

Germline mutation analysis of *hPMS2* gene in Chinese families with hereditary nonpolyposis colorectal cancer

Xia Sheng, Heng-Hua Zhou, Xiao-Yan Zhou, Xiang Du, Tai-Ming Zhang, San-Jun Cai, Wei-Qi Sheng, Da-Ren Shi

Xia Sheng, Xiao-Yan Zhou, Xiang Du, Tai-Ming Zhang, Wei-Qi Sheng, Da-Ren Shi, Department of Pathology, Shanghai Cancer Center, Fudan University, Shanghai 200032, China; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China

Heng-Hua Zhou, Department of Pathology, Shanghai Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200011, China

San-Jun Cai, Department of Abdominal Surgery, Cancer Center, Fudan University, Shanghai 200032, China

Author contributions: Sheng X and Zhou HH contributed equally to this work, participated in the treatment of patients, analyzed the data, performed the research and wrote the manuscript; Zhou XY, Du X, Sheng WQ and Shi DR participated in the treatment of patients, collected the data and designed the research; Cai SJ participated in the treatment of patients, collected the data; Zhang TM contributed to the new reagents/analytic tools.

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Correspondence to: Wei-Qi Sheng, MD, PhD, Associate Professor, Department of Pathology, Shanghai Cancer Center, Fudan University, 270 Dong An Road, Shanghai 200032, China. shengweiqi2006@yahoo.com.cn

Telephone: +86-21-64175590 Fax: +86-21-64046008

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were used as template to amplify the individual exon respectively and DNA sequencing was done. Direct DNA sequencing of the conventional PCR products of exon 6, 7, 8 and 10 of *hPMS2* gene was performed. The same analysis was made in 130 healthy persons without family histories of HNPCC to further investigate the pathological effects of the detected missense mutation.

RESULTS: One HNPCC proband fulfilled Bethesda guidelines and was found to carry the germline mutation of *hPMS2* gene, which has not been reported in Chinese HNPCC families. It was a missense mutation at c.1532C>T of exon 11. It was detected in three controls as well with an occurrence rate of 2.3% (3/130). Since it could not be found in the PMS2-single nucleotide polymorphism (SNP) database, this missense mutation is a new SNP unreported up to date. Meanwhile, 260 reported SNPs of *hPMS2* gene were detected in the 26 HNPCC probands. The 2nd and 5th exons were probably the hot SNP regions of *hPMS2* gene in Chinese HNPCC families involving 53.1% of all reported SNP.

CONCLUSION: The germline mutation of *hPMS2* gene may be rare in Chinese HNPCC families. The 2nd and 5th exons are hot SNP regions of *hPMS2* gene.

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Abstract

AIM: To study the germline mutation of *hPMS2* gene in 26 unrelated Chinese hereditary nonpolyposis colorectal cancer (HNPCC) probands and to fulfill the screening strategy for HNPCC in Chinese.

METHODS: Genomic DNA was extracted from the peripheral blood. To avoid the interference of pseudogene in detection of the remaining 11 exons (exon 1-5, 9, 11-15), long-range polymerase chain reaction (PCR) was conducted to amplify the complete coding region of *hPMS2* gene firstly. Then 1/8 of the PCR products

Key words: Hereditary nonpolyposis colorectal cancer; *hPMS2*; Missense mutation; Single nucleotide polymorphism; Colorectal cancer

Peer reviewers: Henry Thomson Lynch, MD, Department of Preventive Medicine, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178, United States; Minna Nyström, PhD, Department of Biological and Environmental Sciences, PO Box 56 (Viikinkaari 5 D), University of Helsinki, FI-00014 Helsinki, Finland

