# Analysis of hMLH1 Missense Mutations in East Asian Patients with Suspected Hereditary Nonpolyposis Colorectal Cancer

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**Abstract**

**Purpose:** Germ line mutations in the DNA mismatch repair gene *hMLH1* are a frequent cause of hereditary nonpolyposis colorectal cancer and about one-third of these are missense mutations. Several missense mutations in *hMLH1* have frequently been detected in East Asian patients with suspected hereditary nonpolyposis colorectal cancer, but their pathogenic role has not been extensively assessed. The aim of this study was to perform functional analyses of these variants and their association with gastrointestinal cancer in East Asians.

**Experimental Design:** Altogether, 10 *hMLH1* variants were analyzed by yeast two-hybrid and coimmunoprecipitation assays.

**Results:** The carboxyl-terminal replacements Q542L, L549P, L574P, and P581L in hMLH1 resulted in complete loss of activity in both yeast two-hybrid and coimmunoprecipitation tests and thus might be considered as pathogenic. The amino-terminal variants S46I, G65D, G67R, and R217C did not affect complex formation with hPMS2 in coimmunoprecipitation, but partly or fully lost their activity in yeast two-hybrid assay, and we suggested that these variants might reduce the efficiency of the heterodimer to go into the nucleus and thus the mismatch repair function might be blocked or reduced. The V384D and the Q701K variant resulted in the interaction of hMLH1 with hPMS2 at reduced efficiency and might raise the gastrointestinal cancer risk of the mutation carriers.

**Conclusions:** This work availably evaluated the functional consequences of some missense mutations not previously determined in the *hMLH1* gene and might be useful for the clinical diagnosis of hereditary gastrointestinal cancer, especially in East Asians.

The DNA mismatch repair (MMR) system ensures genomic stability in humans. The heterodimers of MSH2 and MSH6 (MutSα) or MSH2 and MSH3 (MutSβ) initiate repair by recognizing single–base pair mismatches and insertion-deletion loops. Similarly, MLH1 dimerizes with PMS2, PMS1, or MLH3 to form three kinds of MutL heterodimers: MutLα, MutLβ, and MutLγ. Upon mismatch binding, the MutS heterodimers recruit MutLα (MLH1-PMS2), which plays an essential role in the MMR system (1–3). Deficiency in MMR causes a mutator phenotype and is linked to cancer, in particular, with hereditary nonpolyposis colorectal cancer (HNPCC). Approximately 50% of germ line mutations identified in patients with HNPCC are in *MLH1*, with about one-third of them being missense mutations. But the functional consequences of missense mutations are difficult to determine. In general, missense mutations are predicted to be pathogenic if they segregate with the disease phenotype, if they are not observed in the normal population, if they are nonconservative changes, or if they occur in evolutionarily conserved amino acids (4). However, segregation studies are not always feasible because of the unavailability of family samples, and rare polymorphisms could also result in nonconservative changes. Therefore, functional assays of missense mutations are necessary to distinguish pathogenic mutations from normal polymorphisms.

As mentioned above, the formation of a hMutLα complex is essential for MMR activity and thus the inhibition of hMLH1-hPMS2 interaction would be one of the primary causes of MMR defects in individuals with HNPCC. Therefore, we carried out an analysis of protein-protein interactions of the hMutLα complexes to assess the functional relation of the *hMLH1* missense mutations with gastrointestinal cancer. We analyzed 10 mutant forms of *hMLH1* which are frequently found in East Asian patients using the yeast two-hybrid system, as well as the coimmunoprecipitation assay to study such protein-protein interactions *in vivo*. Four of these variants have never been previously studied in functional assays. Mutation screening in a normal Chinese population was also carried out to determine whether these *hMLH1* variants were clinically relevant.

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## Materials and Methods

***hMLH1 variants and patients with suspected HNPCC.*** We focused on *hMLH1* missense mutations frequently detected in East Asian patients with suspected HNPCC. The variants and the phenotypic characteristics of the respective patients included in the present investigation are listed in Table 1. Most of the variants were found in HNPCC patients fulfilling the Amsterdam criteria. Three variants selected (S46I, G65D, and P581L) are unique in Chinese patients, and five (R217C, V384D, Q542L, L549P, and L574P) have only been identified in East Asians, although the remaining one (G67R) had been reported in several ethnic patients. In addition, we included Q701K, a variant that has recently been found in 2 out of 105 Chinese gastric cancer patients with low familial recurrence (5). Available clinical data showed that six of the variants displayed high microsatellite instability (MSI-H) and four showed loss of the expression of hMLH1 protein in tumor. Age of cancer onset for most of the patients was <45 years. No second mutation in the MMR genes was detected in the patients who carried 1 of the 10 variants investigated in this work, except for S46I in a Chinese patient with HNPCC, in whom a deletion of 12 bp in exon 11 of hMLH1 was shown (Table 1).

