

Three novel germline mutations in *MLH1* and *MSH2* in families with Lynch syndrome living on Jeju island, Korea

Young Mee Kim¹, Chang Gyu Choe¹, Somi Kim Cho², In Ho Jung^{3,4}, Won Young Chang^{3,4,*} & Moonjae Cho^{1,4,*}

Departments of ¹Biochemistry, ²Faculty of Biotechnology and ³Surgery, Jeju National University School of Medicine, and ⁴Institute of Medical Science, Jeju National University, Jeju 690-756, Korea

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant syndrome characterized by predisposition to early-onset cancers. HNPCC is caused by heterozygous loss-of-function mutations within the mismatch repair genes *MLH1*, *MSH2*, *MSH6*, *PMS1*, and *PMS2*. We genotyped the *MLH1* and *MSH2* genes in patients suffering from Lynch syndrome and in 11 unrelated patients who were diagnosed with colorectal cancer and had subsequently undergone surgery. Five Lynch syndrome patients carried germline mutations in *MLH1* or *MSH2*. Two of these were identified as known mutations in *MLH1*: deletion of exon 10 and a point mutation (V384D). The remaining three patients exhibited novel mutations: a duplication (937_942dupGAAGTT) in *MLH1*; deletion of exons 8, 9, and 10; and a point mutation in *MLH1* (F396I) combined with multiple missense mutations in *MSH2* (D295G, K808E, Q855P, and I884T). The findings underline the importance of efficient pre-screening of conspicuous cases. [BMB reports 2010; 43(10): 693-697]

INTRODUCTION

Lynch syndrome (hereditary non-polyposis colorectal cancer/HNPCC) is one of the most common cancer-susceptibility syndromes and is the most common cause of hereditary early-onset colorectal cancer. It is estimated that HNPCC may account for 5-10% of all colorectal cancers worldwide (1-3). Affected individuals are at increased risk for developing colorectal cancer and extra-colonic carcinomas, including cancers of the endometrium, ovary, urothelium, and stomach (3). HNPCC is characterized by germline mutations in mismatch repair (MMR) genes, including *MLH1* (MIM#120436), *MSH2* (MIM#120435), *PMS1* (MIM# 600258), *PMS2* (MIM #600259),

*Corresponding author. Moonjae Cho, Tel: 82-64-754-3837; Fax: 82-64-702-2687; E-mail: moonjcho@cheju.ac.kr, Won Young Chang, Tel: 82-64-754-1125; Fax: 82-64-725-2593; E-mail: orkorea@jeju.ac.kr DOI 10.5483/BMBRep.2010.43.10.693

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and *MSH6* (MIM #600678) (4-6). About 90% of the germline mutations identified in these MMR genes occur in *MLH1* and *MSH2* (7). To date, more than 300 different mutations have been described in these two genes, collectively accounting for approximately 500 HNPCC cases worldwide (8).

MMR genes are required for the correction of mismatches that occur during DNA replication. Defects in DNA MMR genes result in microsatellite instability (often characterized by replication errors, RER+). It has been suggested that the presence of replication errors may be a useful marker for HNPCC (9, 10). Recently, immunohistochemical techniques have been successfully employed as an alternative strategy for identifying tumors caused by defective MMR (11, 12). These methods provide information on a specific gene that may be defective and therefore may constitute a cost-effective way of limiting the number of genes requiring sequencing.

Here, we describe mutations in the *MLH1* and *MSH2* genes, as well as molecular and clinical findings, in five families with HNPCC-affected members living in Jeju Island, Korea.

RESULTS

As all patients participating the study had been diagnosed with colon cancer and were suspected to have been suffering from Lynch Syndrome (according to Bethesda guideline 7: individual with at least one adenoma diagnosed at age <40 years), we directly sequenced their *MLH1* and *MSH2* genes. We identified five germline mutations in the 11 unrelated patients (Table 1) tested, three of which represented novel germline mutations not listed by the International Collaborative Group on the HNPCC (<http://www.insight-group.org>) website or in the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/search.html>).