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INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome, is an autosomal dominantly inherited disease with cancer-susceptibility. Perhaps it is the most common cause of hereditary colorectal cancer, accounting for 5%-10% of the total colorectal cancers worldwide^[1-3]. People inheriting this predisposition are at a particularly high risk of developing colorectal cancer with an early age of onset^[3,4]. The affected patients always carry germline mutations in DNA mismatch repair (MMR) genes, mostly in *bMLH1*, *bMSH2*, and *bMSH6*^[5,6]. Less commonly, mutations in other MMR genes are present. We analyzed the abnormalities of *bMSH2/bMLH1/bMSH6* genes in a series of Chinese HNPCC families fulfilling different clinical criteria. We studied germline mutation, large genomic variations of the entire coding regions of the three genes and methylation of *bMLH1* promoter in 58 Chinese HNPCC probands, in which 24 fulfilled Amsterdam criteria (AC)^[7], 15 fulfilled Japanese criteria (JC)^[8] and 19 met Bethesda guidelines (BG)^[7]. The total detected gene abnormality rate was only 53.4% (31/58), including 29 cases of germline mutation and 2 cases of methylation of *bMLH1* promoter^[9-14]. So the aberrant MMR genes other than *bMSH2/bMLH1/bMSH6* are suspected to be involved in Chinese HNPCC.

In order to accomplish our serial studies of Chinese HNPCC, we detected *bPMS2* germline mutation in 26 Chinese HNPCC families by long-range polymerase chain reaction (LR-PCR)-based sequencing in this study, and evaluated this manner in the molecular genetics screening of Chinese HNPCC.

MATERIALS AND METHODS

Materials

Twenty-six unrelated HNPCC probands registered from January 1998 to October 2005 at the Department of Abdominal Surgery in Shanghai Cancer Center were retrieved. Five of them fulfilled AC, 10 fulfilled JC and the remaining 11 fulfilled BG. Germline abnormalities of *MSH2/MLH1/MSH6* were excluded in all the 26 probands by PCR-based sequencing. Ten milliliter peripheral blood was collected from each proband for genomic DNA preparation. The peripheral blood samples of 130 healthy volunteers without any family history of hereditary disease or development of colon cancer in early age were obtained for control. The informed consents were signed by all the probands and volunteers before blood drawing. This study was approved by the Medical Ethical Committee of Shanghai Cancer Center, Fudan University. The whole procedures of the study were in accordance with the international rules and regulations.

Table 1 Primer sequences of long-range polymerase chain reaction

Primer name	Sequence (5'-3')	Size (bp)	Exon
LRPCR1			
For	ACGTCGAAAGCAGCCAATGGGAGTT	9964	Exon 1-5
Rev	CTTCCACCTGTGCATACCACAGGCT		
LRPCR2			
For	GGTCCAGGTCCTTACATGCATACTGT	9440	Exon 9
Rev	CTGACTGACATTTAGCTTGTGACA		
LRPCR3			
For	GCGTTGATATCAATGTTACTCCAGA	8812	Exon 11, 12
Rev	AGTAGTCAGGGTAAAACATCCAGT		
LRPCR4			
For	AAAATTAGTCAGACTTGATGGTGTG	9804	Exon 13-15
Rev	CCTTCCATCTCCAAAACCAGCAAGA		

DNA extraction

Genomic DNA was extracted from the peripheral blood using the QIAGEN (Hilden, Germany) DNA extraction kit and following the manufacturer's instructions. Concentrations of the genomic DNA were determined by an ultraviolet spectrophotometer (Beckman, DU640 type).

PCR amplification and DNA sequencing

LR-PCR (exon 1-5, 9, and 11-15): Since exon 1-5, 9, and 11-15 of *bPMS2* genes were severely hampered by the presence of multiple pseudogenes with highly similar sequences. LR-PCR was conducted to preferentially amplify *bPMS2* gene and avoid the interference of the pseudogenes.

Four overlapping sets of primers were designed to amplify the complete coding region of *bPMS2* gene by LR-PCR^[15,16] (Table 1). The LR-PCR amplification profile is also shown in Table 1. Then 1/8 of the four LR-PCR products were used as template to amplify the 11 exons (exon 1-5, 9, 11-12 and 13-15) individually. The primer sequences are listed in Table 2.

PCR (exon 6, 7, 8 and 10): Conventional PCR was performed to detect the four exons (exon 6, 7, 8 and 10) which were seldom influenced by pseudogenes. Four sets of primers and PCR amplification profile are listed in Table 2.

DNA sequencing: The conventional PCR products were subjected to 2% agarose gel electrophoresis, while for LR-PCR products, 1% agarose was used with 9Kb as marker. After observation of clear and expected size bands, the products were purified and used as a template for sequencing reactions with BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing primers were M13F or M13R. Automated fluorescence analysis was performed on a 3700 DNA sequence system (ABI, USA).

