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Unbalanced Germ-Line Expression of *hMLH1* and *hMSH2* Alleles in Hereditary Nonpolyposis Colorectal Cancer[1]

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Abstract

We analyzed the *hMLH1* and *hMSH2* genes in 30 unrelated hereditary nonpolyposis colorectal cancer (HNPCC) patients using mutational and immunohistochemical analyses combined whenever possible with primer extension assays, designed to estimate *hMLH1* and *hMSH2* transcript expression in peripheral blood lymphocytes. Single-strand conformational polymorphism screening and PCR-direct sequencing revealed seven *hMLH1* and five *hMSH2* sequence variants in 14 unrelated HNPCC patients, including three definite pathogenic mutations, four amino acid substitutions of uncertain pathogenic significance, and five polymorphisms. Immunohistochemistry indicated the lack of either hMLH1 or hMSH2 protein expression in tumors from 13 patients, and the absence of both hMLH1 and hMSH2 immunostaining was observed in the tumor from one additional case. The lack of hMLH1 or hMSH2 immunostaining was associated with the presence of microsatellite instability in the corresponding tumor and was also observed in tumors from patients negative for pathogenic mutations by mutational screening. There was a marked unbalance in the allelic expression of either *hMLH1* or *hMSH2* transcripts in three of eight unrelated HNPCC patients that could be analyzed, although a less marked unbalance was detected in two additional patients. Tumors from patients with germ-line unbalance in *hMLH1* or *hMSH2* transcript expression did not express the corresponding mismatch repair protein and displayed microsatellite instability. Our results indicate that constitutional alterations in *hMLH1* and *hMSH2* transcript expression may represent genetic markers for HNPCC carrier status also in cases in which mutational analysis did not detect a definite pathogenic variant. This suggests that transcript deregulation may represent a relevant mode of germ-line inactivation for mismatch repair genes.

Introduction

HNPCC[4] is a genetically heterogeneous disorder that is believed to account for 2–10% of all cases of colorectal cancer and that may be caused by germ-line mutations of at least five genes of the DNA MMR system, including *hMLH1*, *hMSH2*, *hMSH6*, *hPMS1*, and *hPMS2* (1–5). Adding to the genetic complexity of the disease, constitutional mutations in the transforming growth factor β type II receptor gene may result in a superimposable disease phenotype (6). Mutations of the *hMLH1* and *hMSH2* genes seem to be most frequently responsible for HNPCC (7). However, the task of identifying pathogenic mutations in *hMLH1* or *hMSH2* is complicated by the fact that most of the alterations reported in these genes consist in point mutations scattered throughout the coding sequence (7). Moreover, these mutations include a high percentage of missense variants of uncertain pathogenic significance (7, 8), the assessment of which may require the development of specific functional assays (9). In some of the HNPCC cases with undetectable MMR gene mutations, immunohistochemical analyses of colorectal tumors evidenced the loss of the hMLH1 or the hMSH2 protein (10), which suggested an assignment to the *hMLH1 or hMSH2* genes, despite the negative results of mutational analysis. Because mutations were not detected even by sequencing of the entire coding region and of flanking intron-exon borders (10), it is conceivable that, at least in some cases, pathogenic mutations may be located in noncoding regions of the genes. This possibility adds a further level of complexity to the design of efficient strategies for the molecular diagnosis of HNPCC.

Recent studies indicated that somatic events leading to reduced *hMLH1* expression may play a pathogenic role in MMR-defective tumors (11, 12, 13), but the possibility that altered levels of MMR gene transcripts may also be present in the germ-line of HNPCC patients has not yet been evaluated. We analyzed the *hMLH1* and *hMSH2* genes in 30 Italian HNPCC families using complementary techniques based on mutational and immunohistochemical analyses, combined whenever possible with the estimate of *hMLH1* and *hMSH2* transcript expression in PBLs. The analysis of MIN was also performed to assess the presence of MMR deficiency in tumors. Remarkably, this approach allowed the identification of germ-line alterations of *hMLH1* or *hMSH2* transcript expression, associated with the loss of the corresponding protein in tumors and with MIN, in cases in which definite pathogenic mutations could not be identified.

