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# Detection of germline mutations of *hMLH1* and *hMSH2* based on cDNA sequencing in China

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## Abstract

**AIM:** To detect the germline mutations of *hMLH1* and *hMSH2* based on mRNA sequencing to identify hereditary non-polyposis colorectal cancer (HNPCC) families.

**METHODS:** Total RNA was extracted from peripheral blood of 14 members from 12 different families fulfilling Amsterdam criteria II. mRNA of *hMLH1* and *hMSH2* was reversed with special primers and heat-resistant reverse transcriptase. cDNA was amplified with expand long template PCR and cDNA sequencing analysis was followed.

**RESULT:** Seven germline mutations were found in 6 families (6/12, 50%), in 4 *hMLH1* and 3 *hMSH2* mutations (4/12, 33.3%); (3/12, 25%). The mutation types involved 4 missense, 1 silent and 1 frame shift mutations as well as 1 mutation in the non-coding area. Four out of the seven mutations have not been reported previously. The 4 *hMLH1* mutations were distributed in exons 8, 12, 16, and 19. The 3 *hMSH2* mutations were distributed in exons 1 and 2. Six out of the 7 mutations were pathological, which were distributed in 5 HNPCC families.

**CONCLUSION:** Germline mutations of *hMLH1* and *hMSH2* can be found based on cDNA sequencing so as to identify HNPCC family, which is highly sensitive and has the advantages of cost and time saving.

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**Key words:** *hMLH1*; *hMSH2*; Colorectal cancer; Hereditary non-polyposis; Reverse transcription; Germline mutation

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## INTRODUCTION

*hMLH1* and *hMSH2* are the two most important genes for HNPCC, which is the most common hereditary colon syndrome accounting for 10% of all colorectal cancers. It is autosomally dominant with a penetrance rate of 80-90%. HNPCC occurrence is closely associated with deficiency or loss of function of mismatch repair (MMR) genes. Affected individuals have an approximately 70% lifetime risk of colon cancer with a mean onset age of 44 years and an approximately 40% lifetime risk of endometrial cancer in females. At least 5 MMR genes, *hMLH1, hMSH2, hMSH6, hPMS1,* and *hPMS2*, have been implicated in HNPCC[1,2]. Information of genetic linkage analysis shows that germline mutations of *hMLH1* and *hMSH2* account for nearly 90% of all germline mutations found in HNPCC[3]. Germline mutations in MMR genes predispose to colorectal and other HNPCC associated epithelial cancers. Identification of MMR gene germline mutations has direct clinical implications in counseling and management of HNPCC.

Methods such as microsatellite instability (MSI), immunohistochemistry (IHC)[4-6], and sequencing of genes are employed to screen HNPCC. The most specific method is to detect the germline mutations of MMR. Its cost and sensitivity limitations can be overcome at least in part by RNA-based analysis[7]. It is the first time in China that we identified HNPCC families by detecting germline mutations of *hMLH1* and *hMSH2* genes based on cDNA sequencing with special primers and heat-resistant reverse transcriptase.

## MATERIALS AND METHODS

### Subjects

Fourteen anticipants from 12 unrelated families fulfilling Amsterdam criteria II for HNPCC were studied. Personal and family cancer history was obtained from the patients and their relatives. Pathological diagnosis and death were confirmed by review of medical records, pathological reports or death certificates.

### Samples

Three microliters of peripheral blood was taken from each participant. Total RNA was extracted using TRIzol (Sigma Company) according to the manufacturer's instructions.

### *RT-PCR*

cDNA was synthesized with transcriptor reverse transcriptase (Roche Diagnostics) using 0.5 μg of total RNA and specific primers complementary to the 3' end of *hMLH1* (2484-TATGTTAAGACACATCTATTTATTTA-2459) and to the 3' end of *hMSH2* (3145-CCACCAAACTACATGATTTTATTTATAAAATTC-3114). RT was performed at 60 °C for 60 min.

cDNA of *hMLH1* and *hMSH2* was amplified in two overlapping fragments using primers (Table 1) to generate products of ~2 000 bp. PCR was performed using expand long template PCR (Roche Diagnostics) at 94 °C for 5 min; then 10 cycles at 94 °C for 30 s, at 59 °C for 30 s, at 68 °C for 3 min; 32 cycles at 94 °C for 30 s, at 57 °C for 30 s, at 68 °C for 3 min with a final elongation at 68 °C for 7 min.