***Mutation screening of normal individuals.*** One hundred and twelve normal individuals from Jiangsu, China were recruited. Total genomic DNA was extracted from peripheral blood using QIAamp DNA blood mini kit (Qiagen) according to the manufacturer's instructions. Genomic DNA from samples was screened for germ line mutations by PCR of specific exons (as listed in Table 1), followed by denaturing high-performance liquid chromatography analysis done on the WAVE system (Transgenomic). The samples showing abnormal denaturing high-performance liquid chromatography profiles were sequenced on an ABI 3100-Avant automated sequencer (Applied Biosystems). Informed consent was obtained from all subjects. The study was approved by the ethics committee of the Medical School of Nanjing University.

***Plasmid constructions.*** The wild-type *hMLH1* and *hPMS2* cDNAs were a gift from Prof. Robert Brown and Dr. Helen Robinson. Overlapping PCR site-directed mutagenesis was used to introduce the mutations into *hMLH1* cDNA (6). The nucleotide sequences of the PCR primers used are available upon request from the authors. The mutated fragments were cloned into pGADT7 (Clontech) using *Eco*RI and *Bam*HI to produce the transcriptional AD-fused *hMLH1* construct pGADT7-*hMLH1* for yeast two-hybrid assay. Similarly, the *hPMS2* cDNA was ligated into pGBKT7 to produce pGBKT7-*hPMS2,* the DNA BD-fused *hPMS2* construct. Then the *hMLH1* cDNA (wild-type or mutant) was cloned into the *Xho*I and *Sfi*I sites of pCMV-Myc vector (Clontech), whereas the wild-type *hPMS2* cDNA was cloned into the *Sal*I and *Sfi*I sites of pCMV-HA vector (Clontech) to construct the two kinds of plasmids for coimmunoprecipitation assays. The resulting

**Table 1.** Genetic and clinical data of variants in hMLH1 under study

| Exon | Nucleotide change | Amino acid change | Ethnic group (ref.) | No. and character of patients (ref.) | MS status (informative cases/ HNPCC cases) | hMLH1 protein in tumor (informative cases/HNPCC cases) | Mean/earliest age of onset (ref.) |
|---|---|---|---|---|---|---|---|
| 2 | c.137G>T | S46I | Chinese (28) | 1 HNPCC (also mutation in exon 11, hMLH1) (28) | MSI-H (1/24) (ref. 28) | Lost (1/24) (ref. 28) | NA |
| 2 | c.194G>A | G65D | Chinese (28) | 1 CRC-F (28) | MSI-H (1/15) (ref. 28) | Lost (1/15) (ref. 28) | NA |
| 2 | c.199G>A | G67R | Chinese (29) | 5 HNPCC (29–33) | MSI-H (1/61) (ref. 34) | NA | 39 (29–34)/ 26 (31) |
| | | | Japanese (30) | 1 SCRC with MPC (34) | | | |
| | | | Swedish (31) | | | | |
| | | | Swiss (32, 33) | | | | |
| | | | U.S. (34) | | | | |
| 8 | c.649C>T | R217C | Chinese (17, 29, 35) | 2 HNPCC (17, 18) | MSI-H(1/12) (ref. 17) | Lost (1/12) (ref. 17) | 42 (18) |
| | | | Japanese (36) | 2 CRC-F (29, 35) | | | |
| | | | Korean (18) | 1 SCRC with MPC (36) | | | |
| 12 | c.1151T>A | V384D | Chinese (17, 28, 29, 39, 40) | 2 HNPCC (17), 3 CRC-F (28, 29, 40) | MSI-H for CRC-F (1/9) (ref. 40) | NA | 45 (39, 40)/ 32 (39) |
| | | | Japanese (39) | SCRC (39, 40) controls (39) | MSS for SCRC (2/18) (ref. 40) | | |
| 14 | c.1625A>T | Q542L | Korean (19) | 1 HNPCC (19) | NA | NA | 25 (19) |
| 14 | c.1646T>C | L549P | Korean (18) | 1 HNPCC (18) | NA | NA | 46 (18) |
| 15 | c.1721T>C | L574P | Korean (19, 20) | 2 HNPCC (19, 20) | NA | NA | 33 (19, 20)/ 32 (20) |
| 16 | c.1742C>T | P581L | Chinese (17) | 1 HNPCC (17) | MSI-H(1/12) (ref. 17) | Lost (1/12) (ref. 17) | NA |
| 18 | c.2101C>A | Q701K | Chinese (5) | 2 GC-LF (5) | NA | NA | 67 (5)/66 (5) |