Materials and Methods

Patients. Thirty unrelated, cancer-affected HNPCC patients were identified from the files of the Institute of Pathology and of the Department of Clinical Physiopathology of the University of Florence and from the Register of the Regina Elena Cancer Institute, Rome, Italy. Seventeen patients derived from AC+ and 13 from AC− families. The research protocol was approved by the ethical review board of the University Gabriele D'Annunzio, and informed consent was obtained from all of the subjects who participated in the study.

Nucleic Acid Extraction and cDNA Preparation. gDNA was isolated from whole fresh blood using the QIAmp Blood Kit 50 (Qiagen Inc., Chatswort, CA). Total RNA from PBLs was isolated following the acid guanidinium isothiocyanate-phenol-chloroform extraction method (14), and cDNA was prepared by incubating DNase-treated total RNA (5 μg) with 300 units of Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in the presence of random hexamers and RNase inhibitor (Perkin-Elmer, Branchburg, NJ). To obtain specific amplification of reverse-transcribed

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[4] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair; PBL, peripheral blood lymphocyte; AC, Amsterdam criteria; gDNA, genomic DNA; ddNTP, 2′3′-dideoxynucleoside-5′triphosphate; 3′UTR, 3′ untranslated region; MIN, microsatellite instability; SSCP, single-strand conformational polymorphism.

mRNA, cDNAs were amplified using primer pairs directed to sequences located in two different exons.

SSCP and Sequencing. PCR-SSCP analysis was performed as described (15, 16). PCR products corresponding to samples showing unique SSCP conformers were directly sequenced using the Sequenase II sequencing kit (Amersham Life Sciences, Cleveland, OH). Sequence variants were always confirmed using independent DNA preparations. Screening of the two genes was also completed in cases in which a putative pathogenic variant had been identified.

Immunohistochemistry. Immunohistochemical assays were performed in 24 unrelated patients with available paraffin-embedded tumor specimens. Immunoperoxidase staining for the hMLH1 and hMSH2 proteins was performed on 5 μm-thick paraffin-embedded sections mounted on silane-coated slides and dried at 56°C for 30 min. After dewaxing and blocking endogenous peroxidase, sections were rinsed in water and then placed in 10 mM sodium citrate buffer (pH 6.0). The sections were pretreated by microwave at 750 W for 20 min, washed, transferred to PBS, and incubated for 20 min at room temperature with mouse monoclonal antibody against the hMSH2 protein (1 μg/ml; clone FE11, Oncogene Science, Cambridge, MA). In parallel experiments, immunostaining for the hMLH1 protein was performed by applying the specific mouse monoclonal antibody (10 μg/ml; clone 14, Oncogene Science) for 45 min at room temperature. Antigen-bound primary antibody was detected using a standard streptavidin-biotin assay. Sections were lightly counterstained with hematoxylin. In each case, normal tissue adjacent to the tumor was used as an internal control. Sections of normal colonic mucosa from an unrelated non-HNPCC patient affected with diverticulosis and sections without primary antibody were always included as positive and negative controls, respectively. In the case of patient GDLV-52#II-2, affected with mammary carcinoma, the assays were conducted on sections representative of mammary cancer and of normal mammary tissue. Slides were reviewed by the same pathologist (L. M.), who had no knowledge of the results of molecular analyses. Only nuclear immunostaining was considered as positive.

