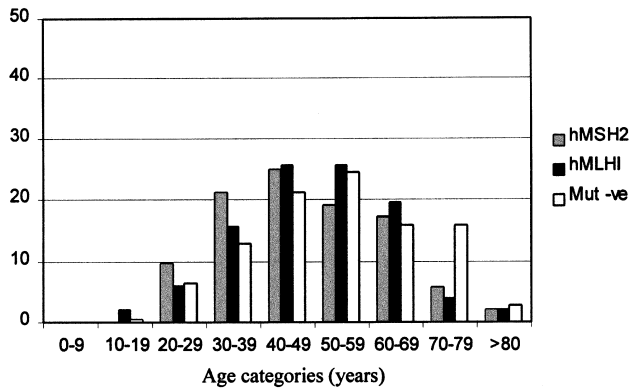
Figure 2 Age at diagnosis in 292 patients with familial colorectal carcinoma, according to mutation status. One to six affected mutation-positive family members were included in this analysis, and only *hMSH2* and *hMLH1* causative mutations were included.

differ between the *hMSH2* mutation–positive group, the *hMLH1* mutation–positive group, and mutation-negative group: lymphoproliferative disease, renal/renal-tract cancers, endometrial/ovarian cancers, and stomach cancer. All these malignancies were overrepresented in all three groups, compared with the expected frequency in the general population (see table 4). Differences in SIR trends were apparent between the groups. Endometrial cancer incidence was significantly increased, compared with that in the general population, as were stomach cancer, CNS tumors (in the mutation-negative group and the *hMSH2* mutation–positive group only), renal/renal-tract cancer, and lymphoproliferative disease. Breast cancer was not overrepresented in the *hMSH2* mutation–positive group, but it was overrepresented in both the hMLH1 mutation–positive group and the mutation-negative group, which was a highly significant difference. The SIR of breast cancer in the mutation-negative group was similar to that observed in the *hMLH1* mutation–positive group. Differences in the SIR of prostate cancer were observed between the mutation-negative group and the *hMSH2* mutation–positive group (no cases were observed in the *hMLH1* mutation–positive group). This difference was significant, as determined by Fisher's exact test ($P = .038$), which indicated that there was a relatively high number of prostate cancer patients in the mutation-negative group, compared with both mutation-positive groups.

The age at diagnosis of breast cancer in the study population does appear to be younger than expected. The average age at diagnosis of breast cancer diagnosis was 54.27 years in the overall population in this study; in the mutation-negative group, it was 55.55 years; in the *hMLH1* mutation–positive group, 51.33 years; and in the *hMSH2* mutation–positive group, 54 years. This age is ~6 years younger than the average age, 60 years,

in New South Wales (Cancer in New South Wales: Incidence and Mortality 1997 database). Furthermore, we determined that this malignancy was significantly more likely to be present in the mutation-negative group and in the *hMLH1* mutation–positive group than in the *hMSH2* mutation–positive group ($P = .006$). Also, breast cancer was more likely to be identified in the *hMLH1* mutation–positive group than in the *hMSH2* mutation–positive group ($P = .024$).

When the occurrence of the cancer types was compared overall, some similarities between the groups could be identified, as shown in table 4. The spectrum and frequency of malignancies in the *hMLH1* mutation–positive group more closely matched those in the mutation-negative group than did those in the *hMSH2* mutation–positive group. Interestingly, in the *hMSH2* mutation–positive group the incidence of kidney and renal-tract cancers was greater than that in either the *hMLH1* mutation–positive group or the mutation-negative group; however, this difference did not reach significance.