NOTE: The HNPCC families shown here fulfilled the Amsterdam criteria. Informative cases indicates number of cases carrying this indexed mutation; HNPCC cases indicates the number of HNPCC (or CRC-F, or SCRC) cases overall tested in the referenced report.
Abbreviations: NA, not available; CRC-F, familial recurrence for colorectal cancer (proband with CRC and at least two other first-degree relatives with CRC or HNPCC-like cancer); SCRC, sporadic colorectal cancers; GC-LF, low familial recurrence for gastric cancer (the presence of one additional affected family member, two GC cases in the family); MPC, multiple primary cancers; MSI, microsatellite instability; MSI-H, two or more markers showed microsatellite instability in the five markers checked; MSS, microsatellite stable, none of the five markers showed instability.

hMLH1 proteins (wild-type or mutants) were tagged at their $NH_2$ terminus with c-Myc, whereas hPMS2 was tagged with HA. All of the plasmid constructs were verified by DNA sequencing on an ABI 3100-Avant automated sequencer (Applied Biosystems).

***Yeast two-hybrid assay.*** Yeast two-hybrid assay was carried out with the matchmaker *GAL4* two-hybrid system (Clontech). Briefly, pGBKT7-*hPMS2* and pGADT7-*hMLH1* (wild-type or mutant) clones were cotransformed into the *Saccharomyces cerevisiae* strain Y187 and selected on SD/-Leu-Trp medium. Positive protein-protein interactions were ascertained by transcription activation of the highly inducible GAL1 UAS-driving *lacZ* reporter gene in the host strain by quantitative liquid culture β-galactosidase activity assay with *o*-nitrophenyl-1-thio-β-D-galactopyranoside (Amresco) as the substrate (7).

***cDNA transfections and Western blot.*** The human embryonic kidney fibroblast cell HEK293T has been reported to not express hMLH1 because of promoter hypermethylation, and thus lacks hPMS2 as it would not be stable without hMLH1. This allows the expression and analysis of MutLα variants in this cell line without the interference of endogenous MutLα (8, 9). The 293T cells (Cells Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Shanghai, China) were transiently cotransfected with pCMV-MYC-*hMLH1* and pCMV-HA-*hPMS2* using a Lipofect Transfection Reagent (RM201-1; Tiangen Biotech Beijing Co. Ltd.) according to the manufacturer's instructions. The cells were lysed and separated on 10% SDS-polyacrylamide gels, followed by electroblotting, and probed with anti-MYC (mouse monoclonal; Clontech) and anti-HA (rabbit polyclonal; Clontech) antibodies and visualized with enhanced chemiluminescence reagents (Applygen Technologies, Inc.).

***Coimmunoprecipitation.*** Total cell protein extracts were incubated with anti-MYC monoclonal antibody (mouse monoclonal; Clontech) followed by immunoprecipitation with protein A-agarose (Invitrogen). The protein-agarose complexes were then collected and resolved through SDS-PAGE. Immunoblots were done as described in the previous section. The densities of the desired protein bands were quantified with the Quantity One-4.5.0 program (Bio-Rad) and the relative amount of hPMS2 was determined (10).

## Results

***Sequence alignment and functional domain analysis.*** As indicated in Fig. 1, the 10 variants now studied cover different regions of the *hMLH1* gene (11, 12). The variants S46I, G65D, G67R, and R217C are located in the conserved $NH_2$-terminal region of hMLH1 which is considered important for ATP-binding. Two $NH_2$-terminal missense mutations, G65D and G67R, affect conserved amino acid residues in the second motif of the four ATP-binding pockets. The S46I variant resides near the first ATP-binding pocket, but this codon is not conserved. R217C is located near the domain interface. V384D is located at the boundary between the $NH_2$-terminal region and the COOH-terminal domain. The remaining mutations are in the COOH-terminal region of the polypeptide which is important for hPMS2 interaction.