Microsatellite Analysis. Microsatellite analysis could be performed in the cases with available immunohistochemistry, with the exception of cases GDLG-32#IV-5 and GDLM-10#III-3. Paraffin-embedded sections were collected on microscope slides. Areas representative of tumor and of normal tissue (*muscularis propria* and/or microscopically normal colonic mucosa) were identified within single deparaffinized sections lightly counterstained with hematoxylin and microdissected into 1.5-ml polypropilene vials, using a H&E-stained step section from the same block as a guide. DNA extractions and microsatellite typings were performed as reported previously (17). DNA extracted from blood of the same patient was used as an additional control for the evaluation of constitutional microsatellite allele pattern. As a first step, we analyzed three microsatellite loci: *D2S123*, *D3S1611*, and *BAT-26*. Cases with no instability at these loci or with instability at a single locus were further analyzed at up to four additional loci (*D9S145*, *D1S158*, *SCZD1*, and *D11S905)*. Paired genotypings positive for microsatellite alterations were confirmed in duplicate or triplicate experiments performed using DNA derived from independent extractions. In the case of patient GDLG-26#II-4, the limited amount of the available biopsy tissue allowed duplicate analyses at only four loci. Typings were scored by three independent investigators (R. P., R. M-C., A. C.) in a blind fashion. Cases were considered MIN-positive when instability was present at $\geq$2 loci.

Primer Extension Assay. To quantitate the relative expression of transcripts in patients heterozygous for nucleotide substitutions, we used a previously described primer extension protocol (18, 19). This method is based on the incorporation of a single ddNTP that is selected to allow the differential extension of an end-labeled primer annealed next to a polymorphic nucleotide marker. The sequence of primers designed for the relative quantitation of each polymorphic allele is available from the authors upon request. Ten pmoles of each primer were 5′-phosphorylated with [γ^{32}P]ATP in the presence of T4 polynucleotide kinase (Amersham Life Science, Cleveland, OH) and purified through H_2O-equilibrated G-25 Sephadex Quick Spin Columns (Boehringer Mannheim, Indianapolis, IN). Primer extension assays were performed in parallel experiments using gDNA and cDNA templates (approximately 30 ng), treated with exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, OH) and annealed with the appropriate ^{32}P-oligonucleotide (0.8 pmol). Reactions (6 μl) were performed at 37°C for 10 min in a buffer containing: 36 mM Tris-HCl (pH 7.5), 18 mM $MgCl_2$, 45 mM NaCl, 3 mM DTT, 0.6 units of Sequenase Version 2.0 DNA polymerase (USB, Cleveland, OH), 300 μM of the appropriate ddNTP and 120 μM of the other three 2′-deoxynucleoside-5′triphosphates. Thereafter, reactions were heat-denatured in a buffer containing formamide (38%) and were electrophoresed through a sequencing gel. The relative expression of transcripts marked by a three bp deletion in *hMLH1* was evaluated using a simplified primer extension protocol, as previously described (20). All of the experiments were confirmed using two independent RNA extractions. The radioactive signals corresponding to each allele were analyzed using the Molecular Imager system (BIO-RAD, Hercules, CA). Relative transcript expression was estimated by comparing the ratio between the signals corresponding to the two alleles using cDNA as primer extension template. This ratio was normalized by the corresponding ratio obtained using gDNA as a template. A 100% expression was arbitrarily assigned to the allele showing higher level of expression.

Results and Discussion

We screened the coding sequence and flanking intron-exon borders of the *hMLH1* and *hMSH2* genes in a series of 30 unrelated HNPCC patients using SSCP followed by PCR-direct sequencing. Twelve different sequence variants of either *hMLH1* or *hMSH2* were detected in 14 unrelated patients (Table 1). The novel deletion of 1 bp at codon 318 in *hMLH1* and the a→t transversion at nucleotide +3 in intron 5 of *hMSH2* (4, 21) seem to have a clear pathogenic role because of their predicted effect on the protein. Moreover, the in-frame deletion of Lys^{618} in *hMLH1* was reported as pathogenic (4, 22, 23) and the loss-of-function of the corresponding protein was demonstrated in yeast (9). Three additional variants, including the novel amino acid substitutions, respectively, at codon 364 of *hMLH1* and at codon 342 of *hMSH2*, and the previously reported amino acid variant at codon 326 of *hMLH1* (4, 7) did not seem to be common polymorphisms inasmuch as they were not detected in the control population (Table 1). An analysis of segregation with disease was not informative for these variants. The amino acid substitution at codon 326 of the *hMLH1* gene had been previously tested in functional assays that provided evidence for a normal function in yeast (9). The amino acid substitution at codon 322 of the *hMSH2* gene was reported in other studies either as a pathogenic mutation (24) or as a polymorphism (25). Of the other sequence variants, the substitution of Val^{219} with Ile and a previously reported 3-bp deletion in the 3′UTR of the *hMLH1* gene seem to be relatively common polymorphisms based on their frequency in the control population (Table 1; Refs. 7, 20). Three other variants at codon 234 of the *hMLH1* gene and at codons 328 and 579 of the *hMSH2* gene are not predicted to result in amino acid substitutions.

