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Microsatellite instability and mutation analysis of *hMSH2* and *hMLH1* in patients with sporadic, familial and hereditary colorectal cancer

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To date, at least four genes involved in DNA mismatch repair, *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2*, have been demonstrated to be altered in the germline of patients with hereditary nonpolyposis colorectal cancer (HNPCC). Additionally, defective mismatch repair is thought to account for the observation of microsatellite instability (MIN) in tumors from these patients. The genetic defect responsible for the MIN+ phenotype in sporadic colorectal cancer, however, has yet to be clearly delineated. In order to better understand the role of somatic and germline alterations within *hMSH2* and *hMLH1* in the process of colorectal tumorigenesis, we examined the entire coding regions of both of these genes in seven patients with MIN+ sporadic colorectal cancer, 19 patients with familial colorectal cancer, and 20 patients meeting the strict Amsterdam criteria for HNPCC. Thirteen germline, two somatic, and four neutral alterations were identified. The two somatic mutations occurred in patients having familial cancer, while the germline mutations were distributed among one sporadic (14%), three familial (16%), and nine HNPCC (45%) cases. All patients with identified mutations in the mismatch repair genes, whose tumors were available for analysis, demonstrated MIN. On the other hand, we could not identify mutations in the subset of clinically defined HNPCC patients with MIN negative tumors nor in the majority (6/7) of MIN+ sporadic tumors.

INTRODUCTION

Colorectal cancer is one of the three leading causes of cancer mortality worldwide with an incidence of approximately one million cases and a mortality of 500 000 annually (1). Early detection by general population screening has not led to a major breakthrough in the prevention of this potentially curable disease. However, identification of individuals with a genetic susceptibility to colorectal cancer will increase the yield of screening procedures and may improve cancer prevention. In several different studies, the proportion of hereditary colorectal cancer (CRC) has been estimated to be 0.5 to 13% (2–5). Hereditary colorectal cancer can be subdivided into the polyposis and the nonpolyposis syndromes. Only a small proportion of colorectal malignancies are caused by the polyposis syndromes, of which familial adenomatous polyposis (FAP) is the most frequent. This autosomal dominantly inherited disorder is characterized by the development of a large number of polyps (at least 100) in the colorectum that, if untreated, will inevitably lead to colorectal cancer. FAP is caused by mutations in the APC gene on chromosome 5q21 (6–8) and, although there is heterogeneity in regards to the age of onset and extracolonic disease manifestations, the colorectum is always involved. As a result, FAP can almost always be diagnosed in an individual without knowledge of the family history.

Hereditary nonpolyposis colorectal cancer (HNPCC), on the other hand, accounts for a much larger proportion of inherited colorectal cancer and, until recently, was defined merely by family history. This disorder is characterized by a smaller number of colorectal polyps, syn- and metachronous colorectal cancers and cancers of other sites. The predominant extracolonic organ affected is the endometrium, but stomach, small intestine, hepatobiliary, kidney, ureter and ovarian cancers may cluster in HNPCC families. These cancers most commonly occur approximately 20 years earlier than in the general population. For comparative studies, the International Collaborative Group defined the Amsterdam Criteria (9) for the identification of HNPCC kindreds, that is: (i) at least three relatives should have histologically verified colorectal cancer; one of them should be a first degree relative to the other two; (ii) at least two successive generations should be affected; and (iii) in one of the relatives, colorectal cancer should be diagnosed under 50 years of age.

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In sporadic colorectal cancer, a number of studies (10) suggests that the process of tumorigenesis proceeds through a series of genetic alterations which include both dominant acting proto-oncogenes (*c-myc* and *ras*) and tumor suppressor genes (*APC*, *p53*, and *DCC*). Recently, a novel type of genomic instability, characterized by alterations in microsatellite repeat size within tumor DNA, was identified in a subset of sporadic colon cancers (11,12) and in tumors from patients with HNPCC (13). Over the past 2 years, this phenomenon of microsatellite instability (MIN) has been reported in an ever increasing number of tumor systems (14–16). Interestingly, recent data suggests that a downstream consequence of this particular type of genomic instability is the alteration of specific genes containing repeat sequences, such as the TGF-β receptor (RII) (17).

