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Mismatch Repair Gene *PMS2*: Disease-Causing Germline Mutations Are Frequent in Patients Whose Tumors Stain Negative for PMS2 Protein, but Paralogous Genes Obscure Mutation Detection and Interpretation

Hidewaki Nakagawa,[1] Janet C. Lockman,[1] Wendy L. Frankel,[2] Heather Hampel,[1] Kelle Steenblock,[3] Lawrence J. Burgart,[3] Stephen N. Thibodeau,[3] and Albert de la Chapelle[1]

[1]Division of Human Cancer Genetics, Comprehensive Cancer Center, and [2]Department of Surgical Pathology, The Ohio State University, Columbus, Ohio; and [3]Departments of Laboratory Medicine and Pathology, Mayo Clinic and Foundation, Rochester, Minnesota

ABSTRACT

The MutLα heterodimer formed by mismatch repair (MMR) proteins MLH1 and PMS2 is a major component of the MMR complex, yet mutations in the *PMS2* gene are rare in the etiology of hereditary nonpolyposis colorectal cancer. Evidence from five published cases suggested that contrary to the Knudson principle, *PMS2* mutations cause hereditary nonpolyposis colorectal cancer or Turcot syndrome only when they are biallelic in the germline or abnormally expressed. As candidates for *PMS2* mutations, we selected seven patients whose colon tumors stained negative for PMS2 and positive for MLH1 by immunohistochemistry. After conversion to haploidy, truncating germline mutations of *PMS2* were found in two patients (2192delTAACT and deletion of exon 8). These mutations abrogated PMS2 protein in germline cells by Western analysis. In two additional patients, PMS2 protein from one allele also was abrogated. Novel or previously described missense variants of *PMS2* were detected, but their pathogenicity is undetermined. We detected and characterized a new transcript, *PMS2CL*, showing 98% sequence identity with exons 9 and 11–15 of *PMS2* and emanating from a locus close to *PMS2* in chromosome 7p. Its predicted protein product was not detected. Thus, in addition to several previously described *PMS2*-related genes resembling the 5′ end of *PMS2*, at least one related gene resembles the 3′ end of *PMS2*. In conclusion, both detectable and presently undefined germline mutations are deleterious and produce susceptibility to cancer by the two-hit mechanism. Paralogous genes interfere with mutation detection, resulting in underdiagnosis of *PMS2* mutations. Mutation detection in *PMS2* requires haploid DNA.

INTRODUCTION

A major component of the mismatch repair (MMR) complex is the MutLα heterodimer between the MMR proteins MLH1 and PMS2 (1). Despite its seemingly crucial role in MMR, germline mutations of *PMS2* have been only rarely reported in the etiology of Lynch syndrome (hereditary nonpolyposis colorectal cancer) or Turcot syndrome. In fact, only five families with a total of six such germline mutations have been published. Furthermore, there is uncertainty regarding the mechanism by which *PMS2* mutations bring about cancer predisposition. In one study (2), a teenage patient with two cancers demonstrated two different germline nonsense mutations: one inherited from the father, the other from the mother (compound heterozygosity). Remarkably, both parents and five other family members who were heterozygous for one of the two mutations, apparently had no increased cancer predisposition. These findings were interpreted to indicate the need for biallelic germline inactivation of *PMS2* to produce cancer predisposition. A similar conclusion was derived from a family described by Trimbath *et al.* (3). In two additional cases, children with cancer were heterozygous for germline mutations and even showed widespread microsatellite instability in normal tissues, but the evidence appeared to support the notion of recessive inheritance (even though a second mutation was not found), in that a parent who had the same mutation had no cancer (4, 5). The fifth patient reported was heterozygous for a *PMS2* germline mutation, but clinical features were not described (6).

The Knudson two-hit model (7) applies to the *MLH1*, *MSH2*, and *MSH6* genes in that heterozygosity for a germline mutation confers a high risk of cancer; however cancer develops only after a second, somatic hit inactivates the wild-type allele in a cell of a target organ, such as the colon. Intuitively, the same should apply to *PMS2*.

Here, we used immunohistochemical analysis of the MMR genes to identify seven patients who were candidates for *PMS2* mutations. Among these, one had a germline frameshift mutation in *PMS2*, and one had a large deletion of exon 8 of *PMS2*. Western blotting showed that these mutations led to the abrogation of PMS2 protein. Similar abrogation of protein from one allele was seen in two additional patients in whom mutations could not be detected. Furthermore, we show that in addition to the previously described family of *PMS2* genes that comprise genes highly homologous to the 5′ region of *PMS2*, there is at least one additional transcribed gene that is highly homologous to the 3′ region of *PMS2*. We conclude that *PMS2* can behave according to the Knudson model and propose that due to the existence of numerous related genes, mutations in *PMS2* may be overlooked.

MATERIALS AND METHODS

Patients and Controls. Patients were selected from an ongoing Institutional Review Board-approved cohort study of all eligible, consenting, consecutively diagnosed colorectal or endometrial cancer patients in the metropolitan Columbus, Ohio, area. A primary screen consisted of the determination of microsatellite instability (MSI) in the tumor using a modification of the Bethesda five-marker panel (8). Germline DNAs of the first 103 patients with at least one unstable microsatellite marker were studied for mutations in *MLH1*, *MSH2*, and *MSH6*. For the purposes of this study, all 103 MSI-positive tumors were immunohistochemically stained for MSH2, MSH6, MLH1, and PMS2. All four cases that stained positively for MSH2, MSH6, and MLH1 but negatively for PMS2 were subjects of this study. Another three patients with the same characteristics (MSI positive; no mutation found in *MLH1*, *MSH2*, and *MSH6*; immunohistochemically positive for MLH1, MSH2, and MSH6; negative for PMS2) diagnosed at the Mayo Clinic were added, for a total of seven patients studied in detail.