To obtain further evidence regarding the potential pathogenic role of the *hMLH1* and *hMSH2* genes in our series of HNPCC patients, we analyzed the expression of the corresponding MMR proteins by immunohistochemistry on paraffin-embedded tumors that could be retrieved for 24 unrelated cases. Examples of immunohistochemical analyses are shown in Fig. 1. In 10 unrelated patients with negative results at mutational screening, immunohistochemical analysis did not provide evidence for the tumor-associated loss of hMLH1 or hMSH2 protein expression (Table 1), which suggests that other genes may have played a pathogenic role. Lack of hMLH1 immunohistochemical staining was observed in tumors from five patients, and tumors from eight patients were negative for hMSH2 immunostaining (Table 1). The absence of hMLH1 and hMSH2 immunostaining was observed in the tumor from one additional patient (Table 1).

To verify whether the lack of hMLH1 or hMSH2 immunostaining in tumors was associated with MMR deficiency, we analyzed microsatellite status on microdissected sections of the same tumors *versus* paired normal tissue. In all of the cases analyzed, the lack of either hMLH1 or hMSH2 immunohistochemical expression in tumors corresponded to the presence of MIN (Table 1). MIN was also detected

Table 1 *Sequence variants, tumor immunostaining, and clinical characteristics of unrelated HNPCC patients*

Unrelated patients	Germ-line allele variants							Tumor analyzed	Tumor immunostaining[b]		Microsatellite instability[c]	Germ-line transcript unbalance		Age at diagnosis[e]	AC[f]
	hMLH1		*hMSH2*		Nucleotide change	Consequence	Allele freq.[a]		hMLH1	hMSH2		Gene	Unbalance[d]		
	Exon	Codon	Exon	Codon											
GDLM-1#III-1								Co Ca[h]	−	+	2/3			<40	+
GDLM-2#III-1			intron 5	+3IVS	gta→gtt	del[g] exon 5		Co Ca	+	−	3/3			<50	+
GDLM-4#III-2								Co Ca	+	+	3/3			<50	+
GDLM-6#III-1								Co Ca	+	+	2/3			<50	+
GDLM-7#III-3								Co Ca[h]	−	−	2/3			<40	+
GDLM-8#II-1			6	328	GCC→GCT	Ala→Ala	0	Co Ca	+	−	3/3			<40	+
GDLM-9#II-2			6	322	GGC→GAC	Gly→Asp	0	Co Ca	+	−	2/3	*hMSH2*	7-fold	<50	+
GDLM-10#III-3								Co Ca	+	+				<50	+
GDLV-11#II-9	8	219			GTC→ATC	Val→Ile	0.06	Co Ad	−	+	3/7	*hMLH1*	None	<40	+
GDLV-12#II-2								Co Ca	+	+	2/3			<40	+
GDLV-13#III-2								Co Ca	+	+	0/6			<50	+
GDLV-14#II-1								Co Ca	+	+	2/3			<50	−
GDLG-15#III-2								Co Ca	+	+	0/6			<40	+
GDLG-18#III-19								Co Ca	+	+	3/3			<40	+
GDLG-19#III-8								Co Ca	+	−	2/3			<40	+
GDLG-20#II-1[i]	9	234			GAG→GAA	Glu→Glu	0	Co Ca	−	+	3/3	*hMLH1*	2-fold	<40	−
	3′UTR				TTC del		0.02					*hMLH1*	2-fold		
GDLG-21#III-2[i]	3′UTR				TTC del		0.02					*hMLH1*	None	>50	−
GDLG-22#III-1								Co Ca	+	+	3/3			<50	−
GDLG-23#II-2														<50	−
GDLG-26#II-4	11	326			GTG→GCG	Val→Ala	0	Co Ad	−	+	2/4	*hMLH1*	17-fold	<50	−
GDLG-27#IV-1								Co Ca	+	−	2/3			<40	+
GDLG-28#II-5														>50	−
GDLG-29#III-8	16	618			AAG del	Lys del								<50	−
GDLG-31#III-11	11	318			C del	Frame-shift								<50	−
GDLG-32#IV-5	8	219			GTC→ATC	Val→Ile	0.06	Co Ca	+	−		*hMLH1*	None	<40	−
GDLV-35#III-5			6	328	GCC→GCT	Ala→Ala	0	Co Ad	+	−	3/3			>50	−
GDLX-39#III-7			11	579	AAA→AAG	Lys→Lys	0							<40	−
GDLG-49#IV-2			6	342	GTT→ATT	Val→Ile	0	Co Ca	+	−	2/3	*hMSH2*	1.4-fold	<40	+
GDLV-50#I-1								Co Ca	+	+	0/6			>50	−
GDLV-52#II-2	12	364			ACA→GCA	Thr→Ala	0	Br Ca	−	+	3/7	*hMLH1*	94-fold	<40	+