***Identification of mutations in 112 normal individuals.*** Out of the 10 single-nucleotide variants examined, only one (c.1151T>A, V384D) appeared in the normal population examined. The allele frequency of this variant was 2.67% (6 of 224) in the Chinese normal individuals checked. None of the other nine variants were detected in this population [although we still found the c.655 A>G (p.Ile219Val) in 3 of the 112 samples, this variant is not contained in the current study].

***Yeast two-hybrid assay.*** Liquid cultures were assayed quantitatively for β-galactosidase activity to verify and compare the relative strength of the protein-protein interactions. R659P and the blank plasmid pGADT7 were used as the pathologic missense mutation control and the blank control, respectively. As expected, the five missense mutations (Q542L, L549P, L574P, P581L, and Q701K) located in the COOH-terminal of hMLH1 necessary for interaction with hPMS2 showed extremely low levels of β-galactosidase activities in comparison with wild-type hMLH1. Their activities are similar to the pathologic missense mutation control R659P. Interestingly, the two $NH_2$-terminal missense mutations, G67R and R217C, and the V384D variant in the region between the $NH_2$-terminal and the COOH-terminal also displayed very weak β-galactosidase activities, whereas the remaining two $NH_2$-terminal missense mutations S46I and G65D retained ~40% of their activities (Table 2).

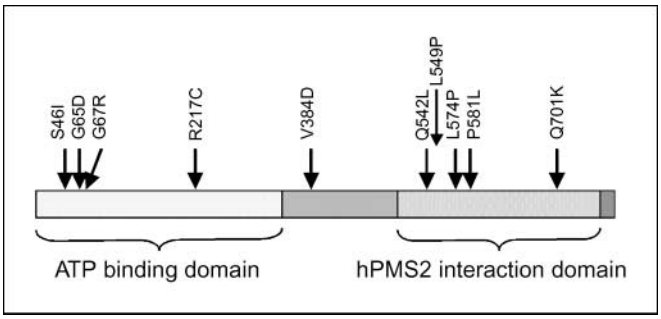


**Fig. 1.** Location of hMLH1 variants studied in this work.

***Western blot and coimmunoprecipitation assays.*** To clarify whether the low levels of β-galactosidase activities in the two-hybrid assay were caused by defects in the physical interaction between hMLH1 variants and a wild-type hPMS2, we next performed a combined coimmunoprecipitation/Western blot analysis (Fig. 2).

Immunoblotting of total cell extracts confirmed that mutant and wild-type hMLH1 proteins were expressed equally well (Fig. 2A and B, *top*). The expression levels of hPMS2 were also similar (Fig. 2A and B, *top*).

As shown in Fig. 2, the four hMLH1 variants carrying missense mutations in the amino-terminal exons 2 (S46I, G65D, and G67R) and exon 8 (R217C) did not exhibit significant changes in their physical interaction with hPMS2 (Fig. 2A, *middle* and *bottom, lanes 2-5*). The results are consistent with other previous observations that mutations in the $NH_2$ terminus of hMLH1 are unlikely to affect the formation of MutLα heterodimer. The missense mutation V384D in exon 12 retained half of its interaction with hPMS2 (Fig. 2A, *middle* and *bottom, lanes 6*). For the five hMLH1 variants carrying mutations at the carboxyl end, the missense mutation in exon 14 (Q542L L549P) and exon 15 (L574P) tended to display defects in their physical interactions with hPMS2 as R659P, the mutant chotrol, in exon 17. The P581L missense mutation in exon 16 yielded a polypeptide that interacted only very weakly with hPMS2 (~16% compared with the wild-type of hMLH1), whereas the missense mutation Q701K in exon 18 retained ~70% interaction with hPMS2 (Fig. 2B, *middle* and *bottom, lanes 2-7*). In those cases, the antibody against MYC-hMLH1 precipitated MYC-hMLH1 but not HA-hPMS2, or only partly, from the total protein extracts.