Discussion

The identification of germline mutations in families with HNPCC remains difficult, despite the fact that there have been improvements in the technology of mutation detection. In this report we have analyzed a large group of patients (and their families, when available) for mutations in the DNA-mismatch-repair genes *hMLH1* and *hMSH2*. Selection of families on the basis of the Amsterdam citeria remains the best method in which the probability of mutation detection remains high, at ~60%. When the criteria are extended to include a number of other facets, which are included among the Bethesda criteria, the mutation-detection rate falls to
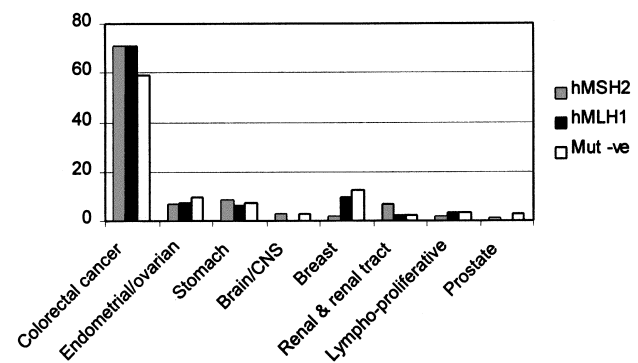
Figure 3 Relative percentage of malignancies within the three groups of families with HNPCC. The tumor spectrum was determined on the basis of 559 patients with cancer, all of whom were from families with a clustering of CRC.

~34%. Given that there have been several reports (Wijnen et al. 1998; Heinimann et al. 1999) indicating that relaxation of the Amsterdam criteria results in a poor rate of identification of mutations, many groups are adhering to the Amsterdam criteria when they select families for genetic-testing purposes. However, since our mutation-detection rate identified mutations in patients who fulfilled the Bethesda criteria but not the Amsterdam criteria, we would advocate that this group of patients should be screened for DNA-mismatch-repair–gene alterations. Of particular note, two separate causative mutations were identified in two Bethesda-criteria patients who presented with multiple colonic primaries over a period of many years and who did not have a family history of disease. There remain, however, in both categories, a substantial proportion of families that have an autosomal dominant disease pattern, which is best accounted for by a single, highly penetrant disease allele. Studies using denaturing gradient gel electrophoresis to detect mutations in various genes suggest detection rates of 90%–95% (Wahlberg et al. 1999). Therefore, we do not believe that, in the identification of genetic change within coding regions of the genes under investigation, methodologies used in the present study are so deficient that many changes in *hMSH2* or *hMLH1* have been missed. Nevertheless, some changes will not be detectable when the strategy is employed; these are likely to be changes in promoter or enhancer regions, cryptic changes within noncoding sequences, and large deletions encompassing either whole exons or the entire gene.

Two mutations in *hMSH2* were of special interest: (1) the 24-bp inversion and substitution, at positions 1311–1334 inclusive in exon 8, and (2) the 66-bp duplication at positions 2048–2114 in exon 13. Neither of these mutations led to any unique phenotype—and, presumably, neither results in altered hMSH2 function. The best explanation for both of these mutations is a recombination event that resulted in the inadvertent duplication of sequence. There was no apparent disease phenotype that could be readily identified with any particular mutation in this study, confirming the notion that a breakdown in DNA mismatch repair is not associated with overt differences in disease phenotype. Disease-spectrum differences associated with *hMSH2* or *hMLH1* were, however, identified on examination of the types of tumors present in families that harbored either a *hMLH1* mutation, a *hMSH2* mutation, or no mutation.

