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Advances in Brief

hMSH2 Mutations in Hereditary Nonpolyposis Colorectal Cancer Kindreds[1]

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Abstract

It has recently been shown that hereditary nonpolyposis colorectal cancer (HNPCC) is caused by hereditable defects in DNA mismatch repair genes. However, the fraction of HNPCC due to defects in any one repair gene and the nature of these mutations are not known. We analyzed 29 HNPCC kindreds for mutations in the prototype DNA mismatch repair gene *hMSH2* by a combination of linkage analysis, polymerase chain reaction-based screening, and sequencing of the coding region. The complete intron/exon structure of the gene was ascertained to facilitate this analysis. The results suggest that at least 40% of classic HNPCC kindreds are associated with germline mutations in *hMSH2* and that most of these mutations produce drastic alterations in the predicted protein product.

Introduction

HNPCC[3] was one of the first familial cancer syndromes described in the biomedical literature (1) but its molecular pathogenesis has only recently become clear. HNPCC appears to be due to hereditary defects in DNA mismatch repair (2–7). In prokaryotes and *Saccharomyces cerevisiae*, the products of the DNA mismatch repair genes *mutS* and *mutL* participate in the recognition and correction of mismatched base pairs resulting from replication errors (reviewed in Ref. 8). These genes have been highly conserved through evolution; five human genes homologous to those responsible for mismatch repair in unicellular organisms have been discovered (2–7, 9, 10). Two of the human genes (*hMSH2* and *DUG*) are related to *mutS*, while the other three (*hMLH1*, *hPMS1*, *hPMS2*) are homologous to *mutL*. Mutations in *hMSH2* or *hMLH1* have been identified in selected HNPCC kindreds.

Cancers that arise in HNPCC patients are genetically unstable (5, 11). It has been shown that nearly all cancers from HNPCC patients (12–14), as well as 12–18% of sporadic colorectal cancers (15–17) and a variable fraction of other tumor types (18–23), have accumulated multiple mutations at repeated sequences distributed throughout their genome. Cell extracts from the tumors of HNPCC patients lack biochemically definable DNA mismatch repair capacity *in vitro*, while normal cells from HNPCC patients are mismatch repair proficient (5). On the basis of the biochemical and genetic analyses, it has been suggested that the wild-type allele of the relevant mismatch repair gene is inactivated during tumor formation, thereby increasing the mutation rate and accelerating tumor progression (4). This scenario is formally equivalent to that proposed by Knudson (24) to explain tumorigenesis associated with inherited mutations of tumor suppressor genes.

Although it is currently believed that all HNPCC patients have hereditary defects in DNA mismatch repair genes, the proportion of kindreds with mutations in any specific gene is not known. Presumably, mutations in any of the DNA mismatch repair genes could lead to HNPCC. Information on the nature and number of these mutations is critical for designing effective strategies to detect the mutations in families with HNPCC and to provide appropriate genetic counseling. In this study, we have evaluated a cohort of classically defined HNPCC kindreds to determine the prevalence of mutations in the prototype human DNA mismatch repair gene *hMSH2*. Our results indicate that at least 40% of classic HNPCC kindreds are caused by mutations in this single gene.

Materials and Methods

Samples. Lymphocytes were obtained from one or more affected members in each kindred. The member(s) chosen for analysis included at least one who was under 50 years of age when diagnosed with colorectal cancer. Two families were from Finland, one was from New Zealand, and the remainder were from North America. RNA and DNA were purified from the fresh lymphocytes or from Epstein-Barr virus-transformed lymphoblastoid cell lines as described previously (25, 26).

Analysis of cDNA. cDNA was generated using random hexamers and RT as described (27). The PCR was used to amplify the *hMSH2* transcript in two overlapping fragments. Fragment A contained codons 1–628 and fragment B contained codons 250–934. PCR was performed using 35 cycles of 95°C (30 s), 58°C (1 min) and 70°C (2 min, 30 s) in the buffer described by Sidransky *et al.* (28). The primers used for RT-PCR included signals for transcription by T7 polymerase and *in vitro* translation at their 5′ ends [codons 1–628 (fragment A): 5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGCGGTGCAGCCGAAGG-3′[4] and 5′-CCTTTCTCCAAAATGGCTGG-3′; codons 250–934 (fragment B): 5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGGAGAGCAGATGAATAGTGCTG-3′[4] and 5′-GCTTATCAATATTACCTTCATTCCATTACTGGG-3′]. Thus, the RT-PCR products could be transcribed and translated *in vitro*, as described by Powell *et al.* (27), to search for the presence of mutations which resulted in an altered size of the encoded polypeptide. Controls for RT-PCR included a cDNA sample processed identically except for the omission of RT.