**Table 2.** Yeast two-hybrid assay of hMLH1 variants

| Structural alteration | Exon | Nucleotide change | β-Galactosidase activity* | Relative activity (%) † |
|---|---|---|---|---|
| MLH1 (wild-type) | | | 13.75 ± 3.48 | 100 |
| S46I | 2 | AGT→ATT | 6.08 ± 0.52 | 44.2 |
| G65D | 2 | GGC→GAC | 4.66 ± 3.02 | 33.95 |
| G67R | 2 | GGG→AGG | 1.90 ± 0.27 | 13.8 |
| R217C | 8 | CGC→TGC | 1.66 ± 0.75 | 12.1 |
| V384D | 12 | GTT→GAT | 2.39 ± 0.90 | 17.4 |
| Q542L | 14 | CAG→CTG | 2.24 ± 0.73 | 16.3 |
| L549P | 14 | CTT→CCT | 1.58 ± 0.07 | 11.5 |
| L574P | 15 | CTC→CCC | 2.27 ± 0.37 | 16.5 |
| P581L | 16 | CCG→CTG | 1.99 ± 0.85 | 14.5 |
| Q701K | 18 | CAG→AAG | 2.28 ± 0.20 | 16.6 |
| R659P ‡ | 17 | CGA→CCA | 2.80 ± 0.38 | 20.4 |
| Blank plasmid§ | | | 1.19 ± 0.31 | 8.65 |

*Results of the yeast two-hybrid assay with liquid assays for β-galactosidase activities. Values are means and SD of β-galactosidase activities obtained from three independent transformants.
† The relative β-galactosidase activity in the yeast two-hybrid assay was calculated for each variant in comparison with yeast cells containing both wild-type *hMLH1* and *hPMS2* plasmids that were scored as 100%.
‡ Mutant control.
§Containing pGBKT7-hPMS2 and pGADT7.

***Evaluation of pathogenicity.*** The evaluation of pathogenicity of the 10 variants based on this study and on literature data is summarized in Table 3.

## Discussion

In this study, the functional consequences of 10 *hMLH1* missense variants detected frequently in East Asians with suspected HNPCC were evaluated mainly by yeast two-hybrid and coimmunoprecipitation, four of the variants have thus far never been tested. The R659P variant was used as a control because the mutation is located in the essential hPMS2-interaction domain and was already predicted to be pathogenic from different laboratory works (refs. 6, 13–16). All variants, except for the V384D variant, were absent from any of the 112 Chinese normal controls.

Our data showed that complex formation between any one of the four hMLH1 protein variants carrying mutations at the carboxyl end (Q542L L549P, L574P, and P581L) and hPMS2 were not detectable or were only weakly detectable, similar to the R659P variant in both assays (Table 2; Fig. 2B, *middle* and *bottom, lanes 2-5*). All four variants were detected in patients from HNPCC families fulfilling the Amsterdam criteria, and available data showed that the probands were young patients with colorectal cancer (CRC; Table 1; refs. 17–20). Out of these variants, P581L in hMLH1 was evaluated, for the first time, for its functional consequence. Proline is an amino acid with a closed ring, whereas leucine is a hydrophobic amino acid. This change might affect the structure of the protein, and thus, its interaction with hPMS2. Our data suggested that this variant was pathogenic due to the functional impairment of the hMLH1 protein in its interaction with hPMS2 by coimmunoprecipitation analysis, and the extremely low level of β-galactosidase activities in comparison with wild-type hMLH1 in yeast two-hybrid assay. Actually, clinical data showed that this variant displayed MSI-H and loss of the expression of hMLH1 protein in tumor (17). No consistent results from previous functional studies have been obtained on Q542L. It was considered to be a loss-of-function mutation by *in vivo* DNA MMR in the yeast assay (14) and showed ~40% reduction in the β-galactosidase activity in yeast two-hybrid assay by Kondo et al. (15). However, it was suggested to be a normal polymorphism in the GST-IVTT assay (16) and a mutator effect in the yeast assay (13). Our results suggested that Q542L in hMLH1 causes a complete inactivation of this gene product either with yeast two-hybrid assay or with coimmunoprecipitation. Hence, we refer to it as a disease-causing mutation. Wanat et al. suggested that L549P was pathogenic in humans, as L549P (corresponding to yeast mutant allele L559P) showed significant MMR defects through examination of the MMR proficiency in the S288c strain of *S. cerevisiae* (21). L574P was previously referred to as pathogenic in mutator effect assay in yeast (13), GST-IVTT assays (15, 16), and yeast two-hybrid assay (15). Our results, obtained by yeast two-hybrid assay and coimmunoprecipitation analysis in human cells, also showed the causative role of these two variants in HNPCC.