An explanation for the MIN$^+$ phenotype has come, in part, from the cloning and identification of several genetic susceptibility loci for HNPCC. To date, at least four genes involved in DNA mismatch repair, *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* have been cloned and characterized (18–22), and these have been demonstrated to be altered in the germline of patients with HNPCC (19–22). Importantly, the cloning of these genes now enables the search for specific intrafamilial mutations and identification of gene carriers. Several reports have suggested that germline mutations in *hMSH2* and *hMLH1* account for the majority of HNPCC families (23,24) and for the MIN$^+$ phenotype exhibited in tumors from these families. The genetic defect responsible for the MIN$^+$ phenotype in sporadic colorectal cancer, however, has yet to be clearly delineated. To better understand the frequency and role of somatic and germline alterations within *hMSH2* and *hMLH1* in the pathogenesis of colorectal cancer, we examined the DNA sequence of both of these genes in a group of patients having sporadic, familial colorectal carcinoma, and HNPCC meeting the Amsterdam criteria.

RESULTS

Microsatellite instability

Individuals were identified for this study based on either the presence of tumor microsatellite instability or the presence of a positive family history. Overall, there were seven patients with sporadic colorectal tumors, 19 patients with familial colon cancer (12 American and seven German), and 20 patients from HNPCC kindreds (11 American and nine German kindreds). Eleven colorectal tumors were previously described as MIN$^+$ (11). The MIN status of the tumors for those patients selected by family history was determined using DNA extracted from either frozen or paraffin-embedded tumor. These data are summarized in Table 1. Of the 35 cases selected because of a positive family history (HNPCC or familial), fresh or paraffin-embedded tissue was available on only 22. Of these, 12 (55%) were MIN$^+$. Of the 11 American HNPCC patients, four of five (80%) were MIN$^+$, whereas this proportion was lower in the German HNPCC population (2/6, 33%) (Table 1).

hMSH2 and *hMLH1* sequence analysis

The DNA sequence of *hMSH2* and *hMLH1* was evaluated in all 46 patients. Our approach was to sequence each of the 16 *hMSH2* and 19 *hMLH1* exons from one affected member of each family. In one American family, the proband (Case 4A) had colorectal polyps, but did not have cancer. In 16 cases, DNA from both fresh frozen tumor and leukocytes was available for sequencing.

A total of 17 alterations, 15 germline and two somatic, were found in 15 patients (Table 1). Eleven (65%) of these were either nonsense or frameshift mutations and, of the 15 germline alterations, nine were in the *hMLH1* gene and six in *hMSH2*. The two somatic mutations occurred in patients having familial cancer, while the germline mutations were distributed among one sporadic (14% of sporadic cases), three familial (16% of familial cases), and nine HNPCC cases (45% of HNPCC cases). Two patients demonstrated more than one germline sequence alteration. In case 4F, a missense mutation in exon 3 of *hMSH2* and a 1 bp deletion in exon 1 of *hMLH1* were identified. In the second case, 4A, a 1 bp deletion leading to a frameshift in exon 13 of *hMSH2* and a missense mutation in exon 16 of *hMLH1* were identified. In case 4F, the frameshift alteration is presumably the causative mutation and the missense mutation a neutral polymorphism, since only the deletion co-segregated with the disease phenotype in one other affected member that was examined in this family. Other family members for case 4A were not available for further testing. In those cases where tumor was available for study, none of the mutations demonstrated a homozygous pattern. These results need to be interpreted cautiously, however, since normal cells are present within the tumor.

The highest number of mutations identified was in the American group of HNPCC patients (6/11, 55%). Although the same clinical criteria were applied, only three (33%) mutations were found in the nine German HNPCC probands. Germline alterations, however, were also found in four other patients not meeting the Amsterdam criteria. The first was a G→A transition (A492T) in exon 13 of *hMLH1* in a 41 year old patient with a MIN$^+$ sporadic rectal cancer. It is unclear at this point whether this alteration represents a neutral polymorphism or a disease causing mutation. The other alterations occurred in three familial cases (3F, 4F, 5F), two of which were suggestive of HNPCC, but did not fulfil the strict Amsterdam criteria. All three of the presumably disease-causing mutations in this group, two frameshift and one nonsense mutation, occurred in *hMLH1*. Of note, all tumors from patients with mutations in *hMSH2* or *hMLH1* (either germline or somatic) were MIN$^+$. However, not all patients with a MIN$^+$ tumor had a demonstrable mutation. This was particularly true for the sporadic group: six of the seven MIN$^+$ tumors lacked a recognizable mutation.