Intron-Exon Borders. The sequences of most intron-exon junctions were ascertained by direct sequencing of the P1 genomic clone M1015 (4) using primers chosen from the cDNA sequence (4). M1015 contained all *hMSH2* exons except exon 1. A P1 clone containing the first exon was obtained by screening a human P1 library (Genome Systems, Inc.) with a PCR product corresponding to nucleotides 12–272 of the cDNA. This clone was then used

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[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; IVSP, *in vitro* synthesized protein; PCR, polymerase chain reaction; RT, reverse transcriptase; cDNA, complementary DNA.

[4] These primers have sequences required for transcription and translation at their 5′ ends.

Table 1 *Primers used for sequencing of RT-PCR products*

Direction[a]	Codon[b]	Sequence
S	9	5′-CTGCAGTTGGAGAGCGCG-3′
AS	74	5′-TCTTTGCTCCTGCCGGCC-3′
S	73	5′-GAATCTGCAGAGTGTTGTGC-3′
S	125	5′-CCTGGCAATCTCTCTCAG-3′
S	190	5′-TCCTCATCCAGATTGGACC-3′
S	252	5′-GATGAATAGTGCTGTATTGCC-3′
S	291	5′-TGACTACTTTTGACTTCAGCC-3′
S	351	5′-CGGATAAGAACAGAATAGAGG-3′
S	404	5′-GTTACCGACTCTATCAGGG-3′
S	460	5′-GGATCAGGTGGAAAACCATG-3′
S	513	5′-CTGGATTCCAGTGCACAG-3′
S	571	5′-GAAGAAGCCCAGGATGCC-3′
S	622	5′-CCAGCCATTTTGGAGAAAGG-3′
S	684	5′-GTGATAGTACTCATGGCCC-3′
S	718	5′-AATTGAAAGGAGTCTCCACG-3′
S	769	5′-ATTGCAACAAAGATTGGTGC-3′
S	811	5′-AACTATGCTTTATCAGGTGA-3′
S	869	5′-AGCAAAGAAGTGCTATCTGG-3′

[a] S, sense; AS, antisense.
[b] Corresponds to codon at 5′ end of each primer.

to define the donor site at the 5′ end of intron 1. In selected patients, the sequence of the exons and flanking intronic sequences were amplified by PCR using the primers described in Table 3.

Sequencing. Sequencing of RT-PCR products, P1 clones, and genomic PCR products was performed with SequiTherm Polymerase (Epicentre Technologies, Madison, WI), using end-labeled primers and the conditions specified by the manufacturer. The primers used to determine the sequence of the RT-PCR products are listed in Table 1. All mutations were confirmed by sequencing of independent PCR products from affected patients or their relatives.

Results

All families chosen for analysis met the standard criteria for HNPCC (29) (*i.e.*, at least three first-degree family members in two generations were affected with colon cancer, at least one patient being under 50 years of age). The involvement of *hMSH2* in these families was ascertained by the following experimental strategy. (*a*) Families sufficiently large for linkage analysis were studied with a variety of polymorphic markers surrounding *hMSH2* on chromosome 2p16. (*b*) Families in whom linkage to *hMSH2* could not be excluded were evaluated for *hMSH2* mutations that altered the size of the encoded polypeptide. In this analysis, lymphocyte RNA was used as a template for RT-PCR and the PCR product was used to generate IVSP. When a shortened *hMSH2* polypeptide was identified in this assay, the sequence of the relevant region of the cDNA was determined to find the responsible mutation. (*c*) When no truncated protein was observed in the IVSP assay, the sequence of the entire coding region was determined by direct sequencing of the RT-PCR products. (*d*) Mutations were verified by sequencing of genomic PCR products.