All missense changes were searched for by single-stranded conformation polymorphism analysis and/or sequencing in a healthy control population of between 91 and 142 individuals, in each case including at least 35 grandparents from the Centre d'Etude du Polymorphisme Humain collection obtained from the Coriell Institute, Camden, New Jersey.

Immunohistochemistry. Paraffin-embedded tissue was cut at 4 μm and placed on positively charged slides. The slides were placed in a 60°C oven for 1 h, cooled, deparaffinized, and rehydrated through xylenes and graded ethanol solutions to water. All slides were quenched for 5 min in a 3% hydrogen

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Requests for reprints: Albert de la Chapelle, Human Cancer Genetics Program, Comprehensive Cancer Center, The Ohio State University, Medical Research Facility, Room 646, 420 West 12th Avenue, Columbus, OH 43210. Phone: (614) 688-4781; Fax: (614) 688-4772; E-mail: delachapelle-1@medctr.osu.edu.

peroxide solution in methanol to block for endogenous peroxidase. Antigen retrieval was performed by a heat method for all four antibodies in which the specimens were placed in a citric acid solution [Dako's Target Retrieval Solution (pH 6.1)] for 30 min at 94°C using a vegetable steamer. After allowing slides to cool for 15 min at room temperature, slides were placed on a Dako Autostainer immunostaining system for use with immunhistochemistry. The primary antibody was incubated for 1 h at room temperature. The primary antibodies used were MLH1, clone G168-728 (1/60; BD PharMingen); PMS2 (1/400; Santa Cruz Biotechnology); MSH2, Ab-2 (1/200; Oncogene Research Products); and MSH6 (1/400; Transduction Laboratories). The detection system used for all antibodies was a labeled streptavidin-biotin complex. This method is based on the consecutive application of (*a*) a primary antibody against the antigen to be localized, (*b*) biotinylated linking antibody, (*c*) enzyme-conjugated streptavidin, and (*d*) substrate chromogen (3,3′-diaminobenzidine). The tissue was protein blocked using Dako's Serum Free Protein Block before the primary antibody application. Endogenous avidin and biotin were blocked before the biotinylated-linking antibody. Slides were then counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions, and coverslipped.

DNA Analysis and Sequencing. Exon-by-exon amplification and sequencing of *PMS2* was performed as described previously (9) with some modifications. Exon 11 was divided into two parts: the first part was amplified by the primers 5′-gtcctctcaccatttcagg-3′ and 5′-agtttggctgaggcaaaactc-3′; and the second part was amplified by the primers 5′-actgcagcagcgaatatgc-3′ and 5′-aaaaaagaaaattttagataaaaagag-3′. The PCR products were purified using QIAquick PCR Purification kit (Qiagen) and sequenced directly by the primer used for PCR or cloned into TA vector pCR2.1 (Invitrogen) and then sequenced, using the ABI sequencing system (Perkin-Elmer Applied Biosystems).

Tumor DNA. To obtain tumor DNA from fixed material for loss of heterozygosity (LOH) analysis and sequencing, paraffin block samples were microdissected using H&E-stained sections, achieving 70% contribution of tumor cells. The markers used for LOH analysis are described in "Results."

Haploid-Conversion and Reverse Transcription-PCR Analysis. The diploid-to-haploid conversion technique was described previously (10, 11). Haploid-converted clones from all patients except case 283 were created by GMP Genetics, Inc. In brief, human fresh lymphocytes or immortalized lymphoblastoid cells were electrofused with a specifically designed mouse cell line (E2). Unfused mouse parental cells were negatively selected by HAT (Life Technologies, Inc.), and unfused human cells were negatively selected by Geneticin (Life Technologies, Inc.). Hybrid cells were maintained in DMEM (Life Technologies, Inc.), including 10% FBS, 0.5 mg/ml Geneticin, 1× HAT, and penicillin-streptomycin, and selected for their content of human chromosomes by the study of several polymorphic markers. Clones having a copy of each homologue of human chromosome 7 were selected to study *PMS2*. RNA was extracted from original lymphocytes and haploid-converted cells by Trizol according to its manufacturer (Invitrogen). Two μg of total RNA were treated with Superscript II (Invitrogen) to produce cDNA, using random hexamers according to its manufacturer. We designed three sets of primers to amplify three overlapping amplicons covering the whole human *PMS2*-coding region. These were designed so as not to amplify mouse *PMS2*. The primers for the first part of *PMS2* were 5′-gaaagcagccaatgggagttc-3′ (Ex1F) and 5′-tagacctcattcacgagtctgc-3′ (Ex9R); for the second part, 5′-catggagttggaaggagttcaa-3′ (Ex8F) and 5′-ttgacaaatgtcagaactggaaa-3′ (Ex11R); and for the last part, 5′-tcagccaactaatctcgcaac-3′ (Ex11F) and 5′-accaattattccatacagtgactacg-3′ (Ex15R). Long-range reverse transcription-PCR was performed by using Expand Long Template PCR system (Roche); and PCR was carried out at 94°C (10 s), 60°C (30 s), and 68°C (1 min) for 10 cycles and at 94°C (10 s), 60°C (30 s), and 68°C (1 min + cycle elongation of 10 s for each cycle) for 27–30 cycles. The PCR products were run on a 1.5% agarose gel to determine their size and sequenced by the PCR primer.