PCR products were size fractionated by agarose gel electrophoresis and analyzed by ethidium bromide staining.

### *Sequencing*

Purified PCR fragments were sequenced directly using a DNA sequencing kit according to Applied Biosystems from USA with BigDye Terminators on an ABI3700 automated DNA sequencer.

cDNA of *hMLH1* (2 484 bp) was sequenced in six overlapping fragments and cDNA of *hMSH2* (3 145 bp) was sequenced in eight overlapping fragments using primers (Table 2).

## RESULTS

The sizes of amplified *hMLH1* and *hMSH2* segments were respected (Figure 1). Seven germline mutations were found in 6 out of 12 families, 4 *hMLH1* and 3 *hMSH2* mutations (4/12, 33.3%); (3/12, 25%). The mutation types involved 4 missense, 1 silent and 1 frame shift mutations as well as 1 mutation in non-coding area, including *hMLH1* mutation in family H2 at 649 codon 217 exon 8: CGC→TGC; *hMLH1* mutation in family H31 at 1742 codon 581 exon 16: CCG→CTG; *hMLH1* missense mutation in family H114 at 1151 codon 384 exon 12: GTT→GAT; family H111 *hMLH1* non-coding area at 2438 exon 19: A→C; family H11 *hMSH2* at 14 codon 5: CCG→CAG; family H38 hMSH2 mutations at 295 and 296 codon 99 exon 2: 295: A→C, 296:del.G (Table 3, Figure 2).

## DISCUSSION

Colorectal cancer (CRC) is one of the most common malignant tumors and its incidence is increasing gradually. According to the different molecular mechanism, CRC is divided into sporadic and genetic types. The latter type HNPCC is characterized by its early onset[8-10], location in the proximal colon and an increased risk of neoplasms in extracolonic organs including endometrium, stomach, urothelium, small intestine, ovary and multiple

**Table 1** Sequence and localization of primers used for amplification of cDNA of *hMLH1* and *hMSH2*

| Sense | Antisense |
|---|---|
| *hMLH1*-1F(1-18) | *hMLH1*-5R(2198-2175) |
| CTTGGCTCTTCTGGCGCC | GAGCGCAAGGCTTTATAGACAATG |
| *hMLH1*-4F(1333-1353) | *hMLH1*-6R(2484-2459) |
| GCTGAAGTGGCTGCCAAAAAT | TATGTTAAGACACATCTATTTATTTA |
| *hMSH2*-1F(1-21) | *hMSH2*-7R(2753-2732) |
| GGCGGGAAACAGCTTAGTGGG | GGGCATTTGTTTCACCTTGGAC |
| *hMSH2*-6F(1898-1920) | *hMSH2*-8R(3145-3114) |
| CGTGTCAAATGGAGCACCTGTTC | CCACAAACTACATGATTTTATTTATAAAATTC |