The results of this strategy were as follows: (*a*) Of the 29 families available, linkage analysis was informative in 10 (30–32). Six of the kindreds were shown to be linked to markers surrounding *hMSH2* on chromosome 2p16, while linkage was excluded in four families. In the other 19 kindreds, too few individuals were available to make definitive conclusions. (*b* and *c*) Mutations of *hMSH2* were sought in the 6 kindreds linked to chromosome 2p markers and in the 19 kindreds in which chromosome 2p linkage could not be excluded. Ten kindreds with *hMSH2* alterations were identified, including seven which have not been reported previously (Table 2). Of these 10, 9 resulted in truncated proteins which could be detected by the IVSP assay (exam-

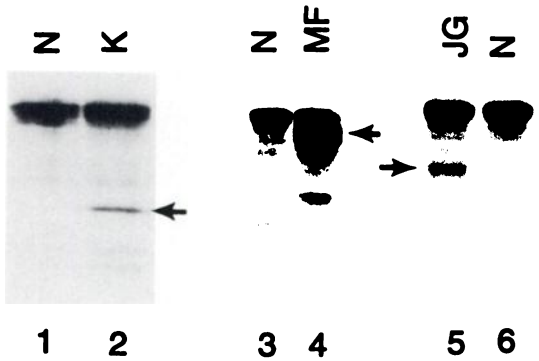

Fig. 1. IVSP Assays on RT-PCR products from HNPCC patients. *In vitro* transcription and translation of RT-PCR products were carried out as described in "Materials and Methods" and the resultant labeled proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel. The products shown were obtained from normal (*N*) individuals (*Lanes 1, 3,* and *6*) or from affected members of kindreds 3106 (*K*; *Lane 2*), MF (*Lane 4*), or JG (*Lane 5*). *Arrows*, truncated proteins (*Lane 2*, M_r 49,000; *Lane 4*, M_r 70,000; *Lane 5*, M_r 60,000). The normal size of these proteins is M_r 69,000 in *Lane 1* (fragment A) and M_r 75,000 in *Lanes 3* and *6* (fragment B); see "Materials and Methods."

Table 2 *hMSH2 mutations in HNPCC kindreds*

Kindred	Exons affected	Codons affected	cDNA change	Genomic DNA change	Predicted protein change
3106	8	458	TTA TO TGA	TTA TO TGA	Truncation at codon 458
MF	5	265–314	Exon 5 Deleted	GTA to GTT at splice donor	In-frame deletion of codons 265–314
C[a,b]	5	265–314	Exon 5 Deleted	GTA to GTT at splice donor	In-frame deletion of codons 265–314
RB	5	265–314	Exon 5 Deleted	GTA to GTT at splice donor	In-frame deletion of codons 265–314
JG	15	820–878	Exon 15 Deleted	GTT to ATT at splice donor	Out of frame deletion of codons 820–878, creating a new termination codon 6 bp[c] downstream of the splice site
JV	12	638–669	nt 1981–2073 deleted	CAT to TAT at codon 639 creating new splice donor site	Out of frame deletion of codons 638–669, creating a new termination codon 17 bp downstream of the splice site
TM	13	669–737	Exon 13 deleted	Not identified	Out of frame deletion of codons 668–736, creating a new termination codon 22 bp downstream of the splice site
DH	8–15	426–878	Exons 8–15 deleted	Not identified	Out of frame deletion of codons 426–878, creating a new termination codon 6 bp downstream of the splice site
J[a]	12	622	CCA to CTA	CCA to CTA	Proline to leucine
8[a]	7	406	CGA to TGA	CGA to TGA	Truncation at codon 406

[a] cDNA or genomic DNA change previously reported (Ref. 4).
[b] This missense mutation perfectly segregated with disease in 21 members of the kindred (Ref. 4).
[c] bp, base pairs; nt, nucleotides.

