

Analysis of human MutS homolog 2 missense mutations in patients with colorectal cancer

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Abstract. Germline mutations of DNA mismatch repair gene human MutS homolog 2 (*hMSH2*) are associated with hereditary nonpolyposis colorectal cancer (HNPCC). A total of one-third of these mutations are missense mutations. Several *hMSH2* missense mutations have been identified in patients in East Asia, although their function has not been evaluated. In the present study, the role of ten *hMSH2* missense mutations in the pathogenesis of colorectal cancer was examined. The hMSH2/hMSH6 protein interaction system was established using yeast two-hybrid screening. Next, the missense mutations were analyzed for their ability to affect the protein interaction of hMSH2 with its partner hMSH6. Additionally, the Sorting Intolerant from Tolerant tool was applied to predict the effects of different amino acid substitutions. The results demonstrated that certain *hMSH2* mutations (L173R and C199R) caused a significant functional change in the human hMutS α complex and were identified to be pathological mutations. The Y408C, D603Y, P696L and S703Y mutations partially affected interaction and partly affected the function of hMSH2. The remaining four variants, T8M, I169V, A370T and Q419K, may be non-functional polymorphisms or could affect protein function through other molecular mechanisms. The present study evaluated the functional consequences of previously unknown missense mutations in *hMSH2*, and may contribute to improved clinical diagnosis and mutation screening of HNPCC.

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is one of the most common inherited cancer susceptibility syndromes, and is characterized by the early development of a number of types of cancer, including colorectal cancer (1,2). HNPCC is associated with germline mutations in one of the six DNA mismatch repair genes (MMR). These are human MutL homolog 1 (*hMLH1*), *hMSH2*, human PMS1 homolog 2 (*hPMS2*), human MutS homolog 6 (*hMSH6*), *hPMS1* and *hMLH3*. The majority of germline mutations in the HNPCC have been identified in *hMSH2* and *hMLH1* (3). Genetic screening for HNPCC has primarily focused on these two genes and it was identified that ~half of the germline mutations identified in patients with HNPCC were detected in *hMSH2*. One-third of the germline mutations are missense mutations (4). The etiology of missense mutations is difficult to be evaluated with respect to nonsense mutations, frameshift mutations and large genomic aberrations. Whether a missense mutation is associated with a disease can be examined by carrier family data analysis or a rigorous case-control study based on a molecular epidemiological survey (5). However, there may be incomplete family data and a difficulty in obtaining epidemiological samples. Evaluation of missense mutations may lack direct experimental functional analysis, thus posing a challenge for the genetic diagnosis of hereditary colorectal cancer. Therefore, assessment of the pathogenicity of missense mutations is required if using the functional assays.

The DNA mismatch repair system aids maintenance of the stability of the genome. The human MSH2 protein is comprised of 934 amino acid residues, and the hMSH6 protein is comprised of 1360 amino acid residues. *In vivo*, hMSH2 and hMSH6 proteins constitute a heterologous heterodimer (hMutS α), which binds to the mismatch site and recognizes single base pair mismatches and small insertion or deletion ring structure (6,7). hMLH1 dimerizes with hPMS2 to form a hMutL heterodimer. Next, the hMutS α heterodimer and the hMutL heterodimer constitute a polymer, which recruits other proteins required for mismatch repair (8). The protein-protein interaction of hMSH2 with hMSH6 is the premise of the hMutS α function. Inhibition of the hMSH2-hMSH6 interaction, which is the potential consequence of a missense mutation, may be a major cause of MMR defects in HNPCC (9).

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A previous study established a protein interaction system for *hMLH1* and *hPMS2* using the yeast two-hybrid system, which was applied for the functional evaluation of missense mutations in *hMLH1* genes (10). In the present study, ten missense mutations of *hMSH2* that were frequently detected in patients with HNPCC in East Asia were selected. The *hMSH2* missense mutations were analyzed for their ability to affect the protein interaction of *hMSH2* with its partner *hMSH6* *in vivo*. Additionally, Sorting Intolerant from Tolerant tool (SIFT) tool was applied to predict the effects of amino acid substitution.

Materials and methods

Strains and plasmids. The wild-type *hMSH2* and *hMSH6* cDNA were kindly provided by Professor Josef Jiricny (Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland). Restriction enzymes and ligase were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). DNA ladders were purchased from GenScript (Piscataway, NJ, USA). The vectors for the yeast two-hybrid system pGADT7 and pGBKT7, the reporter strain of *Saccharomyces cerevisiae* AH109 and the *Escherichia coli* strain Top10 were purchased from Clontech Laboratories, Inc. (Mountainview, CA, USA). *E. coli* strain Top10 was used for the genetic engineering of all plasmids.

Characteristics of the variants. A total of 10 missense mutations localized to the entire coding region of *hMSH2* gene were analyzed. A frame-shift mutation, c.1664delA, was used as a positive control (11-18). Mutations were identified in patients with HNPCC from East Asia (Table I).