Bioinformatics analysis

Each result of sequencing was analyzed by DNASTar 5.08 bioanalysis software. The type of mutations and potential

Table 2 Primer sequences and polymerase chain reaction condition of individual exon of *hPMS2* gene

Exon	Primer sequence (5'-3')	Size (bp)	AT (°C)	CN
1	M13F-ACGTCGAAAGCAGCCAATGGGAGTT M13R-CAGGTAGAAAAGGAAATGCATTCACT	475	66	28
2	M13F-ACAGTGTGAGTCAATTTCCACAGT M13R-TTCITTAGCATAAACACCTGCCTGGCA	455	66	28
3	M13F-TAGTCTGGGCTAGTAAATAGCCAGA	705	68	35
4	M13R-TATGACTTAGATTGGCAGCGAGACA			
5	M13F-CITGATTATCTCAGAGGGATCTCA M13R-TCTCACTGTGTTGCCAGTCTAAT	540	68	35
6	M13F-TGCTTCCCTTGATTTGTGCGATGAT M13R-TGAGGCAGGAGAATGTCTGAATCT	504	67	32
7	M13F-ACCCACGAGTTTGACATTGCAGTGA M13R-GTAGAGTTGCAGTGAGCCAAGATA	498	60	35
8	M13F-AGATTGGAGCACAGATACCCGTGA M13R-TGCGGTAGACTTCTGTAATGCACA	414	61	32
9	M13F-CCTTCTAAGAACATGCTGGTTGGTT M13R-ATCTCATTCCAGTCATAGCAGAGCT	279	64	45
10	M13F-AGCCCTCCGTATTTTGCTATICA M13R-GCTTTAGAAGCTGTTTGACACTGT	719	61	32
11	M13F-TCACATAAGCACGTCCTCTCACCAT M13R-GCAACAGAGCAAGACTCTGTCTCAA	1021	64	45
12	M13F-GCCAAGATTGTGCCATTGCACTGTA M13R-AGTAGATACAAGGCTTGTGCTGTGT	493	64	25
13	M13F-GTGACACTTAGCTGAGTAGTGTGT M13R-ATGTTAGCCAGGCTGGTCTCAAAC	372	64	35
14	M13F-GGCTGTATCTCCTGACCTCATGAT M13R-GCACGTAGCTCTGTGTAATAATGA	473	64	35
15	M13F-GCTGAGATCTAGAACCTAGGCTTCT M13R-ACACACGAGCGCATGCAAACATAGA	522	64	35

AT: Anneal temperature; CN: Cycle number. The sequence of M13F was 5'-GTAAAACGACGGCCAGT-3'. The sequence of M13R was 5'-AACAGCTATGACCATG-3'.

significance were determined by comparing the corresponding amino acids and proteins in the following databases (<http://www.ncbi.nlm.nih.gov/>; <http://www.ensembl.org/homosapiens>; and <http://www.insight-group.org>).

RESULTS

Germline mutation of *hPMS2* gene in HNPCC probands

Among the 26 unrelated HNPCC probands, only one (H13) was found to carry the germline mutation of *hPMS2* gene. She was a 30-year-old female BG patient. The mutation was a missense mutation at codon 511 (ACG>ATG, Thr>Met) (Figure 1). To further investigate the pathological effects of the missense mutation, we analyzed the related exon 11 in 130 controls by PCR-based sequencing. The results showed that the mutation of codon 511, consistent with the HNPCC case at c.1532C>T of exon 11 of *hPMS2* gene, was also found in three healthy controls. The occurrence rate was approximately 2.3% (3/130). It could not be found in the PMS2-SNP database (<http://www.nfdht.nl>; <http://www.insight-group.org>; and <http://www.ensembl.org>). Thus, the mutation at c.1532C>T of *hPMS2* gene which we detected in the HNPCC patient is an unreported new single nucleotide polymorphism (SNP).