[a] Allele frequencies were derived from the screening of 100 chromosomes from unrelated control individuals.
[b] Presence +, or absence − of immunostaining in tumors.
[c] Number of unstable loci/number of loci analyzed.
[d] Expressed as fold-unbalance between transcripts showing higher and lower levels of expression, respectively.
[e] Earliest age at colon cancer diagnosis in the family.
[f] Family fulfilling (+) or not fulfilling (−) Amsterdam diagnostic criteria for HNPCC.
[g] del, deleted.
[h] Co Ca, colon cancer; Co Ad, colon adenoma; Br Ca, breast cancer.
[i] Previously reported for the partial screening of the *hMLH1* gene (TTC del in the 3′UTR) as patients GDLG-20 No. 1 and GDLG-21 No. 7, respectively (20).

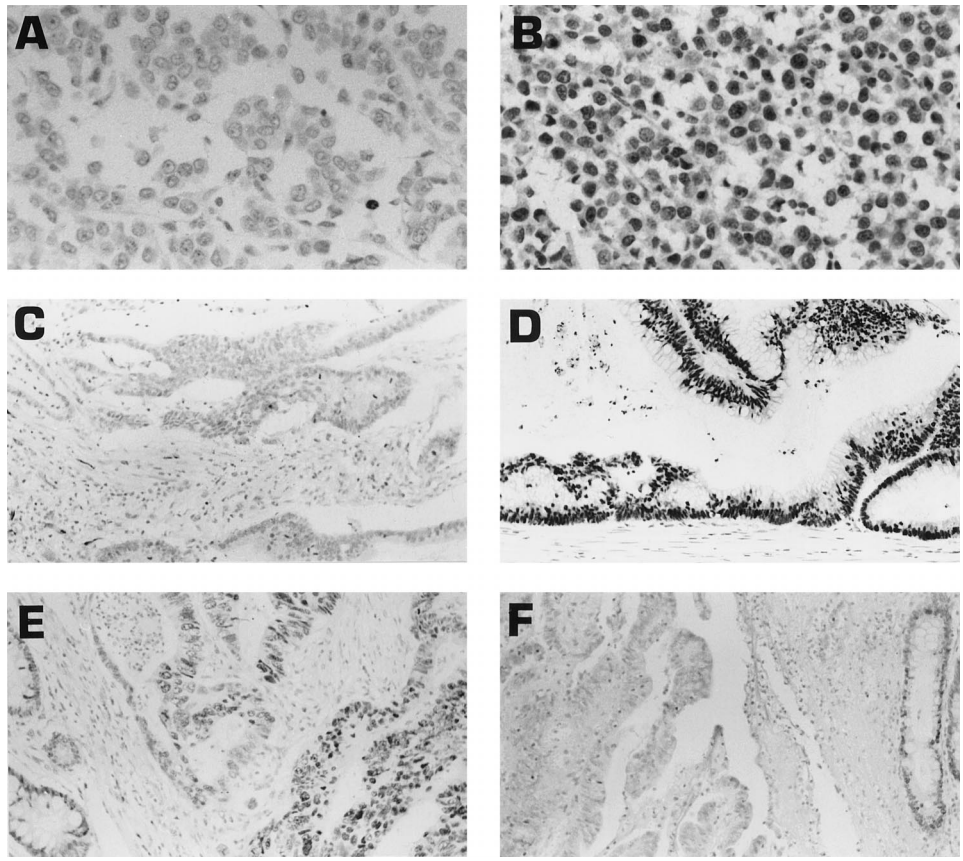

Fig. 1. Immunohistochemical analysis of hMLH1 and hMSH2 expression in three carcinomas from unrelated HNPCC patients. *A-B*, mammary tumor staining negative for hMLH1 (*A*) and positive for hMSH2 (*B*); *C-D*, colonic tumor staining negative for hMLH1 (*C*) and positive for hMSH2 (*D*); *E-F*, colonic tumor staining positive for hMLH1 (*E*) and negative for hMSH2 (*F*). In *F*, a positive-staining normal colonic crypt (*to the right*) serves as an internal positive control.