Mutation scanning of normal individuals. A total of 100 healthy Chinese controls, 55 males and 45 females (age range, 35-75 years old), were randomly recruited in The Jiangsu Cancer Hospital (Nanjing, China) from October 2015 to December 2015. Peripheral blood was obtained from normal individuals. Genomic DNA was isolated from 200 μ l peripheral blood using the QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. DNA was subjected to polymerase chain reaction (PCR) amplification of 16 exons of *hMSH2* gene using specific primers as listed in Table II, respectively. PCR amplification was performed in a volume of 25 μ l that contained 50 ng genomic DNA, 0.5 pmol each primer, 2.5 μ l 10X Taq buffer, 2.5 μ l 25 mM MgCl₂, 2 μ l 2.5 mM dNTPs and 1 U Taq DNA Polymerase (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A thermal cycler (Applied Biosystems Veriti Thermal Cycler; Thermo Fisher Scientific, Inc.) was used for PCR with the following cycling conditions: An initial denaturation step at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 0.5 min; annealing at 55°C for 0.5 min; and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The PCR product was detected on denaturing high-performance liquid chromatography (WAVE System; Transgenomic, Inc., Omaha, NE, USA) to screen the germline mutations of normal individuals of the specific exons. Written informed consent was obtained from all individuals. The study was approved by the Ethics Committee of the Jiangsu Institute of Cancer Research (Nanjing, China).

In silico analysis of *hMSH2* variants. The SIFT tool was used to predict whether an amino acid substitution is deleterious (19,20). SIFT prediction is based on a degree of conservation of amino acid residues in sequence alignments derived from closely associated sequences, obtained from PSI-BLAST (<http://sift.jcvi.org/>). SIFT calculates normalized probabilities for all possible substitutions from the alignment. Positions with normalized probabilities <0.05 are predicted to be deleterious, and those \geq 0.05 are predicted to be tolerated. In the present study, 10 missense mutations in the *hMSH2* gene were analyzed to identify deleterious variants.

Plasmid construction. Wild-type *hMSH2* cDNA was cloned into pGADT7 using the restriction enzymes *Sfi*I and *Xho*I to produce transcriptional AD-fused *hMSH2* construct pGADT7-*hMSH2*. Wild-type *hMSH6* cDNA was ligated into pGBKT7 using the restriction enzymes *Eco*RI and *Sal*I, to produce the transcriptional BD-fused *hMSH6* construct pGBKT7-*hMSH6*. Using pGBKT7-*hMSH6* as the template, various domains of *hMSH6* were amplified with primers containing *Eco*RI and *Sal*I restriction sites (Table III). PCR amplification was performed in a volume of 25 μ l that contained 50 ng pGBKT7-*hMSH6*, 0.5 pmol each primer, 2.5 μ l 10X Taq buffer, 2.5 μ l 25 mM MgCl₂, 2 μ l 2.5 mM dNTPs and 1 U TaKaRa LA Taq Polymerase (Takara Biotechnology Co., Ltd.). The Applied Biosystems Veriti Thermal Cycler was used for PCR with the following cycling conditions: An initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 0.5 min; annealing at 55°C for 0.5 min; and extension at 72°C for 5 min, followed by a final extension step at 72°C for 10 min. The PCR-amplified fragments were then cloned into pGBKT7 using restriction enzymes (*Eco*RI and *Sal*I), to produce recombinant pGBKT7 plasmids with various *hMSH6* domains. Genetic sequencing was then performed using the dideoxynucleotide chain termination method with an automatic sequencer (ABI 3100; Thermo Fisher Scientific, Inc.) (21).

Site-directed mutagenesis and *hMSH2* variant construction. The nucleotide substitutions were introduced into the *hMSH2* cDNA using overlapping PCR site-directed mutagenesis, as described previously (22). Upstream primers Fa, Ra and downstream primers Fb and Rb were synthesized according to the mutation site and PCR reactions were applied. In the first PCR reaction, the upstream fragment was amplified with the upstream primer Fa and the mutant primer Ra, using pGADT7-*hMSH2* as template. In the second PCR reaction, the downstream fragment was amplified with the downstream primer Rb and the mutant primer Fb using pGADT7-*hMSH2* as template. In the third PCR reaction, the final fragment with the target mutant was amplified with primers Fa and Rb using the previous two overlapping PCR-amplified products as the template. Specific primers were listed in Table IV. PCR amplification was performed in a volume of 25 μ l that contained 50 ng template DNA, 0.5 pmol of each primer, 2.5 μ l 10X Taq buffer, 2.5 μ l 25 mM MgCl₂, 2 μ l 2.5 mM dNTPs and 1 U Taq DNA Polymerase (Fermentas; Thermo Fisher Scientific, Inc.). The Applied Biosystems Veriti Thermal Cycler was used for PCR with the following cycling conditions: An initial denaturation step at 95°C for 2 min,

Table I. Clinical and functional characteristics of the 10 *hMSH2* missense variants in the present study.

Exon	<i>hMSH2</i> variation	Nucleotide substitution	Source (Ref.)	Occurrence in healthy individuals	SIFT score
1	T8M	c.23C>T	Chinese, Japanese (11,12)	0/100	0.10
3	I169V	c.505A>G	Chinese (13)	0/100	0.30
3	L173R	c.518T>G	Chinese (13)	0/100	0.00
3	C199R	c.595T>C	Chinese (14)	0/100	0.00
7	A370T	c.1108G>A	Chinese (15)	0/100	1.00
7	Y408C	c.1223A>G	Chinese (13,16)	0/100	0.00
7	Q419K	c.1255C>A	Chinese (13,16)	0/100	0.72
11	Frameshift	c.1664delA	Chinese (11)		
12	D603Y	c.1807G>T	Chinese (17)	0/100	0.02
13	P696L	c.2087C>T	Chinese (15)	0/100	0.00
13	S703Y	c.2108C>A	Chinese (18)	0/100	0.01

hMSH2, human MutS homolog 2; SIFT, Sorting Intolerant from Tolerant.