Primers were designed to amplify the novel *PMS2* homologous gene *PMS2CL* (GenBank accession no. AB116525) specifically and preferentially. The forward primer at exon 9 was 5′-tggtgaatga**c**gtctacc**gcg**t-3′ (PMS2CLf; bold nucleotides distinguish it from *PMS2*), and the reverse primer at exon 15 was 5′-gtaacttaataaaaatgcatgcagcg-3′ (PMS2CLr).

Western Blot Analysis. Protein extracts were made from either lymphoblastoid cell pellets or human-mouse hybrid cells using standard methods. Total protein was determined using Pierce BCA assay system according to the manufacturer's protocol (Pierce Biotechnology, Inc.). Fifty μg of each protein lysate were separated by SDS-PAGE under reducing and denaturing conditions and transferred to polyvinylidene difluoride membrane (Millipore Corp.). Membranes were incubated with PMS2 antibody C-20 (Santa Cruz Biotechnology, Inc.) overnight at a dilution of 1:500, washed, and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:2000. Immune complexes were detected with SuperSignal West Pico Chemiluminescent Substrate and visualized on X-ray film (Pierce Biotechnology, Inc.). β-Tubulin (BD PharMingen) antibody was used as a loading control.

RESULTS

Immunohistochemistry. Among the 103 MSI-positive tumors, loss of PMS2 expression was detected in 24 cases. In 20 of those, the loss of PMS2 expression was concordant with the loss of expression of its heterodimerization partner, MLH1. In these cases, either mutation or promoter methylation of *MLH1* explained the absence of protein. Four MSI-positive tumors from this series and three from the Mayo Clinic series showed loss of expression of PMS2 and retained expression of MLH1. Typical staining patterns of MLH1, MSH2, MSH6, and PMS2 in one tumor (case 94) are shown in Fig. 1.

A Mutation in *MLH1*. One of the seven patients had a missense mutation (Y646C) of *MLH1* (case 283; Table 1) that had been detected before this study was initiated. This amino acid change is expected to disrupt the interaction of PMS2 and MLH1 because the domain of interaction is between MLH1 amino acids 492 and 742 (12, 13). This change was not detected in 280 control chromosomes.

Mutation Analysis using Exon-by-Exon Sequencing of *PMS2*. There were no clear-cut obviously deleterious nonsense or frameshift mutations except in one patient (case 94; Table 1) in whom there was a faint hint of an abnormality. Sequencing of both genomic and cDNA from a lymphoblastoid cell line of this patient suggested the possibility that there might be an admixture of DNA carrying a frameshift change in exon 13 (Fig. 2*B*), but this was too faint to be further verified. This mutation and a deletion found after allele separation are described below.

Many sequence changes of missense or neutral type were detected (Table 1). Some have been described before, and because of high population frequency, are obviously unrelated to disease, in particular P470S, E541K, and G857A (14). We wish to draw attention to our findings in patient 4645 who carried two previously reported missense variants (T485K and T511A). Both were previously reported to be rare polymorphisms (14, 15), and abnormalities of residue 511 displayed dramatically reduced binding capacity to *MLH1 in vitro* (16). These two variants occurred on different chromosomes in this patient. In our control population, we found T485K in 2/90 chromosomes (allele frequency, 0.022) and T511A in 3/90 chromosomes (0.033). We can neither exclude nor confirm that the compound heterozygosity for these two variants might be disease causing. The same argument applies to patient 4460, who had three missense mutations (S46I and E541K on one chromosome; P470S on the other). The patient also had silent change S260S. Codon 46 is located in the ATP-binding domain of MutL; S46I can affect the ATPase activity of MutLα by conformation change (12); moreover, it was not present in 182 control chromosomes (this study). The interpretation of missense mutations in *PMS2* in the light of its numerous paralogous genes is discussed below.

Characterization of Nonsense Mutations after Allele Separation. Of the seven cases, a sample was available from six for conversion analysis. After conversion to haploidy of lymphoblastoid cells from case 94, two clones with one chromosome 7 and two clones with the other chromosome 7 were chosen for additional study. By reverse transcription-PCR, two of the clones (designated W for wild type)