Patient 1

The coding sequences of *MLH1* and *MSH2* were analyzed in two siblings and their mother. All carried a novel GAAGTT duplication (937_942dupGAAGTT) in *MLH1*, which resulted in duplication of Glu313 and Val314 (Fig. 1). No mutations were detected in *MSH2*.

Table 1. Mutations detected in Lynch syndrome patients

Patient number	Gene	Exon (intron)	Mutation	Type
1	<i>MLH1</i>	11	937_942dupGAAGTT Glu313_Val314dup	Insertion of two amino acids
2	<i>MLH1</i>	11	c.912T > C	Polymorphism
		12	c.1185T > A (Phe396Ile)	missense
	<i>MSH2</i>	17	c.1958G > A	Polymorphism
		5	c.884A > G (Asp295Gly)	Missense
3	<i>MLH1</i>	11	c.1666T > C (Lys808Glu)	Missense
		15	c.2564A > C (Gln855Phe)	Missense
		16	c.2651T > C (Ile883Thr)	Missense
4	<i>MLH1</i>	8-10	c.677G > A (Arg226Gln) c.884+386delCTGAGGTG	Deletion of exons 8-10
5	<i>MLH1</i>	10	c.884+4A > G	Deletion
5	<i>MLH1</i>	12	c.1151T > A (Val384Asp)	Missense

Patient 2

Sequence analysis of *MLH1* revealed three single-base mutations. Whereas two of the mutations were silent, the other, a novel T > A substitution at nucleotide 1246, resulted in the replacement of a phenylalanine-encoding codon with an isoleucine-encoding one (TTT > ATT) at codon 396 (Fig. 2A). For *MSH2*, meanwhile, four single-amino acid changes were detected: D295G, K808E, Q855P, and I884T (Fig. 2B). Three of these (D295G, Q855P, and I884T) were previously reported as SNPs, although their significance was not determined. All three mutations have not been previously identified in a single patient.

Patient 3

Sequence analysis of *MLH1* cDNA revealed the novel deletion of exons 8-10 (Fig. 3A). Genomic DNA analyses identified two genetic aberrations: a single-base substitution at the junction between exon 8 and intron 8 (c.677G > A) that results in disruption of a splicing sequence (Fig. 3B) and a deletion in the middle of intron 10 (c.884+386delCTGAGGTG) (Fig. 3C). Comprehensive sequence analysis of the *MLH1* gene revealed no other mutations.

Patient 4

A mutation in intron 10 of the *MLH1* gene, c.884+4A > G, was identified. This mutation caused a splicing defect; as a result, exon 10 was missing and translation was prematurely terminated (13). No mutations were detected in *MSH2*.

Patient 5

Sequence analysis of the *MLH1* gene revealed a T > A substitution at nucleotide 1151, which resulted in the substitution of an aspartate-encoding codon for a valine-encoding one (GTT > GAT). The resulting V384D mutation represents a change in amino acid charge (neutral valine being replaced with acidic aspartate), indicating that a change in protein structure may occur. However, as codon 384 is located in a poorly

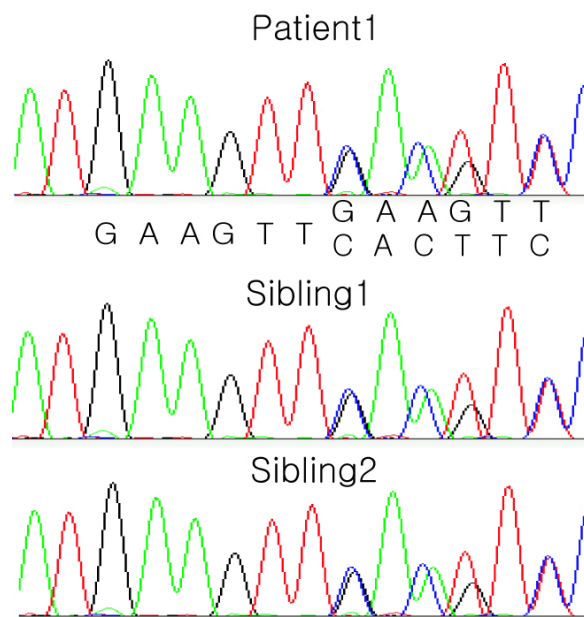


Fig. 1. Novel six-base duplication in *MLH1* in the family of patient 1. *MLH1* cDNA sequencing patterns in patient 1 and two siblings.