In addition to the above sequence alterations, a common polymorphism was detected in exon 8 of *hMLH1* (A→C, I219L), having an allele frequency of 0.69 for isoleucine and 0.31 for leucine. An additional C→T transition in exon 3 of *hMSH2* (S153S) was also detected.

Genotype phenotype correlations

For those familial cases that had a germline mutation, a review of the pedigree for genotype-phenotype correlations was performed (Table 2). Unfortunately, mutation analysis on family members other than the proband was not generally possible. These data, therefore, includes information from individuals who are at 25–100% risk for carrying the genetic defect. In comparing families with *hMSH2* mutations versus families with *hMLH1* mutations, a greater number of endometrial cancers were observed (10 versus 5; $p = 0.11$ using Fisher's exact test) and fewer non-colon/endometrial/skin cancers were observed (5 versus 18; $p = 0.02$).

Table 1. Sequencing, MIN, and clinical data

Case[1]	Tissue[2]	Gene[3]	Exon	Codon	Base changes	Consequence	MIN status[4]	Cancer site (age)
Sporadic								
1S	T/N	*MLH1* (G)	13	492	G →A	missense, Ala→Thr	pos (30/34)	rectum (41)
2S	T/N	neg					pos (29/33)	cecum (78)
3S	T/N	neg					pos (28/32)	asc. colon (85)
4S	T/N	neg					pos (30/34)	asc. colon + hep flex + transv colon (91)
5S	T/N	neg					pos (27/31)	asc. colon (73), prostate (74)
6S	T/N	neg					pos (31/34)	asc. colon + prostate (81)
7S	T/N	neg					pos (28/31)	cecum (75)
Familial								
1F	T/N	*MSH2* (So)	14	749	G→T	nonsense, Glu→stop	pos (21/30)	basal cell (×3), colon-hep flex (85), Squamous cell (×1)
2F	T/N	*MLH1* (So)	13	499	del G	frameshift	pos (7/8)	cecum (35)
3F	T/N	*MLH1* (G)	16	590–591	del TAGA	frameshift	pos (7/8)	colon-hep flex (32)
4F	T/N	*MLH1* (G)	1	21	del G	frameshift		
		MSH2 (G)	3	167	G→C	missense, Asp→His	pos (28/32)	cecum (28)
5F	N	*MLH1* (G)	17	659	C→T	nonsense, Arg→stop	NA[5]	colon (40)
6F	T/N	neg					pos (25/30)	breast (70), cecum (76), multi polyps in asc colon and stomach (76)
7F	T/N	neg					pos (7/9)	bladder (71), breast and asc colon (81)
8F	T/N	neg					pos (9/9)	cecum and transv colon (61)
9F	N	neg					pos (8/9)	cecum (33), end + sig colon (67), polyps in stom (74), and col (77)
10F	N	neg					pos (5/7)	endometrial (46)
11F	N	neg					pos (7/9)	endometrial (51)
12F	T/N	neg					neg (0/9)	rectum (32)
13F	N	neg					neg (0/9)	sigmoid colon (56)
14F	N	neg					neg (0/9)	desc colon (42)
15F	N	neg					neg (0/9)	stomach (48)
16F	N	neg					neg (1/7)	rectal (48)
17F	N	neg					NA	colon (32), sigmoid (65), uterine polyps
18F	N	neg					NA	endometrial (30)
19F	N	neg					NA	sig colon (32)
HNPCC								
1A	T/N	*MSH2* (G)	12	596	del AAT	in-frame del Asn	pos (25/33)	rectal (34)
2A	N	*MSH2* (G)	5		A→T at 943+3	in-frame del of exon 5	pos (9/9)	cecum (39)
3A	N	*MSH2* (G)	13	705	del G	frameshift	NA	endometrial (29)
4A	N	*MLH1* (G)	16	618	A→C	missense, Lys→Thr		
		MSH2 (G)	13	735	del T	frameshift	NA	polyps (transv + sig) (42)
5A	N	*MLH1* (G)	13	495	ins C	frameshift	pos (5/9)	asc col (41), transv col (44), rectal polyps (45)
6A	N	*MLH1* (G)	16	616	del AAG	in-frame del of Lys	NA	ovaries (40)
7A	N	neg					pos (3/6)	endometrial (46)
8A	N	neg					neg (0/6)	ovarian (48), appendiceal (55)