Table II. List of primer sequences used for polymerase chain reaction amplification of the exons of *hMSH2* gene.

<i>hMSH2</i> gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Exon 1	GCATTTTCTTCAACCAGGAG	GTCCCTCCCCAGCACGC
Exon 2	GAAGTCCAGCTAATACAGTGC	CTTCACATTTTTATTTTTCTACTC
Exon 3	TTTTAAAGTATGTTCAAGAGTTTG	ATCTCCTCTACTACTAGACTC
Exon 4	TTTCATTTTTGCTTTTCTTATTCC	TGTAATTCACATTTATAATCCATG
Exon 5	CCAGTGGTATAGAAATCTTCC	CCATTCAACATTTTTAACCC
Exon 6	GTTTTCACTAATGAGCTTGCC	GTGGTATAATCATGTGGG
Exon 7	GACTTACGTGCTTAGTTG	TATGAGGACAGCACATTGCC
Exon 8	GATTTGTATTCTGTAATAATGAGATC	CTACAACTTTCTTAAAGTGGC
Exon 9	TGCTTTACCCATTATTTATAGG	CAACCTCCAATGACCCATTC
Exon 10	TGGTAGTAGGTATTTATGGAATAC	CATCATGTAGAGCATTTAGGG
Exon 11	GTACACATTGCTTCTAGTACAC	AGCCAGGTGACATTCAGAAC
Exon 12	ATTCAGTATTCCTGTGTAC	CGTTACCCCCACAAAGC
Exon 13	CGCGATTAATCATCAGTG	GGACAGAGACATACATTTCTATC
Exon 14	GTTACCACATTTTATGTGATGG	TTCAAGGGTAGTAAGTTTCCC
Exon 15	CTCTTCTCATGCTGTCCC	ATAGAGAAGCTAAGTTAAAC
Exon 16	TAATTACTAATGGGACATTC	TACCTTCATTCCATTACTGG

hMSH2, human MutS homolog 2.

followed by 35 cycles of denaturation at 95°C for 0.5 min; annealing at 55°C for 0.5 min; and extension at 72°C for 2 min, followed by a final extension step at 72°C for 5 min. The final PCR product was cut with appropriate restriction enzymes (Table IV) and cloned into pGADT7-*hMSH2* to replace the wild-type fragment. The recombinant plasmid pGADT7-*hMSH2* with the target mutation was verified by sequencing using an automatic sequencer (ABI 3100; Thermo Fisher Scientific, Inc.).

Yeast two-hybrid screening. The Matchmaker GAL4-based two-hybrid system (Clontech Laboratories, Inc.) was

used in the present study. The recombinant plasmids pGBKT7-*hMSH6* and pGADT7-*hMSH2* (wild type or mutant) were co-transformed into *S. cerevisiae* strain AH109 and plated onto dropout medium SD/-Trp/-Leu (Clontech Laboratories, Inc.) at 30°C for 2-4 days. A total of 10 clones were then selected and inoculated on the dropout medium SD/-Trp/-leu/-His (Clontech Laboratories, Inc.) at 30°C for 2-6 days. The growth status of each transformant in the defect plate was then observed. The clone had normal growth when the transformant grew in two days, whereas slow growth was observed following 2-4 days of culture. No growth was observed >4 days of culture.

Table III. List of primer sequences used for polymerase chain reaction amplification of various hMSH6 domains.

Domain	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
MutS I-IV	CGGAATTCTCTGCCCTCAAATTCTG	GCGTCGACTAAGGACCATCACCCCCTCGAC
MutS I-V	CGGAATTCTCTGCCCTCAAATTCTG	GCGTCGACTATAATTCCTTAATCAAAGTCAG
MutS II-IV	CGGAATTCACCAAGGGTACACAGACTTAC	GCGTCGACTAAGGACCATCACCCCCTCGAC
MutS II-V	CGGAATTCACCAAGGGTACACAGACTTAC	GCGTCGACTATAATTCCTTAATCAAAGTCAG
MutS III-IV	CGGAATTCAGCACTACAAGATCTGGTGC	GCGTCGACTAAGGACCATCACCCCCTCGAC
MutS III-V	CGGAATTCAGCACTACAAGATCTGGTGC	GCGTCGACTATAATTCCTTAATCAAAGTCAG

Results

Plasmid construction. Gene engineering was used to construct the recombinant plasmids pGADT7-hMSH2, pGBKT7-hMSH6 from wild-type *hMSH2* and *hMSH6* cDNA (Fig. 1). Overlapping PCR site-directed mutagenesis was used to construct 11 mutated pGADT7-hMSH2 plasmids. All wild-type and variant plasmids were constructed successfully and verified by sequencing. The hMSH6 protein is comprised of 1,360 amino-acid residues, which can be divided into a N-terminal domain (residues, 1-409), and MutS I, II, III, IV and V functional domains (MutS I is the mismatch-binding domain, MutS II is the connector domain, MutS III is the core domain, MutS IV is the clamp domain and MutS V is the ATPase domain) (6,7). These domains all interact with the corresponding functional domains of hMSH2 protein to complete the mismatch repair function *in vivo* (8) hMSH6 has a large molecular weight which may prevent proper protein folding in yeast. A total of six recombinant pGBKT7 plasmids with various hMSH6 domains were constructed, including pGBKT7-hMSH6-MutS I-IV (residues, 341-1,073), MutS I-V (residues, 341-1,360), MutS II-IV (residues, 518-1,073), MutS II-V (residues, 518-1,360), MutS III-IV (residues, 718-1,073) and MutS III-V (residues, 718-1,360) (Fig. 2) (6,7). All of the pGBKT7 plasmids with different hMSH6 domains were constructed successfully and verified by sequencing.