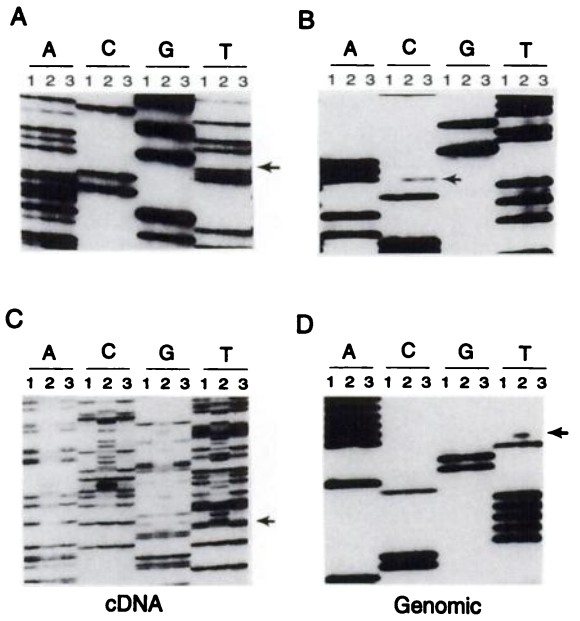

Fig. 2. Sequence analysis of *hMSH2* mutations. RT-PCR products (*A* and *C*) and genomic PCR products (*B* and *D*) were used as templates for direct sequencing as described in "Materials and Methods." The dideoxyadenosine triphosphate mixes from each sequencing reaction were loaded in adjacent lanes to facilitate comparison, as were those for the other nucleotides. In *A* (shown as sense sequence), the T to G change at codon 458 (*arrow*) is barely visible in the cDNA from two affected members of family 3106 (*Lanes 2* and *3*; *Lane 1*, normal) but this change is clearly visible in the genomic PCR product (*B*) (*Lanes 2* and *3*, shown as antisense sequence). A deletion of exon 5 is exhibited in the cDNA of kindred MF (*C*) (*Lane 2*, beginning at *arrow*; *Lanes 1* and *3*, normal). The genomic PCR product from the same patient (*D*) (*Lane 2*, *arrow*) shows the A to T transversion at the splice donor site that caused a deletion in the transcript.

ples in Fig. 1). The IVSP assay can detect truncated proteins even when present at low abundance (27). This sensitivity was important because some of the transcripts encoding truncated proteins were apparently unstable. The best example of this phenomenon was provided by kindred 3106. The presence of a truncated peptide in the RT-PCR products from affected members of this kindred was clear (Fig. 1, *Lane 2*). Sequence of the resultant cDNA, however, revealed only a very faint band corresponding to a termination codon at residue 458 (TTA to TGA) (Fig. 2*A*, *Lanes 2* and *3*). It is known that mRNA encoding truncated proteins is often unstable (33, 34). (*d*) To confirm and extend the results of the RT-PCR analysis, appropriate exons from genomic DNA were amplified and the products were sequenced in the 10 cases with abnormal cDNA. To enable this genomic analysis, the intron-exon structure of the *hMSH2* gene was determined by sequencing P1 clones of normal human DNA. The coding region of *hMSH2* was thereby found to be divided into 16 exons, each with canonical splice acceptor and donor sites. The sequences of the intron-exon borders are listed in Table 3.

Of the 10 cases with cDNA changes, genomic mutations could be identified in 8. In kindred 3106, for example, a termination codon at residue 458 was clearly observed (Fig. 2*B*, *Lanes 2* and *3*). This contrasted with the low intensity of the signal from the RNA product of the mutant allele, as noted above (Fig. 2*A*). In families C, RB, and MF, the same mutation within the splice donor site at the 3′ end of exon 5 was observed, resulting in the deletion of exon 5 from the transcript (Table 2). There was no obvious geographic or ethnic relationship between these three kindreds but we could not exclude shared ancestry. In kindred JG, a similar splice donor mutation (GTT to ATT) at the 3′ end of exon 15 was observed, resulting in the deletion of exon 15 in the transcript. In kindred JV, a new donor site was created within exon 12, resulting in the joining of the middle of exon 12 to the splice acceptor site at the 5′ end of exon 13. In 2 of the 10 kindreds with abnormal cDNA sequences (TM and DH), we could not identify the genomic changes responsible for the cDNA abnormalities from sequencing of the exons and intron-exon borders. In both of these cases, the changes in the transcript were deletions. It is likely that both of these kindreds had relatively large intragenic deletions within *hMSH2*. Kindred TM probably had a deletion which included exon 13 and its surrounding intronic sequences, while kindred DH was likely to have a large deletion encompassing exons 8–15.[5]

Discussion

In the 29 kindreds analyzed, 12 (41%) had evidence for involvement of *hMSH2*. In 10, sequence analysis at the genomic or cDNA levels revealed an alteration of the *hMSH2* gene product. In each of those 10, the predicted protein change was substantial, resulting in a large deletion or truncation of the encoded protein (9 cases) or missense mutation in a highly conserved residue (1 case). In two additional families, statistically significant evidence for linkage to *hMSH2* was observed, but mutations in the *hMSH2* gene could not be found.