SNP detection and analysis of *hPMS2* gene

By DNA sequencing, 27 loci on the exons of *hPMS2*

gene including 260 reported SNP (http://www.ensembl.org/homo_sapiens) were detected in the 26 HNPCC probands. Among them, 30% (78/260) were located in the 2nd exon, 23.1% (60/260) in the 5th exon, 13.8% (36/260) in the 15th exon, 10% (26/260) in the 7th exon, and 9.2% (24/260) in the 11th exon. However, none variant was detected in the remaining exons of the 1st, 3rd, 6th, 8th, 9th and 10th. The 2nd and 5th exons were probably the hot SNP regions of *hPMS2* gene because 53.1% of the reported SNP were located in them. Distribution of the SNP of *hPMS2* gene is shown in Table 3.

DISCUSSION

HNPCC, also called Lynch syndrome, is one of the most common autosomal dominantly inherited cancer syndromes with a high risk of colorectal cancer as well as other tumors occurring in endometrium, stomach, ovary, urinary tract, pancreas, small intestine, brain and skin. People with HNPCC take about 80% risk to develop colorectal cancer in their lifetime. It accounts for 2%-15% of all colorectal cancers. Compared to sporadic colorectal cancer, HNPCC possesses its own characteristics in clinical presentations, treatment, genetic features and management of kindred^[17,18]. Many countries have established the clinical diagnostic criteria for HNPCC, such as AC, JC and BG. Defects in MMR genes, mainly in *hMLH1*, *hMSH2* and *hMSH6* were considered to be closely related to the genetic mechanism of HNPCC. The defection would

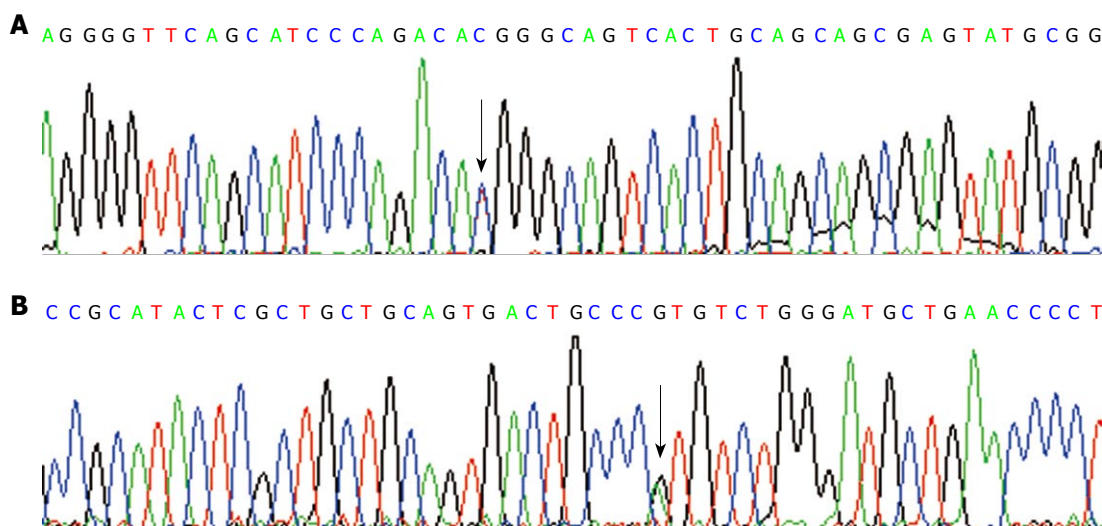


Figure 1 Missense germline mutation of exon 11 of *hPMS2* gene in the proband of H13 hereditary nonpolyposis colorectal cancer kindreds. A: The forward sequence; B: The reverse sequence. Arrow indicates the mutation site, the single basyl substitution was transversed from C to T (C>T) at the codon 511, the codon from ACG to ATG, causing the amiod acid changes from Thr>Met, the change was identified as a new single nucleotide polymorphism.