On average, the age at diagnosis of CRC in the mutation-negative group was 5 years older than that in the mutation-positive group, but the difference was significant only in the *hMSH2* mutation–positive group. In the *hMLH1* mutation–positive group, there was an apparent trend toward ages at diagnosis that were younger than those in the mutation-negative group, but the difference did not reach statistical significance. Interestingly, in terms of the age at disease penetrance, there was no difference between the *hMSH2* mutation–positive group and the *hMLH1* mutation–positive group, which indirectly supports the concept that DNA mismatch repair is a caretaker function and not a gatekeeper function (Kinzler and Vogelstein 1997). This does not mean, however, that differences do not exist between the two groups. The protein MSH2 has been shown to be important in the recognition of DNA mispairs and is an energy-driven process, whereas MLH1 binds to MSH2:DNA complexes and helps to orchestrate endonuclease activity (Fishel 1998). In addition, hMLH1 appears to be involved in meiotic recombination, giving it a multifunctional role (Anderson et al. 1999). Given these subtle differences between the two proteins, we studied the disease profiles of the *hMSH2* mutation–positive group and the *hMLH1* mutation–positive group. There was a tendency for the frequency of CRC in the *hMLH1* mutation–positive group to be greater than that in the *hMSH2* mutation–positive group, with the relative percentages being 12.15 and 8.92, respectively. This suggests that, with regard to CRC risk, there may be some differences between the two groups. A possible explanation for this difference is the greater potential for allelic silencing in *hMLH1*, by hypermethylation of the wild-type allele (Cunningham et al. 1998). Nevertheless, except for breast cancer and prostate cancer, the rates of other cancers were similar in both groups, suggesting the possibility of colon-specific differences in disease expression. The frequency of breast cancer in the *hMSH2* mutation–positive group was significantly less than that observed in either the *hMLH1* mutation–positive group or the mutation-negative group, suggesting a real difference between the three groups. There are conflicting reports as to whether breast cancer risk is increased in HNPCC (Itoh et al. 1990; Nelson et al. 1993; Watson and Lynch 1993; Risinger et al. 1996). Breast cancer incidence overall in our study population was overrepresented within both the *hMLH1* mutation–positive group and the mutation-negative group but was not overrepresented in the *hMSH2* mutation–positive group. Why breast cancer should be underrepresented in the *hMSH2* mutation–positive group is not known, but it may be associated with the proposed roles of hMLH1 and hMSH2 in the BRCA1-associated genome surveillance complex supercomplex involved in the recognition and repair of aberrant DNA structures (Wang et al. 2000). The finding that breast cancer does not appear to be associated with *hMSH2* mutations may explain some of the discrepancies that have been reported in the literature, with respect to breast cancer risk in HNPCC.

As expected, the incidence of prostate cancer was not

overrepresented in the mutation-positive groups considered together. Interestingly, a feature of the mutation-negative group did appear to be the increased incidence of prostate cancer, and this may therefore be a feature of the families with HNPCC that are mutation negative.

Finally, the overall spectrum of disease within the mutation-negative group more closely resembled that observed in the *hMLH1* mutation–positive group than it resembled that in the *hMSH2* mutation–positive group. This, coupled with the similar ages at diagnosis of CRC, suggests either that there are more-subtle changes occurring in the *hMLH1* gene that are not being identified by mutation analysis or that other proteins may interact with hMLH1, such that their disruption results in a disease spectrum similar to that observed in *hMSH2* mutation–positive families.

In conclusion, use of the Bethesda criteria for the selection of families for gene analysis results in a reduced probability of mutation detection. Nevertheless, families or patients that can be identified on the basis of these criteria should be offered genetic testing for genes associated with HNPCC, since this will result in an increased rate of identification of gene-mutation carriers. To improve the probability of mutation detection if the Bethesda criteria are adopted, we would suggest that mutation analysis be performed in conjunction with DNA microsatellite testing and, possibly, immunohistochemical staining for DNA-mismatch-repair proteins. Subdivision of the mutation-positive and mutation-negative groups makes it possible to tease out subtle differences between the various populations, such as similar ages at onset of disease in the mutation-negative and *hMLH1* mutation–positive groups and disease-spectrum differences within each mutation-positive group. Better knowledge of the disease spectrum associated with mutation status will aid in the management of these families. Better classification of the mutation-negative group will aid in identification of additional genes associated with this disorder.

Acknowledgments

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

New South Wales Cancer Council, http://www.nswcc.org.au/pages/ccic/stats/index.htm

International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer, The, http://www.nfdht.nl/database/mdbchoice.htm (for the mismatch-repair-gene–mutation database)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for HNPCC [MIM 120435 and MIM 120436])

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