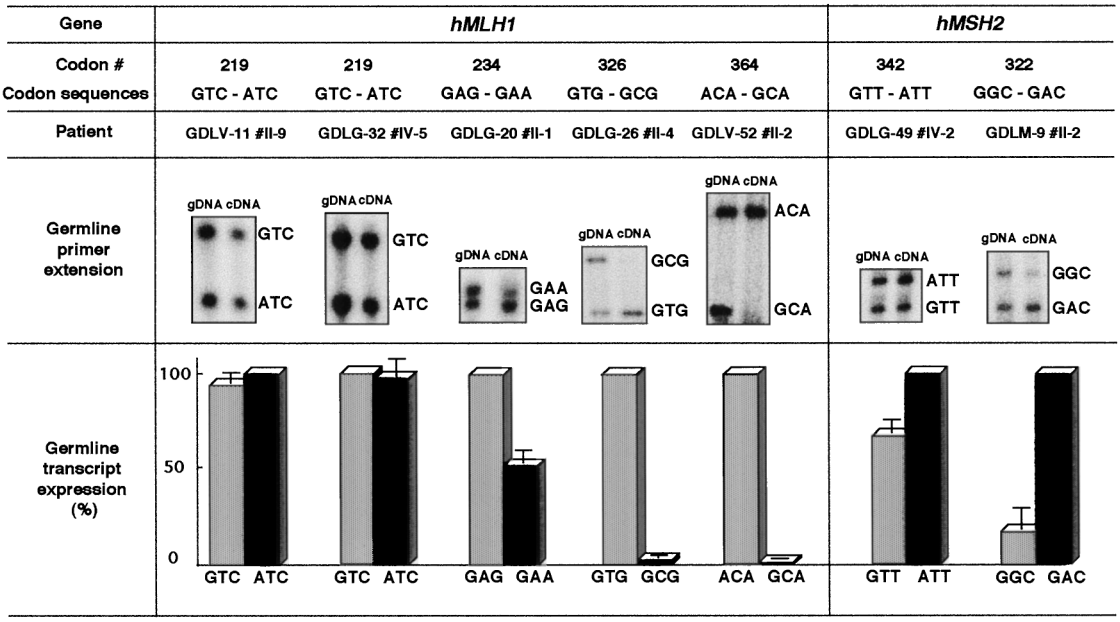

Fig. 2. Analysis of *hMLH1* or *hMSH2* transcripts by primer extension. Regions of *hMLH1* or *hMSH2* bearing polymorphic allele markers were amplified using matched cDNAs and gDNAs derived from PBLs. These templates were used for primer extensions in the presence of a ^{32}P-oligonucleotide and the appropriate ddNTP, as described in "Materials and Methods." *Top*, polymorphic codon sequences used as allele markers; *middle*, examples of primer extensions using gDNA and cDNA templates derived from patients GDLV-11#II-9, GDLG-32#IV-5, GDLG-20#II-1, GDLG-26#II-4, GDLV-52#II-2, GDLG-49#IV-2, and GDLM-9#II-2, respectively; *bottom*, histogram showing the relative expression of transcripts from alleles identified using polymorphic markers. Data represented are the mean ± SE of two to four independent determinations, obtained by comparing primer extension signals derived from gDNA and cDNA.