Thus far, few functional assays have been done on the amino terminal variants of hMLH1. No work has been reported on the functional consequence of the variants S46I and G65D. G67R was shown in MMR analysis in yeast to be a loss-of-function mutation (13, 14, 22). Kondo's work showed that this $NH_2$-terminal missense variant, G67R, in hMLH1 displayed low levels of β-galactosidase activity in a yeast two-hybrid assay, but did not exhibit any change in their physical interaction with hPMS2 in the GST-IVTT assay (15). However, they did not give a clear explanation for this discrepancy. Ellison et al. referred to R217C as an efficiency polymorphism as it functioned in DNA MMR at a reduced efficiency (14). However, it restored MMR efficiency at ~80% in 293T cells and was classified as a rare polymorphism by Trojan et al. (8).

Our results, using coimmunoprecipitation analysis, revealed the normal formation of a complex between the amino-terminal hMLH1 variants (S46I, G65D, G67R, and R217C) and hPMS2 (Fig. 2A, *middle* and *bottom, lanes 2-5*). However, they showed more or less reduced β-galactosidase activities in the yeast two-hybrid assay (Table 2), which was unexpected as they

were not located in the regions of hPMS2 interaction (7, 16). How to interpret the above results?

According to Ban et al., the $NH_2$-terminal region of MutL in *Escherichia coli* contains four putative ATP-binding motifs. The authors further stated that MutL is actually an ATPase and that ATP binding causes conformational changes in MutL, and is likely to regulate the interactions of MutL with other components in the DNA repair machinery (11). This ATPase domain in MutL is highly conserved from bacteria to humans, and in fact, hMutLα has recently been shown to have ATPase activity and this ATPase activity is essential for the MMR system (12, 23). According to structure-based sequence alignment of the conserved $NH_2$-terminal region in the MutL family, G65 and G67 are located in motif II of four highly conserved ATP-binding motifs of MLH1 and both are conserved in the GHL (DNA gyrase B, Hsp90, and MutL) superfamily (11). S46 is not a conserved residue in the MutL family and is not located in the conserved ATP-binding motifs (11). R217 is not a conserved residue and neither is it located in or around the ATP-binding motifs, but it is located near the domain interface. It seems to locate on the exposed surface on the side of the molecule opposite the ATPase activity site. As shown in Table 3, the four variants had all undergone changes in charge or polarity. Charge and polarity are important in the protein's structure and function, especially in the formation of a secondary structure. Thus, the alteration of the amino acids at those positions might affect the proteins' interaction between the helices and the capacity of those mutant proteins to bind and/or hydrolyze ATP, and thus, the ATPase activity of hMLH1.