Mutation identification in 100 normal individuals. A total of 100 individuals were recruited in The Jiangsu Cancer Hospital. Denaturing high-performance liquid chromatography analysis was used to screen the germline mutations of the normal individuals by PCR of the specific exons. None of the missense mutations examined in the present study were identified in the normal controls (Table I).

Prediction of hMSH2 protein activity and structure. The SIFT score of *hMSH2* mutations demonstrated that six of them were considered to be intolerable (L173R, C199R, Y408C, D603Y, P696L and S703Y), indicating that these amino acid substitutions may affect the function of the protein. The remaining four mutations (T8M, I169V, A370T and Q419K) were considered to be tolerable (Table I).

Yeast two-hybrid assay of hMSH2 and hMSH6 domains. Recombinant plasmids pGADT7-hMSH2 (wild type) and pGBKT7 with various hMSH6 domains were co-transformed into *S. cerevisiae* strain *AH109* and selected on nutritional defect

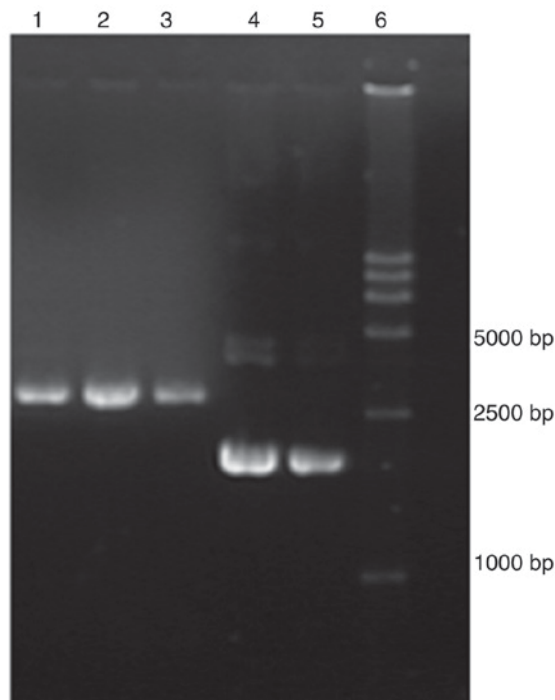


Figure 1. Polymerase chain reaction electrophoresis of plasmid pGBKT7-hMSH6. Lanes 1, 2 and 3, MutS I-V fragments (3.1 kb); lanes 4 and 5, MutS III-V fragments (1.9 kb); lane 6, DNA ladder.

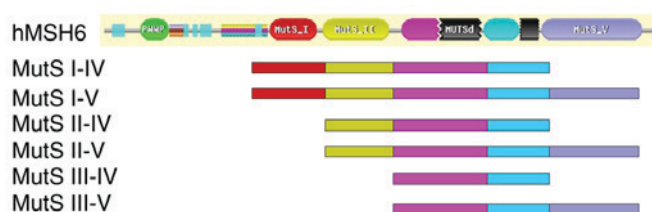


Figure 2. Scope of six hMSH6 domains. hMSH6, human MutS homolog 6. hMSH6 (residues 1-1360), MutS I-IV (residues 341-1073), MutS I-V (residues 341-1360), MutS II-IV (residues 518-1073), MutS II-V (residues 518-1360), MutS III-IV (residues 718-1073) and MutS III-V (residues 718-1360).

plates. The growth status of each transformant was observed (Table V). The negative control was pGADT7/pGBKT7 and the positive control was pGADT7-hMLH1/pGBKT7-hPMS2 (10). It was demonstrated that the *AH109* two-hybrid transformant pGADT7-hMSH2/pGBKT7-hMSH6-MutS II-V and pGADT7-hMSH2/pGBKT7-hMSH6-MutS III-V grew on the histidine-deficient medium (SD/-Trp/-leu/-His). The results

Table IV. List of primer sequences used for polymerase chain reaction amplification of *hMSH2* variants and appropriate restriction enzymes for cloning.