Table 1. Continued

9A	N	neg					NA	sigmoid (32); hep flex (51); sp flex (57), sq cell (59)
10A	N	neg					NA	colon cancer (28)
11A	N	neg					NA	ovarian and endometrial (51), melanoma (62), breast (64)
12A	N	*MLH1* (G)	8	226	C→T	nonsense, Arg→stop	pos (8/9)	asc colon (40), desc colon (43)
13A	N	*MLH1* (G)	16	590–591	del TAGA	frameshift	pos (3/9)	asc colon (33), endometrial (39)
14A	N	*MSH2* (G)	7	409	del AG	frameshift	NA	asc colon (38), transv colon (38), desc colon (51)
15A	N	neg					neg (0/9)	asc colon (56)
16A	N	neg					neg (0/5)	rectal (69)
17A	N	neg					neg (0/5)	rectal (33)
18A	N	neg					neg (1/9)	rectal (23)
19A	N	neg					NA	desc colon (46)
20A	N	neg					NA	asc colon (50), colonic polypectomies every 6 months

[1]S, sporadic; F, familial; A, Amsterdam criteria. Cases 1A–11A are Mayo Kindreds while Cases 12A–19A are German Kindreds. Additionally, 10F, 11F, 14F–16F, 18F, and 19F are German cases.
[2]Tissue available for sequence analysis: T, fresh frozen tumor; N, normal.
[3]So, Somatic mutation; G, Germline mutation.
[4]Pos, positive; neg, negative; number of markers showing instability over total number of markers with successful results.
[5]NA, not available or no amplification.

Table 2. Analysis of *hMSH2* and *hMLH1* kindreds. Results of pedigree analysis for those probands with identified mutations

	hMSH2	*hMLH1*
Number of kindreds	5	7
Number of at risk individuals[a]	124	147
Number of people with colon cancer (number of colon cancers)	28 (33)	34 (42)
Age at diagnosis of first colon cancer (mean)	48.2	42.6
Number of endometrial cancers	10	5
Age at diagnosis of endometrial cancer (mean)	46.1	47.5
Number of cancers, excluding colon, endometrial and skin	5	18

[a]Includes individuals >25 years old who are at 25–100% risk for being a gene carrier by virtue of pedigree position.

The average age of colorectal cancer onset was 48.2 in *hMSH2* kindreds (28 patients with 33 cancers) and 42.6 in families with *hMLH1* mutations (34 patients with 42 cancers).

DISCUSSION

Currently, a minimum of four susceptibility genes have been identified for HNPCC: *hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2* localized to chromosomal arms 2p, 3q, 2q, and 7, respectively (15). Early reports suggested that *hMSH2* and *hMLH1* accounted for up to 90% of HNPCC families (23,24). The actual frequency of involvement for each of the four genes in HNPCC is still uncertain, however, since many of the early reports examined a relatively small number of kindreds, many of which were of Scandinavian origin. It is now known that a significant fraction of Finnish HNPCC kindreds are the result of a founder effect (25,26). Additionally, more recent data suggests that the involvement of *hMSH2* and *hMLH1* in HNPCC may occur at a lower frequency (approximately 60%), and that this frequency may be population dependent (27,28).