in the tumor from case GDLM-7#III-3, displaying a lack of both hMLH1 and hMSH2 immunohistochemical staining (Table 1). This is in agreement with another study that described the presence of MIN and the lack of both hMLH1 and hMSH2 immunostaining in a HNPCC-associated tumor (10).

Among cases with normal hMLH1 and hMSH2 immunostaining in tumors, three cases were MIN-negative and six cases were MIN-positive (Table 1). Our data confirm previous findings that indicated that MIN may also be present in cases without detectable mutations in *hMLH1* and *hMSH2* and with normal hMLH1 and hMSH2 immunohistochemical staining in tumors (10). These findings suggest that other genes affecting MMR function may play a pathogenic role in these MIN-positive familial cases, and that MMR-unrelated cancer predisposing genes may play a role in MIN-negative familial cases.

Notably, the lack of hMLH1 or hMSH2 immunostaining and MIN were observed in tumors from patients in whom a pathogenic mutation could not be identified by SSCP screening of the coding sequence (Table 1). This observation was consistent with data by Thibodeau *et al.* (10), which adopted a mutational screening strategy based on direct sequencing of the *hMLH1* and *hMSH2* coding regions and flanking intron-exon borders. According to the double-hit inactivation model, these observations suggest that, in some cases, tumor-associated loss of either the hMLH1 or the hMSH2 protein may be associated with the presence of germ-line mutations located outside the coding region and intron-exon borders. Such mutations have the potential to affect the transcription, processing, and/or stability of mRNA encoded by the corresponding allele, resulting in germ-line transcript unbalances that should be detectable in normal tissues or PBLs. Moreover, missense mutations or nucleotide variants that are not predicted to alter the amino acid sequence may also affect RNA processing and may result in altered germ-line expression of normal transcripts (26). To test whether germ-line transcript unbalances of MMR genes could represent a marker of HNPCC carrier status, the relative expression of *hMLH1* and *hMSH2* alleles was investigated by primer extension in cases in which heterozygous allelic markers and cDNAs from PBLs were available. These included six cases (GDLM-9#II-2, GDLV-11#II-9, GDLG-20#II-1, GDLG-26#II-4, GDLG-49#IV-2, and GDLV-52#II-2) in which the allele marker was located on the gene showing tumor-associated loss of expression by immunohistochemistry and one case (patient GDLG-32#IV-5), with a marker located on *hMLH1* but with the tumor-associated loss of hMSH2 protein expression (Table 1). One additional case in our series (GDLG-21#III-2), for which immunohistochemical analysis was not available, had been previously screened for *hMLH1* mutations and investigated by primer extension, but no abnormalities in transcript expression were detected (20). As shown in Table 1 and in Fig. 2, marked unbalances in germ-line allele expression were detected by primer extension in patients GDLM-9#II-2 *(hMSH2)*, GDLG-26#II-4 *(hMLH1)*, and GDLV-52#II-2 *(hMLH1)*. This was in agreement with the results of cDNA sequencing, which showed no detectable signal from the corresponding alleles of either *hMLH1* or *hMSH2* (data not shown). Using primer extension, a less marked unbalance in *hMLH1* or *hMSH2* germ-line transcript expression was detected in patients GDLG-20#II-1 and GDLG-49#IV-2, respectively (Table 1; Fig. 2). In these two cases, cDNA sequencing detected signals from both of the alleles, and the relative intensity of the corresponding bands was compatible with a modest transcript unbalance. In case GDLG-20#II-1, the presence of an approximately 2-fold transcript unbalance could be confirmed by primer extension using the additional allelic marker located in the 3′UTR of *hMLH1* (Table 1 and data not shown). The results derived from primer extension, cDNA sequencing, immunohistochemistry, and microsatellite analysis were concordant and indicated that the three cases with a marked germ-line unbalance in transcript expression (GDLM-9#II-2, GDLG-26#II-4, and GDLV-52#II-2) did not express detectable levels of the corresponding MMR protein in tumors and displayed MIN (Table 1; Fig. 2). The lack of hMLH1 or hMSH2 immunostaining in tumors, associated with MIN, was also observed in cases GDLG-20#II-1 and GDLG-49#IV-2, displaying a lower level of germ-line unbalance in allele expression of the corresponding MMR gene (Table 1; Fig. 2). The significance of these modest germ-line unbalances is less definite, and additional studies will be required to assess the role of small variations in transcript expression.