Mutation site	Primer sequence (5'-3')	Restriction enzymes
c.23C>T, T8M	Fa: CTAACGTTTCATGATAACTTCATG Ra: CTCCAAGTGCAGCaTCTCCTTCGGC Fb: GCCGAAGGAGAtGCTGCAGTTGGAG Rb: AATTCTGCATCTTCTACAAAAGC	<i>AclI</i> <i>HindIII</i>
c.505A>G, I169V	Fa: CGGGCCATGGAGGCCCTGGCAATCTCTCTCAGTTTG Ra: GTTTCCTCTGTAcGGAATCCACATAC Fb: GTATGTGGATTCCgTACAGAGGAAAC Rb: AATTCTGCATCTTCTACAAAAGC	<i>SfiI</i> <i>HindIII</i>
c.518T>G, L173R	Fa: CGGGCCATGGAGGCCCTGGCAATCTCTCTCAGTTTG Ra: CACACAGTCCTcGTTTCCTCTGTATG Fb: CATAAGAGGAAACgAGGACTGTGTG Rb: AATTCTGCATCTTCTACAAAAGC	<i>SfiI</i> <i>HindIII</i>
c.595T>C, C199R	Fa: CGGGCCATGGAGGCCCTGGCAATCTCTCTCAGTTTG Ra: CGGGTAAAACACgTTCCTTTGGTCC Fb: GGACCAAAGGAAcGTGTTTTACCCG Rb: AATTCTGCATCTTCTACAAAAGC	<i>SfiI</i> <i>HindIII</i>
c.1108G>A, A370T	Fa: CGGGCCATGGAGGCCCTGGCAATCTCTCTCAGTTTG Ra: GCCTCAATTCTGtATCTTCTACAAAAG Fb: CTTTTGTAGAAGATaCAGAATTGAGGC Rb: GCCTCGAGTCACGTAGTAACCTTTTATTCGTG	<i>SfiI</i> <i>XhoI</i>
c.1223A>G, Y408C	Fa: GAGAGATTGAATTTAGTGGAAAGC Ra: GATTTATACCCTGAcAGAGTCGGTAAC Fb: GTTACCGACTCTgTCAGGGTATAAATC Rb: GCCTCGAGTCACGTAGTAACCTTTTATTCGTG	<i>HindIII</i> <i>XhoI</i>
c.1255C>A, Q419K	Fa: GAGAGATTGAATTTAGTGGAAAGC Ra: GTTTTTCCAGAGCCTtTATAACATTAGG Fb: CCTAATGTTATAaAGGCTCTGGAAAAAC Rb: GCCTCGAGTCACGTAGTAACCTTTTATTCGTG	<i>HindIII</i> <i>XhoI</i>
c.1664delA, frameshift	Fa: GAGAGATTGAATTTAGTGGAAAGC Ra: CATTAAAGAAGTCAATTGCTGTTGGTAAATTTAAC Fb: GTTAAATTTACCAACAGCAATTGACTTCTTTAAATG Rb: GCCTCGAGTCACGTAGTAACCTTTTATTCGTG	<i>HindIII</i> <i>XhoI</i>
c.1807G>T, D603Y	Fa: GAGAGATTGAATTTAGTGGAAAGC Ra: GACAACAGCATaTAGCTGAGCTAAC Fb: GTTAGCTCAGCTaATGCTGTTGTC Rb: GCCTCGAGTCACGTAGTAACCTTTTATTCGTG	<i>HindIII</i> <i>XhoI</i>
c.2087C>T, P696L	Fa: GAGAGATTGAATTTAGTGGAAAGC Ra: GCTGACTCACATaGCACAAAACACC Fb: GGTGTTTTGTGcAtATGTGAGTCAGC Rb: GCCTCGAGTCACGTAGTAACCTTTTATTCGTG	<i>HindIII</i> <i>XhoI</i>
c.2108C>A, S703Y	Fa: GAGAGATTGAATTTAGTGGAAAGC Ra: CAGTCCACAATGtACACTTCTGCTG Fb: CAGCAGAAGTGTaCATTGTGGACTG Rb: GCCTCGAGTCACGTAGTAACCTTTTATTCGTG	<i>HindIII</i> <i>XhoI</i>

Fa, forward primer sequence a; Fb, forward primer sequence b; Ra, reverse primer sequence a; Rb, reverse primer sequence b.

demonstrated that hMSH6-MutS II-V and MutS III-V may interact with hMSH2 in the yeast strain AH109. Additionally,

the two-hybrid transformants pGADT7/pGBKT7-hMSH6-MutS II-V and pGADT7/pGBKT7-hMSH6-MutS III-V did not

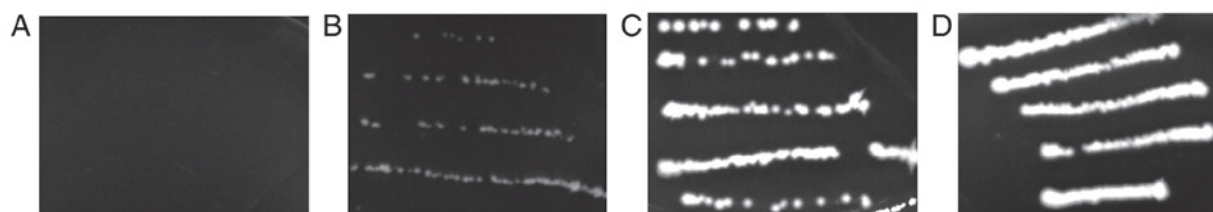


Figure 3. Yeast two-hybrid system for *hMSH2* variants and *hMSH6*-MutS II-V domains. The two-hybrid transformant clones (A) C199R, (B) P696L (C) T8M and (D) wild-type *hMSH2* were selected on SD/-Trp/-leu/-His plate, followed by incubation at 30°C for 4 days. *hMSH2*, human MutS homolog 2; SD, synthetic defined.

Table V. Yeast two-hybrid assay of *hMSH2* and various *hMSH6* domains.