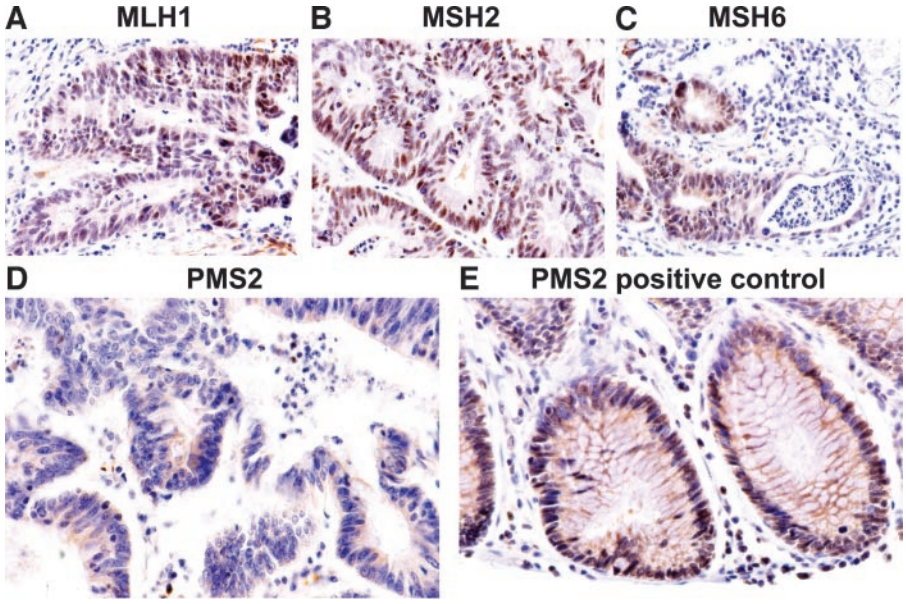

Fig. 1. Immunohistochemical staining of MLH1, PMS2, MSH2, and MSH6 proteins in the tumor from case 94. Malignant cells showed nuclear immunoreactivity with MLH1 (*A*), MSH2 (*B*), and MSH6 protein (*C*), whereas no nuclear staining was seen with PMS2 protein (*D*). *E*, nuclear staining for PMS2 was seen in the adjacent nonneoplastic colon.

showed high transcript levels, whereas the other two clones (M for mutant) showed only faintly detectable transcript levels of *PMS2* (Fig. 2*A*). Sequencing of PMS2 cDNA from the W clones showed normal sequence, except for one previously known common variant (1408C→T; P470S) in exon 11. The M clones showed two changes. There was a 5-bp deletion at codons 731–732 (2192delTAACT) in exon 13 plus a silent C→T change at nucleotide 2466 (L822L) in exon 15. As can be seen in Fig. 2*B*, the deletion is apparent in the M clones and barely visible in cDNA of diploid cells. Importantly, even in the haploid M clones, there was an admixture pattern with a normal-appearing sequence present in the sequencing chromatogram, suggesting that other transcribed sequences homologous to *PMS2* were present (see below). The reverse transcription-PCR product from the haploid M clones was cloned. In 5/12 clones the 5-bp deletion was seen, whereas in 4/12, the entire exon 13 was deleted. In 3/12, only normal transcript occurred. Genomic DNA analysis confirmed that the 5-bp deletion in exon 13 was present in the haploid M clones but barely visible in the original diploid lymphocytes. Genomic DNA further revealed an admixture pattern with the presence of a small amount of a normal allele even in haploidy (Fig. 2*C*).

In patient 4149, haploid conversion demonstrated dramatic improvement of mutation detection as well. Reverse transcription-PCR of *PMS2* transcript from one haploid clone showed an apparently shorter transcript of *PMS2* than the normal transcript (Fig. 3*A*), which was revealed to have exon 8 deleted by sequencing. *PMS2* transcript from the other haploid clone was normal. By genomic DNA analysis, exon 8 could not be PCR-amplified from the haploid clone expressing the shorter transcript, but exons 7 and 9 were amplified (Fig. 3*B*). These findings indicated a heterozygous large deletion of *PMS2* exon 8 in the germline. By sequencing, the breakpoint in intron 7 was at c803 + 384. The breakpoint in intron 8 was at c.904–1533. Thus the deletion comprised 3352 nucleotides. Using primers available on request, this deletion was searched for by PCR in the patient's parents. It occurred in the father, but not in the mother. In the remaining patients, conversion analysis did not disclose other changes than the missense and neutral changes listed in Table 1.

Table 1 *Summary of findings in seven patients with colorectal cancer*

ID	MSI[a]	MLH1	PMS2	MSH2	MSH6	*PMS2* changes likely to be deleterious	*PMS2* sequence changes of unknown significance	Population allele frequency[b]	Age at diagnosis	Family history of cancer
283	5/5	+	−	+	+	*	–		36	Cousin CRC[c] at 30 s
094	5/5	+	−	+	+	2192delTAACT	1408C→T (P470S)	0.48	22	No FH[c]
							2466C→T (L822L)	0.24		
4149	9/9	+	−	+	+	Exon 8 deletion	2466C→T (L822L)	0.24	28	**
4645	6/9	+	−	+	+	–	1454C→A (T485K)	**0.022**	49	Patient had TCC[c] of bladder at 45
							1531A→G (T511A)	**0.033**		No FH
4460	10/10	+	−	+	+	–	137G→T (S46I)	**0.00**	31	No FH
							780G→C (S260S)	0.15		
							1408C→T (P470S)	0.48		
							1621G→A (E541K)	0.11		
178	5/5	+	−	+	+	–	780G→C (S260S)	0.15		
							1866G→A (M622I)	**0.028**	82	No FH
							1970G→C (G857A)	0.22		
969	5/5	+	−	+	+	–	1408C→T (P470S)	0.48	57	Mother bladder ca[c] at 77; Father bladder ca at 82

[a] No. of markers positive/no. studied.
[b] Derived from literature (Ref. 13; no bold) or from this study (bold).
[c] CRC, colorectal cancer; FH, family history of cancer; TCC, transitional cell carcinoma; ca, cancer.
* This patient had a 1937A→G mutation in *MLH1* exon 17 predicting Y646C.
** Father has the exon 8 deletion, healthy at age 60; mother does not have the deletion, healthy at 56; paternal grandfather had CRC at 76; paternal grandmother had ovarian ca at 76.