This study shows that data derived from germ-line allele expression analyses, tumor immunohistochemistry, and MIN may contribute to the assessment of the pathogenic role of *hMLH1* and *hMSH2* alleles, including alleles bearing missense variants of unclear pathogenic significance. In fact, our results indicate that the *hMLH1* missense variant at codon 326 (Table 1), previously reported either as pathogenic (4) or as not pathogenic (9), was associated with a markedly reduced expression of the corresponding allele in PBLs (Fig. 2). Therefore, in patient GDLG-26#II-4, this *hMLH1* allele seems to be pathogenic because of a significant germ-line unbalance in transcript expression, which does not contradict the results of functional assays in yeast demonstrating normal activity of the corresponding protein product (9). Conversely, the *hMSH2* missense variant at codon 322 (Table 1), whose pathogenic role is still debated (24, 25), was normally expressed (Fig. 2), which confirmed previous reports indicating that this *hMSH2* variant may represent a nonpathogenic polymorphism (25). Nevertheless, in patient GDLM-9 #II-2, the presence of the GGC→GAC nucleotide polymorphism at codon 322 of *hMSH2* allowed the detection of a marked unbalance in the germ-line expression of the allele bearing the GGC sequence at the same codon (Fig. 2). Because no sequence variant was detected by mutational analysis of the coding sequence in this allele, the unbalanced expression of the corresponding transcript could derive from a mutation outside the coding region. In patients GDLG-20 #II-1 and GDLG-49#IV-2, the silent nucleotide change at codon 234 of *hMLH1* and the missense variant at codon 342 of *hMSH2*, respectively, were associated with modest unbalances in germ-line transcript expression (Table 1). In these cases, the coding sequence variants could simply represent markers that allowed the detection of transcript unbalances caused by mutations outside the coding sequences. Alternatively, these unbalances could be related to alterations in RNA processing caused by the coding sequence variants. Whatever causes the unbalances, the overall results of the present study indicate that polymorphic sequences represent useful markers for the detection of alterations in *hMLH1* and *hMSH2* transcript expression.

In conclusion, an analysis of transcript expression allowed the identification of genetic markers for HNPCC carrier status in at least three unrelated cases in which SSCP and sequence analyses of *hMLH1* and *hMSH2* did not detect a definite pathogenic variant. Our observations indicate that germ-line transcript unbalances occur in HNPCC and suggest that this mechanism may be an important mode of germ-line inactivation of the *hMLH1* and *hMSH2* genes. Intriguingly, this is consistent with, and complements, recent data indicating that deregulation of transcript levels plays a role in the somatic inactivation of *hMLH1* in MMR-defective colorectal tumors (11, 12, 13). Screening of the coding sequence and testing of missense variants in functional assays are predicted to be ineffective in cases in which pathogenic mutations affect transcript levels. Therefore, assays designed to quantitate *hMLH1* and *hMSH2* allele expression in PBLs may contribute to a more precise definition of the role of these genes as a cause of HNPCC and may also represent a useful diagnostic tool, complementary to mutational screening, immunohistochemistry, microsatellite analysis, and functional assays.

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