**Table 2** *hMLH1* and *hMSH2* primers used for sequencing of cDNA

| Sense | Antisense |
|---|---|
| *hMLH1*-1F CCTGGCTCTTCTGGCGCC | *hMLH1*-1R CTTTTCTCCTCGTGGCTATGTTGT |
| *hMLH1*-2F ATGTGCTGGCAATCAAGGGA | *hMLH1*-2R GGTGCACATTAACATCCACATTCT |
| *hMLH1*-3F CCAAAAACACACACCCATTCCT | *hMLH1*-3R CCTTTGTTGTATCCCCCTCCA |
| *hMLH1*-4F GCTGAAGTGGCTGCCAAAAAT | *hMLH1*-4R CATCTTCCTCTGTCCAGCCACTC |
| *hMLH1*-5F TTGCCATGCTTGCCTTAGATAGTC | *hMLH1*-5R GAGCGCAAGGCTTTATAGACAATG |
| *hMLH1*-6F GCTCCATTCCAAACTCCT | *hMLH1*-6R TATGTTAAGACACATCTATTTATTTA |
| *hMSH2*-1F GGCGGGAAACAGCTTAGTGGG | *hMSH2*-1R CTCTGGCCATCAACTGCGGAC |
| *hMSH2*-2F GGCTTCTCCTGGCAATCTCTCTCA | *hMSH2*-2R CTTGATTACCGCAGACAGTGATGAAAC |
| *hMSH2*-3F GCAAAAAGGGAGAGCAGATGAATAGTG | *hMSH2*-3R GGCAAGTCGGTTAAGATCTGGGAAT |
| *hMSH2*-4F AGATGCAGAATTGAGGCAGACTTTACA | *hMSH2*-4R GGACTTTTTCTTCCTTACAGGTTACACG |
| *hMSH2*-5F CAGAGATCTTGGCTTGGACCCT | *hMSH2*-5R TTCAACACAAGCATGCCTGGAT |
| *hMSH2*-6F CGTGTCAAATGGAGCACCTGTTC | *hMSH2*-6R GATTGGCCAAGGCAGTAAGTTCAT |
| *hMSH2*-7F AATCATAGATGAATTGGGAAGAGGAACT | *hMSH2*-7R GGGCATTTGTTTCACCTTGGAC |
| *hMSH2*-8F CTATCTGGAAAGAGAGCAAGGTGAA | *hMSH2*-8R CCACAAACTACATGATTTTATTTATAAAATTC |

**Table 3** *hMLH1* and *hMSH2* mutations detected by cDNA sequencing

| Families | Genes | Exon | Codons affected | DNA change | Amino acid change | Mutation types |
|---|---|---|---|---|---|---|
| H31 | *hMLH1* | 16 | 581 | T>C, at 1742 | Pro→Leu | Missense |
| H111 | *hMLH1* | 19 | Non-coding area | A>T, at 2438 | | |
| H114 | *hMLH1* | 12 | 384 | T→A, at 1151 | Val→Asp | Missense |
| H2 | *hMLH1* | 8 | 217 | T->C, at 649 | Arg→Cyc | Missense |
| H11 | *hMLH1* | 1 | 5 | A>C, at 14 | Pro→His | Missense |
| H38 | *hMLH1* | 2 | 99 | A>C, at 295 | Arg→Arg | Silent |
| H38 | *hMLH1* | 2 | 99 | Del G, at 296 | Frame shift | Frame shift |