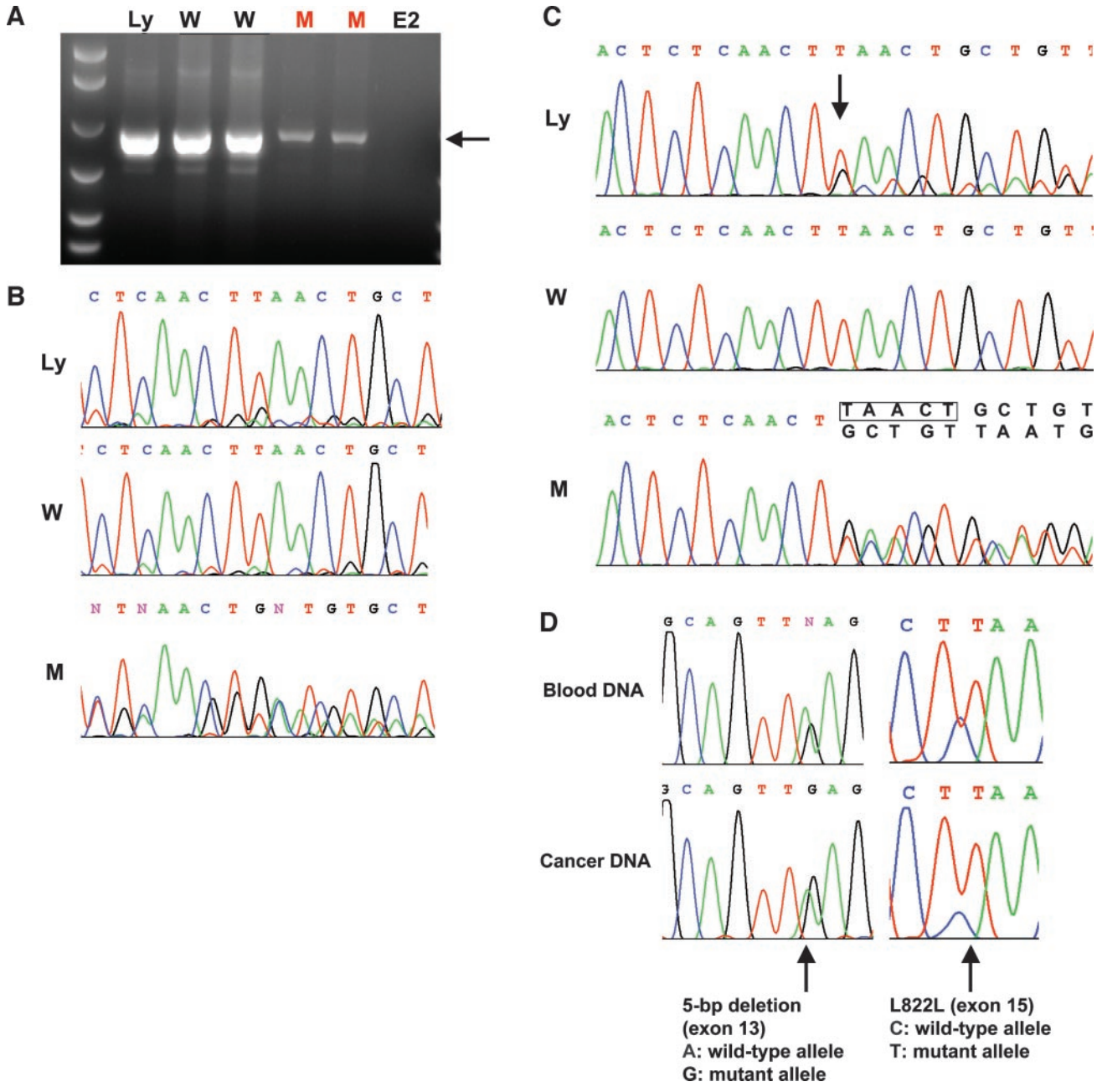

Fig. 2. Haploid analysis of a frameshift mutation in case 94. *A*, reverse transcription-PCR analysis of the hybrid-converted cells and the original lymphocytes (*Ly*) revealed that presumably due to nonsense-mediated decay, the hybrid cells with the mutant allele (*M*) displayed very weak expression compared with the hybrid cells with the wild-type allele (*W*). *E2* is the recipient mouse cell line used in the conversion. *B*, sequence analysis of the *PMS2* transcript expressed in the haploid-converted cells and diploid cells revealed a 5-bp deletion (TAACT) at codons 731–732 in exon 13 in the haploid-converted cells, whereas the transcript from the other allele was normal and the sequence of the transcript from the original diploid cell did not show this mutation, presumably due to nonsense-mediated decay. *C*, sequencing chromatogram of human *PMS2* in the diploid and haploid-converted cell DNA. In diploid cells, the signal from the mutant allele (5-bp TAACT deletion) was so weak that computer analysis ignored it, presumably because of the presence of the wild-type allele plus homologous sequences from the paralogous gene. However, in the haploid-converted cells, the deletion was more evident (in the M clone), whereas the W clone showed only normal sequence. *D*, LOH of *PMS2* in the colon cancer of patient 94 with a heterozygous *PMS2* mutation. In the chromatograms of sequences at the germline mutation (codon 731 in exon 13, complementary sequences) and one common variant (2466C→T at exon 13) of *PMS2*, LOH was seen in the tumor in which the signal from the wild-type allele was decreased compared with the signal in blood DNA.

Somatic Changes in the Tumors. In search of a second mutation, tumor DNA from the fixed paraffin blocks of case 94 was subjected to exon-by-exon sequencing, but no additional mutations were noted. However, by using the germline 5-bp deletion mutation (codon 731 in exon 13) and single nucleotide polymorphisms (1408C→T in exon 11 and 2466C→T in exon 15) as markers, we detected LOH in the tumor in which the signal from the wild-type allele was decreased (Fig. 2*D*).