Table 3 Distribution of single nucleotide polymorphism of *hPMS2* gene in 26 probands

Exon	Nucleotide change	Amino acid change	n	SNP (%)
2	c.24-4C>T	-	14	78 (30)
	c.89A>C	Gln30Pro	15	
	c.117A>G	Val39Val	15	
	c.120G>A	Lys40Lys	4	
	c.121G>A	Glu41Lys	15	
	c.124T>A	Leu42Ile	8	
4	c.288C>T	Ala96Ala	8	18 (6.9)
	c.295A>C	Thr99Pro	10	
5	c.406A>G	Met136Val	10	60 (23.1)
	c.418A>G	Asn140Asp	10	
	c.429T>C	Ile143Ile	10	
	c.452G>A	Arg151His	10	
	c.478C>A	Gln160Lys	10	
	c.492C>T	Ser164Ser	10	
11	c.1408C>T	Pro470Ser	7	24 (9.2)
	c.1454C>A	Thr485Lys	11	
	c.2006+6G>A	-	7	
12	c.2007-4G>A	-	11	12 (4.6)
	c.2007-7C>T	-	1	
13	c.2253T>C	Phe751Phe	1	1 (0.4)
14	c.2324A>G	Asn775Ser	3	5 (1.9)
	c.2340C>T	Pro780Pro	2	
15	c.2466T>C	Leu822Leu	12	36 (13.8)
	c.2570G>C	Gly857Ala	2	
	c.92dupA	-	17	
	c.17G>C	-	5	

SNP: Single nucleotide polymorphism.

consequently lead to the dysfunction of MMR system, ultimately resulting in the development of neoplasm. So, detection of MMR gene mutation is the only gold criteria to make a diagnosis of HNPCC.

Within the family of MMR genes, germline mutations in the coding region of *bMSH2* and *bMLH1* could be detected in up to 45%-64% of all HNPCC families, while *bMSH6* about 10%. Previously we analyzed germ-

line mutations and large genomic variations of the entire coding regions of *bMSH2/bMLH1/bMSH6* genes and the methylation of *bMLH1* promoter in 58 Chinese HNPCC probands, resulting in 29 germline mutations and 2 exhaustive inherited methylations of *bMLH1* promoter (excluding 3 part-methylations of *bMLH1* promoter). The total gene abnormality rate was only 53.4% (31/58). We suspected that the other MMR gene mutations might be associated with the remaining probands without *bMSH2*, *bMLH1* or *bMSH6* gene abnormalities.

The *hPMS2* gene is a member of a set of human mismatch repair genes, located on chromosome 7. It encodes the protein that plays an essential role in repairing DNA by forming an active protein complex with the MLH1 protein which interacts with MSH2 bound to mismatched bases. In 1994, Nicolaides *et al*^[19] firstly found the germline mutation of *hPMS2* gene in a HNPCC patient. Since then, more and more data have proved that *hPMS2* germline mutation is involved in the development of HNPCC. In some reports, it could be detected in as high as 62% of HNPCC probands^[20]. The *hPMS2* gene was suggested as the first candidate gene for testing germline mutations in HNPCC families in which *bMSH2*, *bMLH1* and *bMSH6* aberrant was excluded. However, genetic testing for germline mutation of *hPMS2* gene was technically challenging because they were severely hampered by a large family of highly homologous pseudogenes located on the same chromosome as the true *hPMS2*, such as *PMS2CL*. They shared similar sequences to *hPMS2* but had no functions. Data from literature indicated that the exon 6 to 8 and exon 10 of *hPMS2* could be easily screened by direct sequencing of genomic DNA without interference of pseudogenes. But detection of exon 1-5, 9 and exon 11-15 was complicated due to the interference of *PMS2CL*. LR-PCR was recommended as a useful method to preferentially identify *hPMS2* but not the pseudogenes. In this study, we used LR-PCR to investigate the germline mutation of *hPMS2* gene in those