These results have substantial implications for genetic diagnosis of HNPCC, especially in relationship to two questions. What is the fraction of HNPCC caused by mutations in *hMSH2* and how can these mutations best be detected? In regard to the first question, our data suggest that a minimum of 41% of classic HNPCC kindreds are associated with *hMSH2* mutations. The methods we used to screen for mutations would not detect several kinds of mutations that could inactivate the *hMSH2* gene. For example, we would not have detected mutations in the promoter, intronic, or 3′-untranslated regions of the *hMSH2* gene that resulted in reduced transcription, nor mutations in the coding region that resulted in very unstable transcripts. This point was emphasized by the fact that in two of the six kindreds tightly linked to the *hMSH2* locus, we could not identify a mutation in *hMSH2*, despite sequencing of the entire coding region. Taking our imperfect methods into account and ignoring the possibility that there is another causative gene located close to *hMSH2*, our data suggest that approximately 50% of classic HNPCC is caused by mutations of this gene. This estimate is consistent with earlier estimates based solely on linkage analysis (12, 31). However, it is difficult to select HNPCC families in a totally unbiased fashion, so larger studies will be needed to confirm this estimate.

The second question concerns the most efficacious way to test for *hMSH2* mutations in probands of kindreds with HNPCC. It is apparent from Table 2 that there are few mutational "hot spots," although three kindreds shared the same mutation. Additionally, the type of mutation seemed to bear little relationship to disease phenotype. Kindreds 3106 and RB, for example, had skin tumors characteristic of the Muir-Torre form of HNPCC, shown previously to be linked to chromosome 2 and associated with microsatellite instability (35, 36), while other kindreds with similar mutations of *hMSH2* had no evidence of such skin lesions. It would also appear that evaluation by genomic PCR analysis will not be efficient, when used alone, for detecting mutations in this gene. In kindreds DH and TM, for example, clear alterations of the transcripts were observed on RT-PCR analysis (Table 2) but no mutations could be observed in the genomic DNA. One possible explanation may be that there is large intragenic deletions which included sequences required for amplification of the mutant alleles. Our current strategy for detecting *hMSH2* mutations is therefore based on RT-PCR analysis. We first screen these products using the IVSP assay. Nine of the 10 germline mutations of *hMSH2* in HNPCC

[5] B. Liu, R. E. Parsons, K. W. Kinzler, and B. Vogelstein, unpublished data.