A Novel PMS2-Related Gene. As indicated above, the sequencing of exon 13 in case 94 disclosed an admixture pattern, suggesting the low level presence of DNA of normal-appearing sequence. Because this was noted both in diploid genomic DNA and in cDNA from haploid-converted clones, it suggested that an additional, transcribed PMS2 or PMS2-like sequence occurred somewhere on chromosome 7. In a BLAST search, we noticed a PMS2-similar gene located in chromosome 7p22–23 close to *PMS2*. This genome sequence contains exon 9 and exons 11–15 of *PMS2* and shows 97% sequence identity, having an identical exon-intron structure in a region spanning approximately 19 kb. We named this likely duplication *PMS2-COOH-*

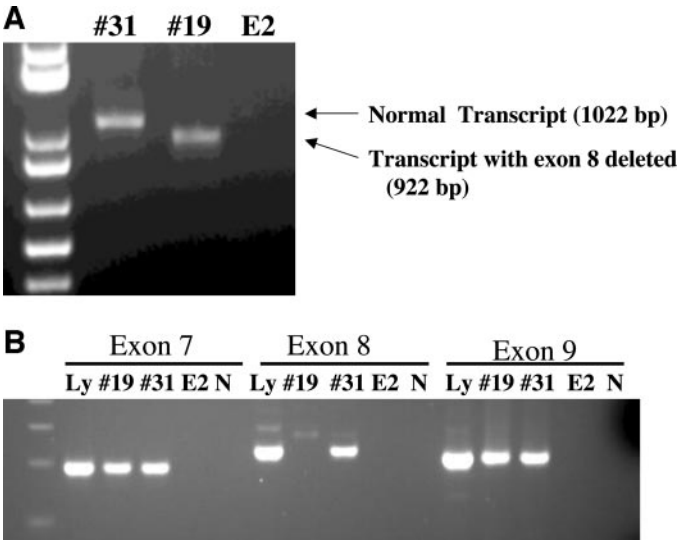

Fig. 3. Haploid analysis of exon 8 deletion in case 4149. *A*, reverse transcription-PCR analysis of the hybrid-converted cells revealed that the hybrid cells with one allele (19) displayed a shorter transcript (922 bp) of *PMS2* than did the hybrid cells with the other allele (31; 1022 bp). The shorter reverse transcription-PCR product was shown to have exon 8 deleted by sequencing. *E2* is the recipient mouse cell line used in the conversion. *B*, DNA analysis of the haploid-converted cells demonstrated that exon 8 of *PMS2* was not amplified from one haploid clone DNA (19), but exons 7 and 9 were amplified from this clone. In the haploid clone with the other allele (31), all exons of *PMS2* were amplified. *Ly* is the original lymphocyte DNA of case 4149; *E2* is the recipient mouse cell line used in the conversion; and *N* is no template PCR as a negative control.

terminal-Like (*PMS2CL*). To assess whether this paralogous gene is transcribed, we designed *PMS2CL*-specific primers in exon 9 and exon 15 to amplify preferentially the *PMS2CL* transcript by taking advantage of nucleotide differences (see "Materials and Methods"). Using these primers, reverse transcription-PCR products were sequenced. As expected, *PMS2CL* was transcribed and lacked exon 10 (156 bp) of *PMS2*. The transcript of *PMS2CL* was 98% identical with exons 9 and 11–15 of *PMS2*, but due to a 2-bp deletion and 1-bp insertion in the exon corresponding to exon 11 of *PMS2*, its predicted translated product will consist only of a 191-amino acid peptide 99% identical to the COOH-terminal portion of PMS2 protein (codons 672–862; Fig. 4). This COOH-terminal region of the PMS2 protein coincides with the PMS2 region interacting with MLH1 protein (codons 675–850; Refs. 16 and 17). However, as shown by Western blot hybridization (see below), the transcript is not translated into protein.

PMS2 Protein Analysis by Western Blot Analysis. As can be seen in Fig. 5*A*, antibody C-20 recognizing the COOH-terminal end of PMS2 gave a strong signal at the expected PMS2 molecular size. Two control samples ("CEPH") showed strong signals, whereas all four patient samples produced bands of approximately one-half the strength of the controls. Two of the patients had truncating mutations (4149 and 94), and two of the patients had no proven deleterious mutation (4460 and 178).

Fig. 5*B* shows the results of Western analysis of human-mouse clones from the same four patients described in Fig. 5*A*. In the hybrid clones, each containing a different copy of the patient's chromosome 7, the following was seen: In patient 4149, hybrid 1 (containing the *PMS2* allele with a deleted exon 8) PMS2 protein was not detected, whereas in hybrid 2 (wild-type *PMS2*), there was a strong PMS2 signal. In patient 94, hybrid 1 (with the 5-bp deletion of PMS2) showed no staining; no wild-type hybrid was available. Interestingly, in patients 4460 and 178, in whom no deleterious *PSM2* mutation has been found, one allele showed staining, and the other one did not, suggesting abrogation of PMS2 protein by unknown mechanisms. This finding is in full agreement with the results from the corresponding diploid cells (Fig. 5*A*). The Western blots revealed no evidence of PMS2 protein corresponding in size to the open reading frame of *PMS2CL*, so this polypeptide was either not produced or not stable.