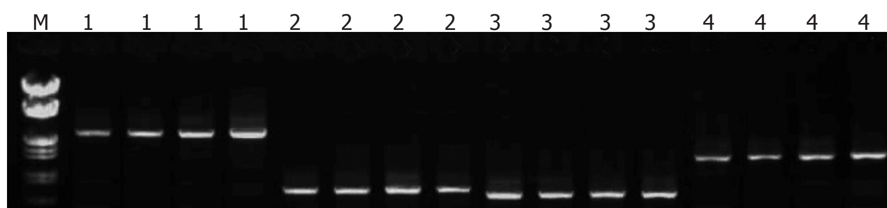


**Figure 1** Amplified segments of *hMLH1* and *hMSH2*. "M" is mark. Lanes 1-4 sizes of segments.

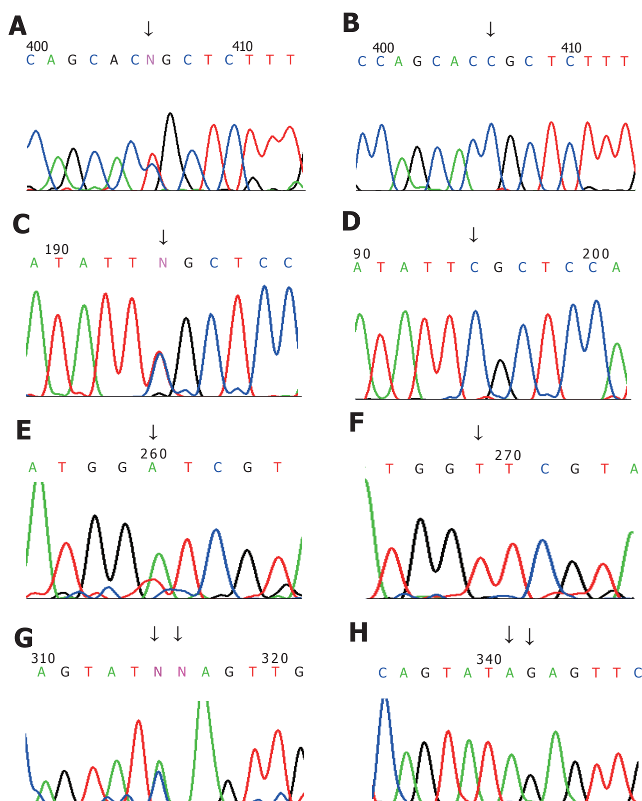


**Figure 2** *hMLH1* and *hMSH2* mutations (**A, C, E, G**) and wild-type sequence (**B, D, F, H**) in families H31, H2, H114, and H38 at different codons. Arrows indicate the corresponding sites of mutation.

metachronous CRCs[9,11-14]. Its prognosis is better than sporadic type of CRC[15]. HNPCC is closely associated with the deficiency or loss of MMR gene function. Identification of MMR gene germline mutations has direct clinical implications in counseling and management of HNPCC.

Methods are available for the identification of HNPCC. The most specific method is to detect the germline mutations of MMR genes. Up to now, the germline mutations are mainly detected by genomic DNA-based sequencing (gDNA). A lot of information shows that the germline mutations of MMR genes associated with HNPCC are mainly localized in exons[3,5,7,13]. The gDNA-based sequencing is invariably affected by introns. cDNA-based sequencing of MMR genes has been reported recently. The new technique utilizes specific primers and heat-resistant reverse trancriptase to specifically synthesize cDNA of MMR genes, then full-length cDNA is amplified in two over fragments using specific primers followed by sequencing analysis of cDNA. The technique can successfully avoid the influence of introns. Additionally, it is well known that RNA is easily decayed. If RNA samples are stored too long, reverse transcription with random primers and common reverse transcription enzyme often fails, while the new technique employs specific primers and heat-resistant reverse trancriptase, the limitations can be overcome at least in part, thus improving the specificity and efficiency. Anna *et al*[7] compared the two techniques and found that cDNA-based sequencing not only has the advantage of specificity and efficiency, but also a lower cost, being 2.5-3 times less expensive than gDNA-based sequencing. We used 35 pair primers to amplify the two genes in the past, and only 4 pair primers were used in the present study, the procedure is greatly simplified.

We detected 7 germline mutations in 14 anticipants with HNPCC from 12 different families employing the new technique. The 3 mutations, at sites 1151, 14, and 217 in *hMLH1* reported, the first two have been verified to be pathological. Moreover, the mutation at 1151 in *hMLH1* has been found only in Japan and Korea, which is likely to be a hot mutation site in East Asia. The mutation at site 217 in *hMLH1* occurs at a less conserved region is in 80 healthy Japanese. Whether it is pathological or not needs further study. None of the 4 unreported mutations belongs to polymorphism[17]. The 6 pathological mutations (2 reported, 4 unreported) were distributed in 5 HNPCC families in our study.

Of course, all mutations cannot be detected by the improved technique. For example, mutations in the promoter and 3'-untranslated regions of *hMLH1* and *hMSH2* cannot be detected. Sequencing of individual exons of gDNA also has such limitations.

Up to now, there is no optimal method to screen HNPCC patients or their families. The new technique can be utilized to screen HNPCC patients and their families, which may achieve a better result.

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