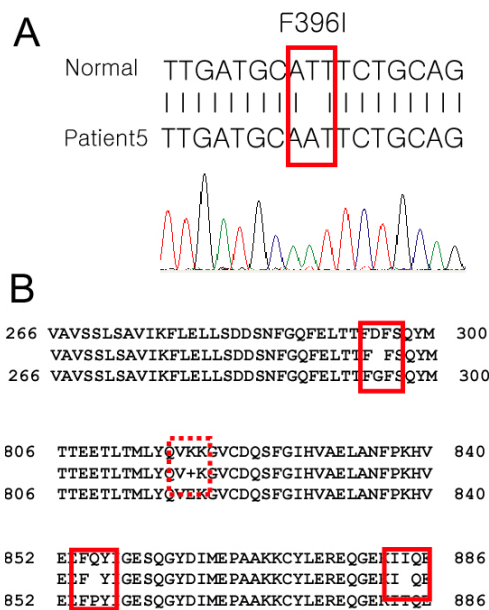


Fig. 2. Characterization of *MLH1* and *MSH2* in patient 2. Sequence analysis of *MLH1* (A) and *MSH2* (B) in patient 2. A single-base substitution (T > A) in *MLH1* caused a Phe residue to be replaced with an Ile (A). Four single-base substitutions were detected in *MSH2* (B).

conserved region of the gene (14, 15), the functional significance of this amino acid substitution remains difficult to evaluate. No mutations were identified in *MSH2*.

DISCUSSION

Here, we describe germline mutations of *MLH1* and *MSH2* in families of suspected Lynch syndrome patients living in Jeju, Korea. In patient 1, a novel six-base insertion resulting in the duplication of two amino acids (Glu313 and Val314) was observed (Fig. 1). This mutation did not cause truncation or premature termination of transcription or translation as a result of the frame shift. The significance of this novel duplication of two amino acids in the ATP-binding domain remains to be determined. However, all point mutations (31, 37, 38, 44, 67, 93, 109, 111, 265) in the ATP-binding domain tested by Drost *et al.* (16) caused sufficient loss of MMR activity and were thus considered pathogenic.

Patient 2 carried multiple mutations in *MSH2* (Fig. 2). These mutations (D295G, Q855P, K808E, and I884T) were localized to the connector domain and the helix-turn-helix domain through which *MSH2* interacts with *MSH3* and *MSH6*. The amino acid changes were rather dramatic. The D295G and Q855P substitutions resulted in changes in charge. Whereas aspartic acid is a charged amino acid, proline is an imino acid with a closed ring, which can alter the direction of the peptide chain. Though the above missense mutations were not validated for loss of activity, and the possibility of functional mutations in *MSH6*, *PMS1*, and *PMS2* remains, the presence of multiple mutations in a single patient is highly likely to reduce MMR activity.

Patient 3 carried deletions (Fig. 3) in exons 8-10 accompanied with the consequent creation of a premature stop codon and the synthesis of a truncated, nonfunctional protein. The patient's genomic DNA contained two mutations: a missense mutation at the junction between exon 8 and intron 8 (c.677G > A) and an eight-base deletion in intron 10 (c.884+386delCTGAGGTG). The c.677G > A missense mutation has been reported to cause deletion of exon 8 (17). We assume that the two mutations we detected caused the deletion of exons 8-10. However, we cannot explain why intact exon 9 and intron 9 were affected by these mutations. We speculate that they perhaps disrupted exonic splicing enhancers (ESEs) or created exonic splicing silencers (18).