Transformant	Growth status
pGADT7/pGBKT7 (negative control)	-
pGADT7- <i>hMSH2</i> /pGBKT7- <i>hMSH6</i>	-
pGADT7- <i>hMSH2</i> /pGBKT7- <i>hMSH6</i> -MutS I-IV	-
pGADT7- <i>hMSH2</i> /pGBKT7- <i>hMSH6</i> -MutS I-V	-
pGADT7- <i>hMSH2</i> /pGBKT7- <i>hMSH6</i> -MutS II-IV	-
pGADT7- <i>hMSH2</i> /pGBKT7- <i>hMSH6</i> -MutS II-V	+
pGADT7- <i>hMSH2</i> /pGBKT7- <i>hMSH6</i> -MutS III-IV	-
pGADT7- <i>hMSH2</i> /pGBKT7- <i>hMSH6</i> -MutS III-V	+
pGADT7- <i>hMLH1</i> /pGBKT7- <i>hPMS2</i> (positive control)	+
pGADT7/pGBKT7- <i>hMSH6</i> -MutS II-V	-
pGADT7/pGBKT7- <i>hMSH6</i> -MutS III-V	-

hMSH2, human MutS homolog 2; +, two-hybrid transformant grew normally in histidine-deficient medium; -, two-hybrid transformant did not grow in the histidine-deficient medium.

grew on the histidine-deficient medium. These results precluded the self-activation of pGADT7/pGBKT7-*hMSH6*-MutS II-V and pGADT7/pGBKT7-*hMSH6*-MutS III-V for His reporter gene in yeast strain *AH109*.

Yeast two-hybrid assay of *hMSH2* variants and *hMSH6*-MutS II-V. Mutant pGADT7-*hMSH2* and pGBKT7-*hMSH6*-MutS II-V plasmids were co-transformed into *S. cerevisiae* strain *AH109* and selected on a SD/-Leu-Trp plate. The selected clones were then inoculated on a SD/-Trp/-leu/-His plate. The growth status of each transformant was observed (Table VI). The negative control was pGADT7/pGBKT7 and positive control was pGADT7-*hMSH2*/pGBKT7-*hMSH6*-MutS II-V. It was demonstrated that the yeast two-hybrid transformants T8M, I169V, A370T and Q419K grew normally, the transformants Y408C, D603Y, P696L and S703Y grew slowly, whereas the

Table VI. Yeast two-hybrid Assay of *hMSH2* variants and *hMSH6*-MutS II-V.

<i>hMSH2</i> variation	Growth status
Wild-type <i>hMSH2</i>	+
T8M	+
I169V	+
L173R	-
C199R	-
A370T	+
Y408C	+/-
Q419K	+
c.1664delA	-
D603Y	+/-
P696L	+/-
S703Y	+/-

hMSH2, human MutS homolog 2; +, two-hybrid transformant grew normally in histidine-deficient medium; -, two-hybrid transformant did not grow in the histidine-deficient medium; +/-, two-hybrid transformant had slow growth on the histidine-deficient medium.

transformants L173R, C199R and c.1664delA did not grow on the histidine-deficient medium in yeast strain *AH109* compared with wild-type *hMSH2* (Fig. 3). The Y408C, D603Y, P696L and S703Y mutations may partly affect the function of *hMSH2* in yeast strain *AH109*. c.1664delA is a frameshift mutation, thus *hMSH2* c.1664delA can not bind *hMSH6*-MutS II-V. The yeast two-hybrid transformant pGADT7-*hMSH2*-c.1664delA/pGBKT7-*hMSH6*-MutS II-V did not grow in the histidine-deficient medium in yeast strain *AH109*. The L173R and C199R mutants gave similar results to those of c.1664delA, indicating that the mutations prevented the interaction between *hMSH2* and *hMSH6*.

Discussion

In the present study, 10 missense mutations to *hMSH2* were examined in patients with HNPCC from East Asia. The functional consequences of the variants were analyzed based on their genetic, epidemiological and functional characteristics.

In vitro measurement of MMR function has been used as a direct method to evaluate the variants in the *hMSH2* gene (23). It was difficult to perform this complex experiment since it required expression and purification of proteins. The

yeast two-hybrid system has been used as a method to study the interaction between proteins (24). A previous study established a protein interaction system for hMLH1 and hPMS2 using the yeast two-hybrid system, which was applied for the functional evaluation of missense mutations in *hMLH1* genes (10). The yeast two-hybrid system has advantages for detecting protein binding: Proteins may maintain a natural conformation to mimic the physiological state *in vivo*, and this system eliminates the time-consuming steps necessary for protein purification *in vitro*. A previous study used a similar system to evaluate the partial *hMSH2* missense mutations I169V, L173R, Y408C, Q419K and S703Y (25). In the present study, the yeast two-hybrid experimental method has been improved and the results have been evaluated using SIFT to evaluate the enlarged *hMSH2* missense mutations. The yeast two-hybrid system was used to analyze the ability of *hMSH2* missense mutations to affect the interaction of hMSH2 with its partner hMSH6 *in vivo*. The growth status of each transformant provided an indication of the protein interaction strength, as the enzyme activity of reporter genes was associated with the degree of protein binding.