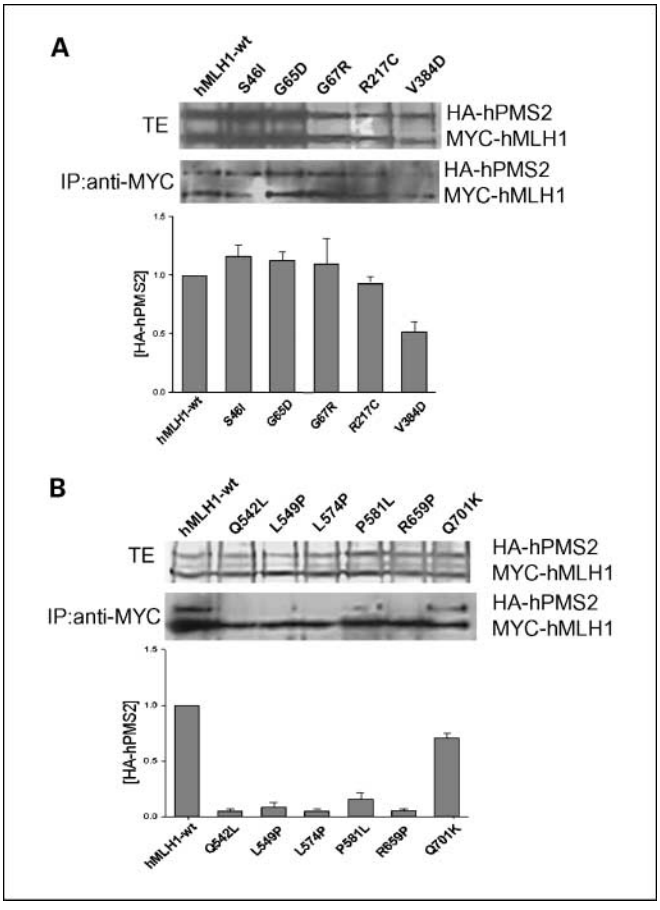


**Fig. 2.** Interaction studies between hMLH1 variants and hPMS2. *A* and *B*, Western blot of total protein extracts (*TE*; 35 μg each) of 293T cells transfected with pCMV-HA-PMS2-wt with either wild-type pCMV-MYC-MLH1 (*hMLH1-wt*) or pCMV-MYC-MLH1 variants (*top*). Immunoprecipitates (*IP*; 500 μg of TE in each) obtained with anti-MYC antibody (*middle*). Following transfer on the membrane, the proteins were visualized with a mixture of the MYC and HA antibodies. Relative quantity of hPMS2 coprecipitated with different hMLH1 proteins (*bottom*). The densities of the desired protein bands in immunoprecipitation were quantified with the Quantity One-4.5.0 program. The relative amount of hPMS2, which bound to wild-type or mutant hMLH1, was determined by normalizing the intensity of hPMS2 band to the intensity of the corresponding hMLH1 band. The amount of hPMS2 bound to wild-type hMLH1 is set to 1. Columns, mean; bars, SD.

It has been known that DNA MMR takes place in the nucleus. Nuclear import and export of MMR proteins is determined by nuclear localization signals and nuclear export sequences, respectively (24). Both nuclear localization signals and nuclear export sequences are present in hMLH1 and hPMS2, yet when expressed alone, hMLH1 and hPMS2 fail to localize into the nucleus. On the contrary, coexpression of hMLH1 and hPMS2 resulted in their nuclear localization. Although some $NH_2$-terminal mutants in hMLH1 did not affect the formation of MutLα, and were also unable to translocate into the nucleus (25–27). These phenomena suggest that COOH-terminal dimerization of hMLH1 and hPMS2 is necessary for nuclear import, but MutLα also undergoes a conformational transformation upon ATP binding that includes $NH_2$-terminal dimerization. The $NH_2$-terminal dimerization-defective mutants might fail to localize in the nucleus despite the fact that they are dimerized by the COOH-terminal domains.

The above hypothesis could be one explanation for the discrepancy in the results of the two assays. The COOH-terminal dimerization of hMLH1 and hPMS2 may not be sustained in the nucleus because the $NH_2$-terminal dimerization-defective hMLH1 mutant (S46I, G65D, G67R, and R217C) proteins binds normally to hPMS2 in coimmunoprecipitation analysis, but the dimers were not shown or only partly shown in a yeast two-hybrid assay that only detects protein interactions in the nucleus *in vivo*. The present results suggest that those kinds of variants might act similarly in human cells as in the yeast cells, that is, reducing the efficiency of the heterodimer to enter the nucleus and thus block the MMR process. Available clinical data showed that all the four variants displayed MSI-H, and three showed loss of the hMLH1 protein expression in tumor. These variants occurred either in patients from HNPCC families fulfilling the Amsterdam criteria, or in patients with familial recurrence for CRC but did not meet the Amsterdam criteria, or in sporadic CRC patients with multiple primary cancers. The reported age of cancer onset was young. No second mutation in the MMR genes was detected in the patients with one of the three variants (G65D, G67R, and R217C) except for S46I (Table 1; refs. 17, 18, 28–36). With both of the functional analysis results and clinical data, we suggest that the three $NH_2$-terminal variants (G65D, G67R, and R217C) might affect the MMR process at different levels and might have functional relation to gastrointestinal cancer. It is interesting to refer to the variant S46I in a Chinese HNPCC patient who carried the second mutation in hMLH1, a deletion of 12 bp in exon 11 (Table 1; ref. 28). In the current yeast two-hybrid assay, this $NH_2$-terminal missense mutation retained 44.2% of the activities and showed normal interaction with hPMS2 in coimmunoprecipitation analysis (Table 2; Fig. 2). We classify this variant as partly loss of function, but its relation to cancer is