Table 3 *Intron-Exon borders*

Exon	Exon size (base pairs)	Codon[a]	Sequence[b]
1[c]	>279	1–71	5′-GGCGGGAAACAGCTTAGTGGGTGTGGGGTCGCGCATTTTCTTCAACCAGGAGGTGAGGAGGTTTCGACATG ....CAAGTACATGGGGCCGGCAGgtgagggccgggacggcgcgtgctggggagggacnngggccttgtggcgcggctcctttccc-3′
2	155	71–122	5′-cttgaacatgtaatatctcaaatctgtaatgtactttttttttttttGAGCAAAGAATCTGCAGAGT ....ATTGGTATTTGGCATATAAGgtaattatcttccttttaatttacttatttttttaagagtagaaaaata-3′
3	279	123–215	5′-aaagtatgttcaagagtttgttaaatttttaaaatttttatttttacttagGCTTCTCCTGGCAATCTCTC ....ACATGGGGAAACTGAGACAGgtaagcaaattgagtctagtgataggaggattccaggcctaggaaaggc-3′
4	147	216–264	5′-ttttcttattccttttctcatagtagtttaaactatttctttcaaaatagATAATTCAAAGAGGAGGAAT ....TGCCAGAAATGGAGAATCAGgtacatggattataaatgtgaattacaatatatataatgtaaatatgtaa-3′
5	150	265–314	5′-actggcaccagtggctataggaaatcttcgatttttaaattctttaattttagGTTGCAGTTTCATCACTGTC ....GAGCCCT TAACCTTTTTCAGgtaaaaaaaaaaaaaaaaaaaaaaaaaaaaggggttaaaaatgttgaatggtt-3′
6	134	315–359	5′-ttttcactaatgagcttgccattctttctattttatttttttgtttactagGGTTCTGTTGAAGATACCAC ....AAGAACAGAATAGAGGAGAGgtatgttattagtttatactttcgttagttttatgtaacctgcagttacc-3′
7	200	359–426	5′-....ttttatactaaaatattttacattaattcaagttaatttatttcagATTGAATTTAGTGGAAGCTT ....GGCTCTGGAAAAACATGAAGgtaacaagtgattttgttttttttgttttccttcaactcatacaatatata-3′
8	110	426–462	5′-..ctgnaaaatgagatcttttttatttgtttgttttactacttttcttttagGAAAACACCAGAAATTATTG ....CAACTTTAGATATGGATCAGgtatgcaatatacttttttaatttaagcagtagttatttttaaaaagcaaa-3′
9	124	463–504	5′-ctgtctttacccattatttntaggattttgtcactttgttctgtttgcagGTGGAAAACCATGAATTCCT ....AAGTGCAGCCAGAGATCTTGgtaagaatgggtcattggaggttggaataattcttttgtcctatacac-3′
10	151	504–554	5′-tggtagtaggtatttatggaatacttttttcttttcttcttgattatcaagGCTTGGACCCTGGCAAACAG ....GGTGTTAAATTTACCAACAGgtttgcaagtcgttattatatttttaacccctttattaattccctaaatgc-3′
11	98	554–587	5′-tctagtacacattttaatatttttaataaaactgttatttcgatttgcagCAAATTGACTTCTTTAAATG ....AATTGTCAATATTTCTTCAGgtaaacttaatagaactaataatgttctgaatgtcacctggcttttggta-3′
12	246	587–669	5′-tttattattcagtattcctgtgtacanttttctgtttttatttttatacagGCTATGTAGAACCAATGCAG ....GATGTTCCACATCATTACTGgtaaaaaacctggtttttgggctttgtgggggtaacgttttgttttttttt-3′
13	205	669–737	5′-gtagcagaaagaagtttaaaacttgctttctgatataatttgttttgtagGCCCCAATATGGGAGGTAAA ....GAAACTGCTTCTATCCTCAGgtaagtgcatctcctagtcccttgaagatagaaatgtatgtctctgtcct-3′
14	248	737–820	5′-tgttaccacattttatgtgatgggaaatttcaggtaattatgtgcttcagGTCTGCAACCAAAGATTCAT ....GCTTTATCAGGTGAAGAAAGgtatgtactattggagtactctaaattcagtanctttggtaatgggaaact-3′
15	176	820–878	5′-tgctgtctcttctcatgctgtcccctcacgcttccccaaatttcttatagGTGTCTGTGATCAAAGTTTT ....AGTGCTATCTGGAAAGAGAGgtttgtcagtttgttttcatagtttaacttagcttctctattattacata-3′
16[d]	>284	879–934	5′-ttttanaaaaganattttaattactaatgggacattcacatgtgtttcagCAAGGTGAAAAAATTATTCA ...TGAAAAATCCCAGTAATGGAATGAAGGTAATATTGATAACCTATTGTCTGTAATAGTTTTATATTGTTTTATATTAACCCTT TTTCC ATAGTGTTAACTGTCAGTGCCCATGGGCTATCAACTTAACATAGTGTTAACTGTCAGTGCCCATGGGCTATCAACTTAA-3′

[a] No sequence variations were detected in the samples analyzed other than the mutations listed in Table 2 and a G1A polymorphism of the second position of codon 322.
[b] Upper case letters correspond to exons, lower case to introns.
[c] Includes 69 base pairs of untranslated sequences upstream of the initiating ATG (underlined).
[d] Includes untranslated sequences downstream of the stop codon (underlined).

kindreds thus far observed (Table 2) can be detected by this assay. If a presumptive mutation resulting in an altered size of the polypeptide produced from the RT products cannot be identified, we then recommend single stranded conformation polymorphism, denaturing gradient gel electrophoresis, or direct sequence analysis of either the RT-PCR products or genomic PCR products to detect missense mutations.

In future experiments, we will examine the same cohort of HNPCC kindreds to determine the incidence of mutations in other mismatch repair genes. This should eventually give us a more complete picture of the mutational spectrum in this disease. HNPCC is a relatively common cause of hereditary cancer in western populations, affecting as many as 1 in 200 individuals. Therefore, the study of mismatch repair gene mutations has significant ramifications for the management of the many families afflicted with this disease.

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