DISCUSSION

Our results highlight several aspects of *PMS2* in the causation of Lynch syndrome (hereditary nonpolyposis colorectal cancer) and spo-

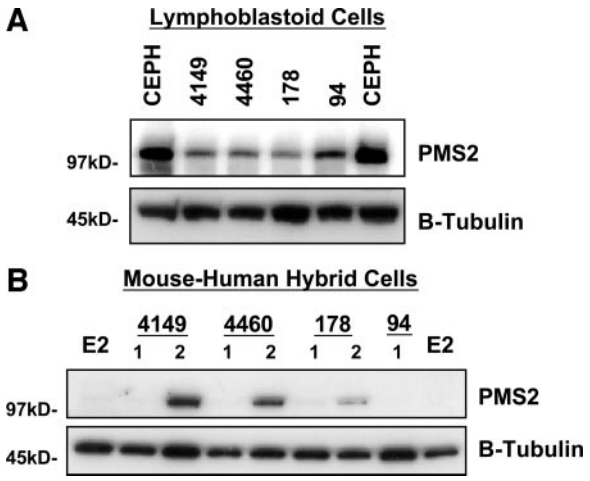

Fig. 5. Western blot analysis of PMS2 protein in germline lymphoblastoid and hybrid culture cells. *A*, using PMS2 antibody C-20, a band at approximately M_r 100,000 was produced. The dosage in two normal controls (*CEPH*) was approximately twice that in the four patients (*4149*, *4460*, *178*, and *94*). There was no evidence of any protein emanating from the open reading frame of the *PMS2CL* gene. β-Tubulin served as a loading control. *B*, the PMS2 antibody C-20 does not react with mouse Pms2 as shown in *Lanes E2* containing material from the parental mouse E2 cell line used in the fusion with human cells. Haploid-converted clones containing one copy of human chromosome 7 from patients 4149, 4460, 178, and 94 are shown. In patient 4149, hybrid *clone 1* contains the PMS2 allele with a deleted exon 8 (no product), whereas *clone 2* contains the chromosome with wild-type PMS2 (strong band). In patients 4460 and 178, the *clones 1* show no PMS2 protein suggesting a mutation. In patient 94, only the hybrid clone with an abnormal PMS2 allele (5-bp deletion) was available for study; it shows no PMS2 protein.

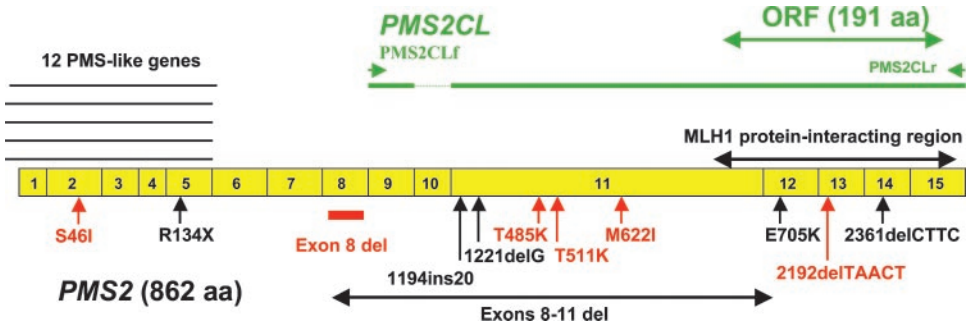

Fig. 4. The structure of human *PMS2* and *PMS2CL* transcript and germline mutations. The transcribed *PMS2CL* (GenBank accession no. AB116525) is 98% identical with exons 9 and 11–15 of *PMS2* but does not contain the sequence corresponding to exon 10 (156 bp) of *PMS2*. Due to a 2-bp deletion and 1-bp insertion in the sequence corresponding to exon 11 of *PMS2*, the *PMS2CL* transcript has an open reading frame for only 191 amino acids of peptide. The locations of the germline mutations reported thus far are shown with *arrows* or *bars*. *Red arrows* or *bars* are the definitely or potentially deleterious sequence changes detected in this study [*2192delTAACT*, exon 8 deletion (*Exon 8 del*), *S46I*, *T485K*, *T511K*, and *M622I*].

radic MSI-positive colorectal cancer. By applying immunohistochemistry staining for PMS2 protein in MSI-positive tumors, we were able to select seven patients that we considered to be candidates for *PMS2* mutations. MLH1 and PMS2 form the MutLα heterodimer that is an important component of the MMR mechanism, and inactivation of MLH1 leads to instability of both proteins (18). Therefore, immunohistochemistry staining for PMS2 was negative in many additional cases in which MLH1 protein expression was negative due to mutation or promoter methylation. We therefore focused on cases in which staining for PMS2 was negative, whereas MLH1 was not affected. MLH1 is thought to have other partners for its heterodimerzation, such as MLH3 and PMS1 (19, 20), which may explain why the loss of PMS2 does not always lead to the instability of MLH1 protein. Among the seven cases that we analyzed for *PMS2* mutations in the germline, we found one frameshift mutation, one large deletion, and two patients with rare missense variants of unknown significance but that were potentially deleterious. In addition, one patient was known to have a missense variant of *MLH1* in the domain interacting with PMS2. In the remaining two patients, the defect underlying the MSI and abnormal immunohistochemistry staining is not known, and the PMS2 protein defect in their tumors may be caused by somatic events. Alternatively, we cannot rule out the possibility that additional germline mutations were missed because of the large number of pseudogenes and "like" genes that complicate the analysis. In addition to the 3′ *PMS2CL* gene described here, there are at least 13 paralogous genes resembling the 5′ end of *PMS2*. Obviously, these sequences complicate all methods of DNA analysis that are in common use. For instance, in experiments not shown, we tested greater than 30 different primer pairs in the 5′ region of *PMS2* in search of PCR conditions unique to *PMS2* itself, but all failed. The problem was the same even after conversion to haploidy, because all of the paralogous genes are located on chromosome 7.