HNPCC probands who did not carry *bMLH1/bMSH2/bMSH6* germline mutations investigated by the previous studies. Four overlapping sets of primers were designed to amplify the complete coding region of *hPMS2* gene by LR-PCR firstly. Then, exon-specific amplifications from the LR-PCR products were performed to obtain a clear sequence with no evidence of pseudogene contamination. We only found one missense mutation in 26 probands, which has not been reported in Chinese HNPCC families. This mutation could also be detected in the 130 control persons with an occurrence rate of about 2.3%. Since it could not be found in the PMS2-SNP database (<http://www.nfdht.nl>; <http://www.insight-group.org>; and <http://www.ensembl.org>), the mutation at c.1532C>T of *hPMS2* gene in our HNPCC case was an unreported new single nucleotide polymorphism (SNP). Our results showed that the germline mutation of *hPMS2* gene was probably a rare event in Chinese HNPCC, even in those probands without *bMLH1/bMSH2/bMSH6* mutations. It was consistent with the results of some other studies^[21]. Interestingly, another mutation was found in the same nucleotide, c1532_1533 delCGinsAC, causing the amino acid changes from Thr to Asn (<http://www.insight-group.org>). So, the exon 11 may be a hot SNP or mutation region of *hPMS2* gene.

The frequency of germline mutation in *hPMS2* gene was reported to be up to 62% if patients whose tumor tissues lacked protein expression of *hPMS2* or had MSI-H features, were selected^[22]. Among the HNPCC families with monoallelic mutation in *hPMS2*, 65.5% were complied with BG. Recently, Niessen *et al.*^[23] identified 4 patients with pathogenic mutation of *hPMS2* among 97 patients with suspected Lynch syndrome who carried no germline mutation in *bMLH1*, *bMSH2* or *bMSH6*. All these 4 patients fulfilled BG and their corresponding tumor cells showed MSI-H and loss of expression of *hPMS2*. Clendenning *et al.*^[24] reported that a kind of frame-shift mutation of *hPMS2* occurred in 12 ostensibly unrelated Lynch syndrome patients with 20% being the deleterious mutation. However, those families with pathogenic mutation did not have significantly high incidence of Lynch syndrome associated malignant tumors, indicating that the germline mutation of *hPMS2* and occurrence of HNPCC were not concurrent sometimes. The patient with *hPMS2* gene mutation in our group also met the requirements of BG. By reviewing the family history of our mutation positive patient, we found that in her first-degree relatives, three suffered from colorectal cancer but diagnosed at age over 60 years, not in accordance with the typical feature of HNPCC. Although we are not so certain about this, the non-classical presentation of her family history, to some extent, represents the phenomenon of separation of HNPCC occurrence and *hPMS2* gene mutation.

At the same time, we detected the reported SNP in these 26 probands and found some interesting results. Most of the SNP (21/27) were in the exons and 12 were non-synonymous coding SNP(cSNP). Since these non-synonymous cSNP can induce the change of amino acid

and the relationship between cSNP and pathogenesis of HNPCC still remains unclear, whether they are involved in the development of HNPCC and HNPCC related tumors needs to be further investigated.

In conclusion, the germline mutation of *hPMS2* gene is rare in the probands of Chinese HNPCC families. Since the testing of *hPMS2* gene mutation is costly and complicated, it may be not reasonable to be included in the screening strategy of Chinese HNPCC. However, the frequency of SNP of *hPMS2* gene is high and further studies are needed to identify its relationship with HNPCC.

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COMMENTS

Background

Germline mutations in mismatched repair genes can lead to hereditary nonpolyposis colorectal cancer (HNPCC). Previously, the authors had analyzed the abnormalities of *hMSH2/hMLH1/hMSH6* genes in a series of Chinese HNPCC families and the total abnormality rate was only 53.4% (31/58). So the aberrant MMR genes such as *hPMS2* were suspected to be involved in Chinese HNPCC.

Research frontiers

HNPCC or Lynch syndrome, is an autosomal dominantly inherited disease with cancer-susceptibility. The testing of *hPMS2* gene mutation is costly and complicated, it may be not reasonable to be included in the screening strategy of Chinese HNPCC. However, the frequency of single nucleotide polymorphism (SNP) of *hPMS2* gene is high and further studies are needed to identify its relationship with HNPCC.

Innovations and breakthroughs

One HNPCC proband was found to carry the germline mutation of *hPMS2* gene. It was a new unreported coding SNP, which could also be detected in the control with an occurrence rate of 2.3% (3/130).

Applications

Germline mutations in genes can be used to diagnose early HNPCC and enrich the databases about HNPCC and SNP.

Peer review

This is an interesting article which deals with a remarkably rare germline mutation, namely PMS2, which is important in the etiology of Lynch syndrome (HNPCC). Their science appears to be sound.

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