In this study, 20 patients from kindreds fulfilling the Amsterdam criteria for HNPCC were examined and only nine (45%) were found to have a germline mutation. Of the 11 HNPCC kindred identified at the Mayo Clinic, six (55%) had a germline mutation while this was the case for only three of the nine German kindreds (33%). The clinical selection criteria (Amsterdam criteria) were identical for both groups. Of note, tumors (when available) from each of the HNPCC patients harboring a germline mutation were MIN$^+$, while all but one of the remaining HNPCC cases lacking a germline mutation failed to demonstrate tumor microsatellite instability. Of the six HNPCC cases that had MIN$^+$ tumors, five had a germline mutation.

Although the overall frequency of germline mutation in the HNPCC kindreds is somewhat lower than anticipated, there are

a number of explanations that might account for this observation. First, deletions of entire exons will not be detected by our sequencing strategy. Although large deletions have been identified in a number of kindreds (28), the true frequency of such abnormalities is still unknown. Second, we cannot rule out the involvement of other mismatch repair genes, such as *hPMS1* and *hPMS2*. Other studies, however, have shown that their involvement in HNPCC kindreds is infrequent (28). Other explanations include the chance occurrence of familial clustering, the possibility of shared environmental carcinogens, and the presence of HNPCC phenocopies. Finally, a subset of HNPCC patients may have an underlying molecular genetic mechanism that is not due to mismatch repair. A recent study by Lewis *et al.* (33) suggest that other less highly penetrant genes for colorectal cancer exist and these may be responsible for a greater proportion of colorectal cancer than the mismatch repair genes so far identified. This latter possibility is particularly intriguing in that all but one of the mutation negative HNPCC kindreds lacked microsatellite instability. The kindreds with clinically defined HNPCC and MIN^- tumors was particularly apparent in the German cohort of patients. It will be important to further test this particular group of patients with linkage analysis to examine the role of defective mismatch repair versus the presence of other novel genetic alterations. Compared to the German kindreds, the frequency of germline alterations in the American cohort of patients was 55%, a frequency more consistent with recent reports (27,28).

In addition to the germline mutations found in the Amsterdam positive families, five of the 19 Amsterdam-negative families also had *hMSH2* and *hMLH1* alterations, two of which were somatic only. The three Amsterdam-negative kindreds with germline mutations included 13 individuals with colon cancer (mean age at diagnosis of 48 years), three prostate cancers, three unknown cancers, two leukemias, and one each of the following: bladder, throat, bone, cervix, and breast. These families failed to meet the Amsterdam criteria because of an apparently nonpenetrant gene carrier in one family, and unknown cancer type in a parent in one family, and nonrecognition of uterine cancer by the strict Amsterdam criteria in the third family. Although strict adherence to the original criteria serves to enrich a study population with gene mutation positive individuals, clinicians need to be aware that these criteria are not diagnostic criteria in the usual sense, and are overly restrictive for clinic purposes. This is especially the case if other HNPCC associated cancers, such as endometrial and stomach cancers, are not taken into account.

The majority of mutations described in this study represent unique alterations. Four of the alterations, however, have been previously described. These include: (i) two alterations in *hMSH2*, a splice mutation resulting in a deletion of exon 5 (29,30) and a 3 bp deletion resulting in an in-frame deletion of asparagine (28,31); and (ii) two alterations in exon 16 of *hMLH1*, 1853A→C (Lys→Thr) (32) and a 3 bp deletion resulting in an in-frame deletion of lysine (27). Of interest, the missense mutation identified in exon 16 of *hMLH1* by Han *et al.* (32) co-segregated with other affected family members suggesting that this was the disease causing mutation. Although such information was not available for this alteration in our study, this particular individual (case 4A) also had a frameshift mutation in *hMSH2*. The clinical significance of this missense mutation, therefore, remains to be established. Also of interest is the high frequency of mutations observed in exon 16 of *hMLH1*. This region of exon 16 has previously been noted as a hot-spot for mutations in *hMLH1* (27).