**Table 3.** Summary of functional analysis results

| Variants | Conserved residue | Change polarity | Functional significance (present results) | | | | Other functional data reported previously (ref.) |
|---|---|---|---|---|---|---|---|
| | | | Presence in 112 controls | β-Galactosidase activity* | Complex formation with hPMS2 † | Functional classification | |
| S46I | No | Yes | No | Partly lost | Normal | Partly loss of function | — |
| G65D | Yes | Yes | No | Partly lost | Normal | Partly loss of function | — |
| G67R | Yes | Yes | No | Extremely low | Normal | Mutant | Mutant (13–15, 22) |
| R217C | No | Yes | No | Extremely low | Normal | Mutant | Efficiency polymorphisms (14), polymorphism (8) |
| V384D | No | Yes | Yes | Extremely low | Half retained | Partly loss of function | Efficiency polymorphisms (38) |
| Q542L | Yes | Yes | No | Extremely low | Lost | Mutant | Mutant (14), polymorphism (13, 15, 16) |
| L549P | Yes | No | No | Extremely low | Lost | Mutant | Mutant (21) |
| L574P | Yes | No | No | Extremely low | Lost | Mutant | Mutant (13, 15, 16) |
| P581L | Yes | No | No | Extremely low | Weak | Mutant | — |
| Q701K | No | Yes | No | Extremely low | Largely retained | Partly loss of function | — |

*Results by yeast two-hybrid.
† Immunoprecipitation results.

uncertain, on account of the fact that the proband also carried another mutation in the *hMLH1* gene. Additional analyses are needed to determine the relationship between the structural abnormalities at the $NH_2$ terminus of hMLH1 and the exact localization of the heterodimer of hMLH1 and hPMS2, and thus, their roles in the MMR process.

V384 is located in the central region of the hMLH1 protein between the $NH_2$-terminal ATP-binding domain and the COOH-terminal hPMS2 interaction domain. The V384D mutation results in charge differences (the neutral hydrophobic amino acid valine is replaced by aspartate, a weak acidic amino acid with a negative charge) and is likely to disrupt the structure formation, and hence, the stability of the hMLH1 protein. V384D has been frequently detected in the East Asian population (Table 1). Additional investigations in Chinese cohorts showed that a higher frequency of this variant was detected in young CRC patients (<45 years) or GC patients with a family history when compared to that in normal controls (37). In a newly reported work, V384D showed ~65% *in vitro* MMR activity (38). Our current assay with a yeast two-hybrid assay detected low levels of β-galactosidase activity, although hPMS2 interaction was still half-retained in coimmunoprecipitation analyses. It is possible that this variant partially reduced the interaction between hMLH1 and hPMS2, and the interaction was not strong enough for the BD and AD domains of the *Gal4* system to come close to activating the reporter gene *lacZ*. Combined with the above results, we suggest that this is partly a loss of function variant and might raise the risk of gastrointestinal cancer in the mutation carriers.

We have identified the Q701K variant in 2 out of 105 Chinese gastric cancer cases, but not in 82 patients with CRC (5). This variant was not detected in 112 normal individuals. Both patients were from families with low GC familial recurrence, which is the presence of one additional affected family member (two GC cases in the family). Unfortunately, samples from patients with the familial disease phenotype were unavailable, and we were not able to perform a cosegregation analysis of the mutation. Glutamine and lysine belong to different polarity groups. Our result first showed that this hMLH1 variant largely retained the interaction with hPMS2 from coimmunoprecipitation analyses, whereas the β-galactosidase activity was significantly reduced in yeast two-hybrid assays. The reason for the contradiction between the two assays might be the similar V384D variants used, which has been stated in the previous section. Therefore, this variant is also classified as partly loss of function and it might have a role in the occurrence of gastric cancer.

In conclusion, our data provide, for the first time, the functional evaluation of the variants S46I, G65D, P581L, and Q701K which is frequently detected in East Asians with suspected HNPCC. Our results are consistent with previous analyses on the functional consequences of such variants as G67R, L549P, and L574P, suggesting that they are pathogenic in humans. Moreover, we provide information on the consequences of the variants R217C, V384D, and Q542L which have not received consistent results in previous studies. This work evaluates the real contribution of germ line missense mutations in the *hMLH1* gene to gastrointestinal cancer predisposition, at least, in East Asians. This information might be useful for mutation screening and clinical diagnosis of East Asians at high risk for gastrointestinal cancer.

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