Patient 4 carried deletions in exon 10 (13). Frameshift mutations within *MLH1* and *MSH2* that inactivate one allele are considered to cause disease. Subsequent complete functional disruption of one MMR locus caused by somatic mutations affecting the wild type homolog preceded the development of cancer.

The majority of MMR gene mutations in HNPCC patients cause truncation and thus loss of function of the affected polypeptide. However, amino acid alterations comprise a significant proportion of these mutations (~15% in *MSH2*, ~30% in *MLH1*, and ~40% in *MSH6*) (19), the consequences of which remain unclear regarding DNA repair. A single amino acid change does not necessarily result in a dysfunctional

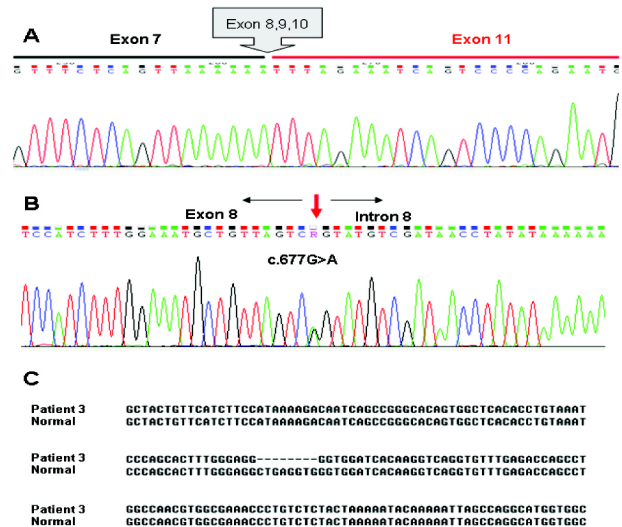


Fig. 3. Characterization of *MLH1* in patient 3. Sequencing pattern of the region from exon 7 to exon 11 of the *MLH1* gene in patient 3 by RT-PCR revealed the deletion of exons 8, 9, and 10 (A). Partial sequencing pattern of exon 8 and intron 8 of the *MLH1* gene in patient 3 (B). Deleted sequence in intron 10 of the *MLH1* gene in patient 3 (C). Partial genomic DNA was amplified by PCR, cloned into a TA cloning vector, and then sequenced.

protein. However, certain single-amino acid substitutions can yield partially active, dominant-negative, unstable, or nonfunctional proteins, depending on the nature of the individual mutations. Defective MMR caused by missense mutations can result from loss of enzymatic activity (ATP binding/hydrolysis) [1]; defective protein-protein interaction (complex formation) [2]; defective protein-DNA binding (mismatch recognition) [3]; aberrant MMR protein subcellular localization [4]; altered MMR protein expression (stoichiometry of MMR complexes) [5]; or [6] altered MMR protein stability. Recently, Drost *et al.* (16) described a new cell-free assay for functional analysis of missense mutations in *MLH1*. They assayed 25 known VUS (variants of uncertain significance) cases, 19 of which exhibited sufficiently poor repair activity to be considered pathogenic (16).

Patient 5 carried the V384D mutation, which has only been identified in East Asians (20). V384 is located in the central region of the *MLH1* protein between the NH₂-terminal ATP-binding domain and the COOH-terminal PMS2 interaction domain. The V384D mutation results in a change in overall charge (neutral, hydrophobic amino acid valine is replaced by aspartate, a weakly acidic and negatively charged amino acid) and is likely to disrupt the structure and thus the stability of *MLH1*. In a recent study, *MLH1* carrying the V384D mutation exhibited reduced MMR activity *in vitro* (about 65% of normal activity) (21). Elsewhere, a yeast two-hybrid assay detected low levels of β -galactosidase activity, although the interaction with PMS2 was partially maintained in co-immunoprecipitation

analyses (20). It is possible that this mutation weakened the interaction between *MLH1* and *PMS2* such that it was not sufficiently strong (in Gal4 system) for the reporter gene (*lacZ*) to be activated. Charge and polarity are important to protein structure and function, especially the formation of secondary structures. Thus, amino acid changes in these positions may affect the interaction between helices in *MLH1*, and thereby, its capacity to bind to and/or hydrolyze ATP.