These patients seem to have clinical features similar to most sporadic MMR-deficient tumor patients with the possible exception of age (median age at diagnosis in the probands is only 36 years). All of the patients had proximal colon cancer with high MSI. However, none of them fulfilled the Amsterdam criteria, and significant family history of hereditary nonpolyposis colorectal cancer-related tumors was conspicuously absent. We propose that germline mutations of *PMS2* mainly predispose to sporadic MSI-positive colorectal cancer in young adults. Another presentation is Turcot syndrome in children bearing the hallmarks of MMR deficiency (4, 5).

How is the pathogenicity of germline mutations of *PMS2* determined? Among the cases with PMS2 mutation reported by others, two (2, 3) behaved as recessive gene mutations in that two different mutations segregated in each of the families. The probands who were compound heterozygous or homozygous for the mutations showed drastic phenotypes of cancer, whereas individuals who were heterozygous for one of the two mutations had no apparent predisposition to cancer. Thus, these nonsense or frameshift mutations showed no or very low penetrance when heterozygous. The same apparent nonpenetrance or low penetrance can be inferred from other cases (4, 5) in which a child with the mutation had early-onset cancer, whereas a parent with the same mutation had no cancer and from our patient 4149 whose father carried the exon 8 deletion but was unaffected at age 60. In the absence of definitive evidence, we do not wish to speculate about the possible pathogenicity of the missense mutations that we detected in all patients but one. At the present time, it is not always possible to determine whether a detected change is in *PMS2* itself or in a paralogous gene. This is particularly problematic in the 5′ region of the gene. In contrast, case 94 (with a truncating mutation) and case 4149 (with a deletion of exon 8) provide evidence that *PMS2* can act in concert with the Knudson principle in that germline heterozygosity for the mutation predisposes to cancer, with the second hit being loss of the wild-type allele in the tumor, at least in case 94.

It remains to be explained why germline heterozygosity for a mutation sometimes appears to predispose to cancer, but often does not. Fig. 4 shows the location of all mutations described thus far. The mutations are distributed relatively evenly over the gene, including the COOH-terminal domain that is responsible for the interaction between PMS2 and MLH1. Thus, the location of the mutation does not seem to determine the pathogenicity *per se*. We speculate that the observed differences in penetrance are not directly related to the paralogous, *PMS2*-like genes that have been described previously (9, 21) and the novel gene, *PMS2CL*, described in the present report. In the National Center for Biotechnology Information database (assembly April 2003), there are 13 sequences highly homologous to the 5′ end of *PMS2* exons 1–5. The transcript of *PMS2CL* is 98% identical to exons 9 and 11–15 of *PMS2*. Crucial to the question of penetrance of *PMS2* mutations is not only the transcription, but also the translation of these paralogous genes. We show here that the *PMS2CL* transcript is only partially composed of an open reading frame; and this is not translated, or the polypeptide is unstable. The paralogous sequences that resemble the 5′ part of *PMS2* are believed to extend over exons 1–5; some of these sequences are transcribed, but whether they are translated is not known. In this regard, our results with Western blots give important clues. We show (Fig. 5) that in diploid "germline" lymphoblastoid cells, the dosage of PMS2 protein is reduced by approximately one-half, not only in the two patients with truncating mutations, but also in two patients in whom only missense mutations occurred. Because PMS2 staining in the tumors of all of these patients was absent (Table 1), an obvious explanation is that each had one germline "hit" and a second "hit" in the tumor. Because of the paucity of tumor material, we were only able to search for the second hit in one case (case 94) in which indeed LOH was seen. Thus we propose that undetectable changes (or the missense changes we found) in cases 4460 and 178 are deleterious at the protein level. The penetrance of all changes will depend on whether and when a second somatic hit occurs. It remains to be determined if the paralogous genes play a role in this regard.

To fully understand how the existence of the various paralogous sequences might affect the penetrance of *PMS2* mutations, more must be learned about their behavior in different individuals. In the case described by Nicolaides *et al.* (22), the allele carrying the nonsense mutation showed exceptionally high levels of expression, leading the authors to propose that it acted as a dominant-negative allele. In contrast, in cases 94 and 4149, we demonstrated low levels of expression of the mutated allele and low levels of protein by Western analysis. The situation is compatible with recessive inheritance at the cellular level, the second hit also being inactivating (LOH), at least in case 94. Finally, paralogous genes and pseudogenes can show variation in copy number that can have a profound effect on function and protein stability. It has already been demonstrated that a polyadenine tract, $(A)_8$ in the proximal part of exon 11, displays interindividual differences in dosage (23). These in all likelihood emanate from *PMS2CL*, which, as shown here, exhibits a low level of transcription. More needs to be learned about the paralogous *PMS2* sequences at the population level. They obviously hamper our ability to detect mutations in *PMS2*. In this report, we show how the presence of *PMS2CL* obscured the detection of a frameshift mutation by genomic sequencing. The same problem may well account for the fact that few mutations have been seen in exons 1–5. It is entirely possible, therefore, that *PMS2* mutations are more common than hitherto thought (24).

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