Two individuals were found to have mutations in both *hMSH2* and *hMLH1*. Both had one missense and one frameshift mutation, the latter more likely to be the causative mutation. In at least one of these cases, the missense mutation is likely to have little clinical significance, since it did not co-segregate with disease in one other affected individual in this family. Had mutation screening stopped upon finding a missense mutation in a DNA mismatch repair gene, the likely causative mutations would have been undetected. Although there will undoubtedly be individuals who are double heterozygotes at the DNA mismatch repair complex, HNPCC is most often recognized as a Mendelian autosomal dominant disorder indicating that inheritance of a mutation in a single gene is sufficient to cause the disease. For those mutations not previously characterized, co-segregation of disease with mutation in a given kindred should ideally be demonstrated prior to undertaking presymptomatic genetic testing for at-risk relatives in HNPCC kindreds. This is especially important when the identified mutation is a missense mutation, the biologic effect of which may be difficult to project.

Microsatellite instability is present in about 15% of unsolicited colon cancers (11–13) but, in this study, was present in 100% (10/10) of tumors (whether from familial or HNPCC cases) in which mutations in either *hMLH1* or *hMSH2* were subsequently found. These results suggest that MIN may be a very sensitive method for distinguishing a subset of HNPCC colon cancers (i.e., those resulting from defective mismatch repair) from sporadic colon cancers. As indicated above, however, defective mismatch repair may not be involved in all clinically defined HNPCC kindreds. Furthermore, since a germline mutation was found in only one of seven MIN^+ sporadic cancers and two of 10 MIN^+ familial cases, MIN status alone is likely to have low specificity for HNPCC. Similar results have been reported for MIN^+ sporadic colon (31,34) and endometrial cancers (35). Although, MIN status bears a strong correlation with a positive family history, the role of the mismatch repair genes in MIN^+ sporadic colorectal cancer and familial cases is still unclear. It seems likely, therefore, that other genes will play a role in the etiology of MIN in these cases. A large scale prospective study is underway to more adequately explore the relationship between MIN status, family history, and clinical disease.

Because of the modest numbers involved, no obvious differences in phenotype could be appreciated between individuals with *hMLH1* versus *hMSH2* germline mutations. However, in comparing the extended pedigrees of probands, some possible distinctions began to emerge (Table 2). The at-risk relatives in the kindreds with *hMLH1* mutations had first colon cancers diagnosed at younger ages (42.6 years versus 48.2 year mean), had fewer endometrial cancers (five versus 10) and had greater numbers of non-colonic/non-endometrial/non-skin cancers (18 versus five) compared to kindreds with *hMSH2* mutations. Given the small number of families, evaluation of additional kindreds will be required to validate these early impressions.

Two unrelated patients presented with an identical mutation in exon 16 of *hMLH1* (del TAGA). The German patient met the Amsterdam criteria, while the American patient was classified as a familial case. Their disease presentation was quite similar, having had a right-sided colon cancer at ages 32 and 33, respectively. The female German patient had a follow-up of 12 years and died at age 45 due to metastatic endometrial cancer diagnosed two years earlier. The male American patient died of metastatic colon cancer two years after diagnosis. Both patients,

therefore, had a short survival of two years after colon cancer and endometrial cancer diagnosis respectively. Identification of more patients with identical mutations may eventually lead to a better understanding of the clinical correlation to underlying genetic alterations. In our example the similar age of onset and course of disease may or may not be coincidental. If this type of observation is confirmed by other investigators more effective and less invasive screening procedures or prophylactic surgery may be considered for proven mutation carriers.

In summary, a high proportion of HNPCC kindreds have germline mutations in *hMSH2* and *hMLH1*. Furthermore, all patients with a mutation in *hMSH2* and *hMLH1* were found to have MIN$^+$ tumors. On the other hand, several patients meeting the Amsterdam criteria for HNPCC were found to have MIN$^-$ tumors suggesting that alternate pathways, not caused by defective mismatch repair, may lead to the syndrome. Patients with MIN$^+$ tumors that do not meet the clinical criteria for HNPCC are less likely to harbor germline mutations in *hMLH1* and *hMSH2*. It is possible that other, as yet undefined mismatch repair genes lead to this phenomenon. Few genotype-phenotype correlations of clinical relevance for the individual patient have yet been established. As more mutations are reported, however, it is hoped that future studies will identify correlations between site of mutation and course of disease.