Genetic engineering was used to construct recombinant plasmids pGADT7-hMSH2 and pGBKT7-hMSH6. Next, the recombinant plasmids pGADT7-hMSH2 and pGBKT7-hMSH6 were co-transformed into *S. cerevisiae* strain AH109. The results demonstrated that the yeast two-hybrid transformant couldn't grow in histidine-deficient medium, indicating that the interaction between hMSH2 and hMSH6 may be inhibited in yeast AH109. The possible reason behind this may be the inappropriate folding of hMSH6 protein when expressed in yeast strain AH109, owing to its size. Additionally, six recombinant pGBKT7 plasmids with various hMSH6 domains were constructed. The results demonstrated that the transformant pGADT7-hMSH2/pGBKT7-hMSH6-MutS II-V and pGADT7-hMSH2/pGBKT7-hMSH6-MutS III-V were able to grow on the histidine-deficient medium (Table V). These results indicate that hMSH6-MutS II-V and MutS III-V may interact with hMSH2 in yeast strain AH109. The hMSH6 MutS II-V domain contains amino acid residues 518-1,360 of the hMSH6 protein, which comprises the core regions of the full protein. The yeast two-hybrid transformant pGADT7-hMSH2/pGBKT7-hMSH6-MutS II-V was used to construct the interaction system of hMSH2-hMSH6 and to evaluate *hMSH2* missense variants (Table VI).

SIFT scores revealed that T8M, I169V, A370T and Q419K were tolerant substitutions. The results of the present study indicated that these variants may be nonfunctional polymorphisms or may affect the protein function through other molecular mechanisms.

SIFT scores revealed that Y408C, D603Y, P696L and S703Y were intolerable substitutions. In the Y408C mutant, tyrosine and cysteine belong to neutral amino acids, but their stereochemistry differs. For the D603Y mutant, aspartic acid is an acidic amino acid, whereas tyrosine is an aromatic amino acid, meaning their reactivities may differ. P696L was associated with a loss of hMSH2 protein expression and high level of microsatellite instability in tumors (15). For S703Y, homology modeling revealed that steric hindrance was increased because of the serine-to-tyrosine mutation at residue 703. Finally, the hydrogen bonds of Ala700 and Glu706 were not formed, affecting

the whole molecular structure and leading to inactivation of hMSH2 function (18). The yeast two-hybrid transformant of the four variants grew slowly on the histidine-deficient medium. Therefore, these variants may affect the partial interaction and partly affect the function of hMSH2.

L173R and C199R are highly conserved from yeast to humans (6). SIFT scores revealed that they were intolerable substitutions. For the L173R mutation, leucine is an uncharged hydrophobic amino acid, whereas arginine is a positively charged, weak-basic amino acid. Their functions may differ as a result of differences in solubility and ionization properties. For the C199R mutation, cysteine is an uncharged hydrophilic amino acid, whereas arginine is a positively charged, weak-basic amino acid. Their functions may also differ as a result of differences in stereochemistry and ionization properties. The yeast two-hybrid transformant of the two variants, including c.1664delA, did not grow on histidine-deficient medium. c.1664delA is a frameshift mutation, thus hMSH2 c.1664delA is not able to bind hMSH6-MutS II-V. L173R and C199R may have a similar effect as c.1664delA. Therefore, it was considered that they these mutations may be pathological.

In the present study, segregation studies were not conducted because of the unavailability of family samples. Therefore, the synergistic effect of low-risk alleles of MMR genes, which was previously described (26), was not evaluated in the present study.

In conclusion, the present study established the preliminary hMSH2/hMSH6 protein-interaction system using a yeast two-hybrid system. Combined with SIFT analysis and amino acid analysis, an evaluation system for missense mutations in *hMSH2* was established. Using this system, 10 missense mutations in *hMSH2* were examined. The results demonstrated that the *hMSH2* mutations L173R and C199R caused a functional change in the hMutSa complex and were identified as pathological mutations. The Y408C, D603Y, P696L and S703Y variants partially affected interaction, and may partly affect the function of hMSH2. The remaining four variants, T8M, I169V, A370T and Q419K, may be non-functional polymorphisms or may affect the protein function through other molecular mechanisms. The present study evaluated the functional consequences of several previously unknown missense mutations in the *hMSH2* gene, which could contribute to clinical diagnosis and mutation screening of HNPCC.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MZ designed the study, performed the research and wrote the manuscript. XZ and SC constructed all the plasmids. JY analyzed the data. YZ performed the yeast two-hybrid assay. ML collected the blood samples and performed mutation scanning of normal individuals. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of the Jiangsu Institute of Cancer Research (Nanjing, China) and written informed consent was obtained from all individuals.

Consent for publication

Informed consent for the publication of any associated data and accompanying images was obtained from all the normal individuals.

Competing interests

The authors declare that they have no competing interests.