MATERIALS AND METHODS

Patients

The patients included in this study were recruited at Jeju National University Hospital, Korea. Of the 56 patients diagnosed with colon cancer who subsequently underwent surgery in 2009, 11 suspected to have been suffering from Lynch Syndrome (based on Bethesda guideline 7: individual with at least one adenoma diagnosed at age <40 years) were enrolled in this study. This study was approved by the Institutional Review Board of Cheju National University Hospital, and informed consent for genetic analyses was obtained from all patients and, where applicable, their parents.

Isolation of total RNA and RT-PCR

Total RNA was extracted from white blood cells (WBCs) using an RNeasy[®] Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. First-strand cDNA was then synthesized from 2 µg of RNA using 25 ng/ml of oligo (dT) (Promega, Madison, WI, USA), 0.5 mM dNTP mix, 0.2 U/µl of Omniscript (Qiagen), and 0.5 U/µl of RNase inhibitor. Reactions were incubated at 37°C for 60 min. The resulting cDNA was amplified in a 50 µl PCR reaction containing 0.2 mM dNTP mix, 0.4 µM primer, and 1.25 U of Taq polymerase (Promega) using the primers used in the *MLH1* cDNA analysis; Forward (1)-ATTGGCTGAAGGCACTCCGTTG, Forward (2)-TGGCTTTTCGAGGTGAGGCTTTG, Forward (3)-ACTACCCAATGCCTCAACCGTGG, Forward (4)-AGAGCAAGCTCCTGGGCTCCAAT, Forward (5)-GGATTACCCCTTCTGATTGACAAC, Reverse (1)-ACAGCATTTCCAAAGATGGAGCG, Reverse (2)-CTTCCACCATTTCACATCAGAA, Reverse (3)-CTGGCT CCGAT AACCTGAGAACA, Reverse (4)-CCGGAATACTATCAGAAGGCAAG and the primers used in the *MSH2* cDNA analysis; Forward (1)-GGCGGGAAACAGCTTAGTGGGTG, Forward (2)-TCCCTGATAATGATCAGTTC TCC, Forward (3)-AGATTGTTA CCGACTCTATCAGGG, Forward (4)-CTTCAGGCTATGTAGA ACC AATGC, Forward (5)-GCCCTGGAAGCTTGAGGAGTTTCA, Reverse (1)-CCATGAGAGGCTGCTTAATCCAC, Reverse (2)-ACTCGGGCTAAGATGCAGTCCAC, Reverse (3)-GTGTAACTGTCAGTGCCCA TGGG, Reverse (4)-TGCA AACAGT CCT CAGTTACAGC. The following thermal conditions were applied: 35 cycles of denaturation at 94°C for 15 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min. PCR products were separated on 2% agarose gels and visualized using ethidium bromide.

Isolation and amplification of genomic DNA in region of exon 11 of *MLH1*

Genomic DNA was extracted from peripheral blood using a Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. Genomic DNA in the region of exon 11 of *MLH1* was PCR-amplified in a 50 ml reaction mixture containing 10 pM each of the forward and reverse primers (forward, CACAGCTCTTAAGTAGCAGTGCC; reverse, CCTA GGAACAACAGCACAATACC), 0.2 mM dNTPs, 100 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, and 2 U of Taq polymerase (Promega). The following thermal conditions were applied: 35 cycles at 95°C for 1 min, 59°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were separated on 1.5% agarose gels and visualized using ethidium bromide.

Sequence analysis

The products of PCR amplification were purified using a QIAquick PCR Purification Kit (Qiagen) and analyzed by direct sequencing in both directions using an ABI-Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit in conjunction with an ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The primers used in the sequencing reactions were the same as those used in the PCR analysis (Table 1). Sequences obtained from both HNPCC patients and healthy controls were compared with those listed in the GenBank database by BLAST analysis.

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