MATERIALS AND METHODS

Patients

Based on the presence of either tumor microsatellite instability or the presence of a positive family history, 46 patients were identified for this study. Eleven patients with colorectal cancer were previously identified as MIN$^+$ by screening paired normal/tumor tissue (11). In a retrospective chart review, one of these patients met the strict 'Amsterdam criteria' for HNPCC (9), three patients were classified as familial and seven patients were classified as sporadic. Familial cases were defined as those individuals having at least one other family member (first or second degree) with colon cancer. The number of HNPCC and familial cases may be an underestimate, however, since some charts contained scant family history information.

An additional 35 individuals, 19 fulfilling the Amsterdam criteria for HNPCC and 16 with familial colorectal cancer, were selected for this study. Ten HNPCC and nine familial cases were randomly identified through the Department of Medical Genetics at the Mayo Clinic, while nine HNPCC and seven familial cases were identified at the Department of Surgery of the University in Düsseldorf. Familial cases were again defined as those individuals having at least one other family member with colon cancer; in 12 of the 16 cases, this was a first degree relative. For the familial cases, the average number of affected (all cancers) individuals per kindred was seven with a range of three to 16 (including the proband), while the average number of colorectal cancers was three with a range of two to seven (including the proband). When available, medical records were obtained on other affected family members to verify the presence of cancer. In most instances, however, such records were not available. Other than the country of origin, the ethnic background of individuals was generally unknown.

Tissue samples and DNA extraction

Frozen tissue samples. Harvested tumor-tissue was immediately frozen at –70°C and stored until time of DNA extraction. Paired noncancerous DNA was obtained from either normal mucosa or peripheral blood leukocytes at the same time that cancer tissue was obtained. Frozen tissue processing and DNA extraction was performed as previously described (36). Briefly, frozen tissue was first mounted and examined microscopically with hematoxylin and eosin-stained cryosections. Microdissection of the specimen was then performed for removal of normal tissue to assure that tumor tissue contained >70% cancer cells. Tumor DNA was obtained from multiple 10 µm thick cryosections, while paired noncancerous DNA was obtained from either normal mucosa or peripheral blood leukocytes.

Paraffin-embedded tissue samples. When available, paraffin-embedded colorectal tumor was obtained on these patients in order to determine MIN status. Paraffin-embedded tissue was cut into 10 µm thick sections that were then mounted on slides. One reference slide was stained with hematoxylin and eosin and examined microscopically for determination of exact tumor tissue localization and percent cancer cells. Tumor and noncancerous DNA was obtained from multiple slides stained with toluidine blue. Tumor or normal tissue was scraped from the toluidine blue stained slide and then placed into a microfuge tube. For approximately 1 cm^2 of tissue, 100 µl of digestion buffer (50 mM Tris, pH 8.0) was added together with 1 µl of proteinase-K (20 mg/ml). The tubes were then incubated with shaking at 55°C for 48 h, adding an equal amount of proteinase K after 24 h. After removal from the incubator, the samples were boiled for 8 min at 95°C and put into an ice/water slurry for 5 min.

Microsatellite analysis

Paired normal and tumor DNA were analyzed for MIN with a common set of 34 microsatellite markers. The number used ranged from a minimum of nine to a maximum of 34 markers. All specimens were examined initially with the same group of nine markers. As part of other studies, however, additional markers were evaluated for some of the tumors and the result of these analysis are included in this study. PCR and gel electrophoresis were performed essentially as described by Thibodeau *et al.* (11). MIN was defined by the presence of additional bands in the PCR amplified product derived from tumor DNA (either expansion or contraction in repeat length) compared to that derived from the paired normal DNA from that patient. Tumors were considered MIN$^+$ if 30% of the markers or greater showed bands of altered mobility in the tumor compared to the normal (37). A list of the 34 markers utilized in this study is available upon request.

Sequence analysis

For all patients, all exons of both *hMSH2* and *hMLH1* were sequenced with use of the fmol sequencing kit (Promega, Madison, WI), regardless of whether a mutation was found. Paraffin-embedded tissue was used to determine the MIN status only, while DNA from leukocytes was used for sequence analysis. When available, genomic DNA from fresh frozen colorectal cancer tissue was also sequenced. Mutations were confirmed by repeating both the exon amplification and sequencing steps. The DNA sequence of the intron-exon boundaries

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