References

- Brosens LA, Offerhaus GJ and Giardiello FM: Hereditary colorectal cancer: Genetics and screening. *Surg Clin North Am* 95: 1067-1080, 2015.
- Lynch HT and de la Chapelle A: Genetic susceptibility to non-polyposis colorectal cancer. *J Med Genet* 36: 801-818, 1999.
- InSiGHT: InSiGHT variant databases. <https://www.insight-group.org/variants/databases/>. Accessed June 29, 2017.
- Woods MO, Williams P, Careen A, Edwards L, Bartlett S, McLaughlin JR and Youngusband HB: A new variant database for mismatch repair genes associated with Lynch syndrome. *Hum Mutat* 28: 669-673, 2007.
- Peltomaki P and Vasen HF: Mutations predisposing to hereditary nonpolyposis colorectal cancer: Database and results of a collaborative study. *The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. Gastroenterology* 113: 1146-1158, 1997.
- Obmolova G, Ban C, Hsieh P and Yang W: Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. *Nature* 407: 703-710, 2000.
- Lamers MH, Perrakis A, Enzlin JH, Winterwerp HH, de Wind N and Sixma TK: The crystal structure of DNA mismatch repair protein MutS binding to a GxT mismatch. *Nature* 407: 711-717, 2000.
- Jiricny J: The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol* 7: 335-346, 2006.
- Geng H, Sakato M, DeRocco V, Yamane K, Du C, Erie DA, Hingorani M and Hsieh P: Biochemical analysis of the human mismatch repair proteins hMutS α MSH2(G674A)-MSH6 and MSH2-MSH6(T1219D). *J Biol Chem* 287: 9777-9791, 2012.
- Fan Y, Wang W, Zhu M, Zhou J, Peng J, Xu L, Hua Z, Gao X and Wang Y: Analysis of hMLH1 missense mutations in East Asian patients with suspected hereditary nonpolyposis colorectal cancer. *Clin Cancer Res* 13: 7515-7521, 2007.
- Wang XL, Yuan Y, Zhang SZ, Cai SR, Huang YQ, Jiang Q and Zheng S: Clinical and genetic characteristics of Chinese hereditary nonpolyposis colorectal cancer families. *World J Gastroenterol* 12: 4074-4077, 2006.
- Nomura S, Sugano K, Kashiwabara H, Taniguchi T, Fukayama N, Fujita S, Akasu T, Moriya Y, Ohhigashi S, Kakizoe T and Sekiya T: Enhanced detection of deleterious and other germline mutations of hMSH2 and hMLH1 in Japanese hereditary nonpolyposis colorectal cancer kindreds. *Biochem Biophys Res Commun* 271: 120-129, 2000.
- Fan Y, Liu X, Zhang H, Dai J, Zhang X, Zhu M, Gao X and Wang Y: Variations in exon 7 of the MSH2 gene and susceptibility to gastrointestinal cancer in a Chinese population. *Cancer Genet Cytogenet* 170: 121-128, 2006.
- Leung SY, Chan TL, Chung LP, Chan AS, Fan YW, Hung KN, Kwong WK, Ho JW and Yuen ST: Microsatellite instability and mutation of DNA mismatch repair genes in gliomas. *Am J Pathol* 153: 1181-1188, 1998.
- Tang R, Hsiung C, Wang JY, Lai CH, Chien HT, Chiu LL, Liu CT, Chen HH, Wang HM, Chen SX, *et al*: Germ line MLH1 and MSH2 mutations in Taiwanese Lynch syndrome families: Characterization of a founder genomic mutation in the MLH1 gene. *Clin Genet* 75: 334-345, 2009.
- Zhang Y, Liu X, Fan Y, Ding J, Xu A, Zhou X, Hu X, Zhu M, Zhang X, Li S, *et al*: Germline mutations and polymorphic variants in MMR, E-cadherin and MYH genes associated with familial gastric cancer in Jiangsu of China. *Int J Cancer* 119: 2592-2596, 2006.
- Yuen ST, Chan TL, Ho JW, Chan AS, Chung LP, Lam PW, Tse CW, Wyllie AH and Leung SY: Germline, somatic and epigenetic events underlying mismatch repair deficiency in colorectal and HNPCC-related cancers. *Oncogene* 21: 7585-7592, 2002.
- Jin HY, Yan HL, Song LH, Cui L, Ding YJ and Sun SH: Identification and functional analysis of a novel germ-line mutation in hMSH2 from a Chinese hereditary nonpolyposis colorectal cancer family. *Acad J Sec Mil Med Univ* 26: 888-891, 2005 (In Chinese).
- Ng PC and Henikoff S: Predicting deleterious amino acid substitutions. *Genome Res* 11: 863-874, 2001.
- Kumar P, Henikoff S and Ng PC: Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 4: 1073-1081, 2009.
- Vander Molen J, Frisse LM, Fullerton SM, Qian Y, Del Bosque-Plata L, Hudson RR and Di Rienzo A: Population genetics of CAPN10 and GPR35: Implications for the evolution of type 2 diabetes variants. *Am J Hum Genet* 76: 548-560, 2005.
- Nystrom-Lahti M, Perrera C, Raschle M, Panyushkina-Seiler E, Marra G, Curci A, Quaresima B, Costanzo F, D'Urso M, Venuta S and Jiricny J: Functional analysis of MLH1 mutations linked to hereditary nonpolyposis colon cancer. *Genes Chromosomes Cancer* 33: 160-167, 2002.
- Clark AB, Cook ME, Tran HT, Gordenin DA, Resnick MA and Kunkel TA: Functional analysis of human MutS α and MutS β complexes in yeast. *Nucleic Acids Res* 27: 736-742, 1999.
- Rodríguez-Negrete E, Bejarano ER and Castillo AG: Using the yeast two-hybrid system to identify protein-protein interactions. *Methods Mol Biol* 1072: 241-258, 2014.
- Zhu M, Fan YM, Zhu YB and Wang YP: Establishment of a hMSH2/hMSH6 protein interaction system and functional evaluation of hMSH2 gene missense mutations. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 30: 559-564, 2013 (In Chinese).
- Duratturo F, Liccardo R, Cavallo A, De Rosa M, Grosso M and Izzo P: Association of low-risk MSH3 and MSH2 variant alleles with Lynch syndrome: Probability of synergistic effects. *Int J Cancer* 129: 1643-1650, 2011.



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