

Short Report

Low mutation rate of *hMSH2* and *hMLH1* in Taiwanese hereditary non-polyposis colorectal cancer

Wei S-C, Yu C-Y, Tsai-Wu J-J, Su Y-N, Sheu J-C, Wu C-HH, Wang C-Y and Wong J-M. Low mutation rate of *hMSH2* and *hMLH1* in Taiwanese hereditary non-polyposis colorectal cancer.

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Hereditary non-polyposis colorectal cancer (HNPCC), the most common type of hereditary colorectal cancer, is thought to be a simple Mendelian disease involving DNA mismatch repair genes. The majority of mutations associated with HNPCC occur in the *hMSH2* and *hMLH1* genes. The reported incidence of mismatch repair gene mutations in HNPCC kindreds varies considerably (from 22 to 86%), and most mutations are unique. This study aimed to determine the genetic basis of Taiwanese HNPCC kindreds, focusing on the two major genes involved in this disease. A total of 15 Taiwanese HNPCC kindreds meeting the Amsterdam criteria, including 72 affected individuals among a total of 266 individuals, were analyzed using both RNA- and DNA-based methods. The mutation rate of *hMSH2* and *hMLH1* in these 15 kindreds was 0% and 20%, respectively, which is lower than that reported in other countries. Two novel mutations were discovered in *hMLH1*: one was an allelic loss of a 5.2-kb genomic fragment causing exon 16 deletion; and the other was a two-nucleotide deletion that resulted in a frameshift mutation of exon 3. We also identified one *hMLH1* exon 4 mutation (a C to T transition in codon 117), which had been reported previously in western countries. This is the first genetic study of HNPCC from Taiwan.

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The prevalence of colorectal cancer (CRC) in Taiwan has shown a gradual increase, in recent years, and is now the third highest cause of cancer-related death (1). Although CRC has long been considered a major public health concern in most western nations, its status as the leading cause of cancer-related death in eastern countries has only been established recently. It has been estimated that at least 10% of the CRC burden can be attributed to genetic factors (2). The most common hereditary CRC disorder is hereditary non-polyposis colorectal cancer (HNPCC) (3).

The diagnosis of HNPCC is based on the Amsterdam criteria (4), which require:

1 three or more relatives with histologically verified CRC, one of whom must be a first-degree relative of the other two persons;

2 a CRC history involving at least two generations; and
3 at least one CRC case diagnosed before 50 years of age.

Several studies have shown that HNPCC is a simple Mendelian disease involving DNA mismatch repair (MMR) genes (*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMLH3* and *hMSH6*) (5–13). The majority of the HNPCC-associated DNA MMR gene mutations occur in the *hMSH2* and *hMLH1* genes (14–16). Approximately 38% of HNPCC kindreds may have associated mutations in *hMSH2*, and 49% may have *hMLH1*-associated mutations (15, 16). The reported incidence of MMR gene mutations in HNPCC kindreds reported in previous series varies considerably, from 22 to 86% (17, 18). According to

the database of the International Collaborative group (ICG)-HNPCC, the majority of the mutations affected either *hMLH1* or *hMSH2* and were evenly distributed along the genes. Most *hMSH2* mutations were frameshift (60%) or nonsense (23%), whereas mutations in *hMLH1* were mainly frameshift (40%) or missense (31%). Most mutations were unique (14). Establishment of mutation spectra in the DNA MMR genes in HNPCC kindreds is beneficial to any potential carrier for early genetic screening and surveillance.

According to our previous report, the clinical characteristics of Taiwanese HNPCC were similar to those observed in western countries, although the Taiwanese cohort had a younger age of disease onset and lack of small bowel and urinary tract cancer (19). This study aimed to determine the genetic basis of Taiwanese HNPCC kindreds, focusing on the DNA MMR genes *hMSH2* and *hMLH1*.

Materials and methods

Patients

Fifteen Taiwanese HNPCC families that fulfilled the Amsterdam criteria were enrolled in the study. Initial screening was performed on a single proband from each of the 15 unrelated families. Additional family members, if available, were tested for the segregation of specific molecular variants. After completing genetic counseling and obtaining informed consent for participation, a 20-ml blood sample from each individual was drawn into EDTA anticoagulant for the preparation of genomic DNA and RNA samples.

DNA and RNA purification

DNA was purified from 200 µl of whole blood using the Viogene blood and tissue genomic DNA extraction kit (Viogene, Taipei, Taiwan), according to the manufacturer's instructions. Lymphocytes were isolated from 20 ml of whole blood by using the

Ficoll-Paque™ Plus system (Amersham Pharmacia Biotech, Uppsala, Sweden). Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

RNA-based analysis

Reverse transcription–polymerase chain reaction (RT–PCR)

Two micrograms of total RNA was reverse transcribed using 1 µg of oligo-dT, 4-mmol/l deoxynucleoside triphosphates, and 300 U of Superscript II reverse transcriptase (Life Technologies, Arlington Heights, IL), following the manufacturer's suggested reaction conditions. PCR amplifications were performed in 25-µl reaction mixes containing the following: 1–2 µl of complementary DNA mix, 2.5 µl of 10 × PCR buffer [50-mmol/l Tris–HCl (pH 9.1), 16-mmol/l ammonium sulfate, 3.5-mmol/l MgCl₂, and 150-µg/ml bovine serum albumin], 0.8-mmol/l deoxynucleoside triphosphates, 5 U of OptiPol DNA polymerase (GeneTeks BioScience Inc., San-Chung, Taipei), and 5 pmol of each PCR primer. Primers for both *hMSH2* and *hMLH1* were designed to amplify the genes into three overlapping segments of about 1 kb in length. The upstream primer of each segment contained signals for efficient transcription and eukaryotic translation (T7 promoter sequence and a translation initiation site). The primers used for the PCR are shown in Table 1. The reactions were programmed for thermal cycling as follows: 35 cycles of 95 °C for 30 s, 58–62 °C for 30 s, and 70 °C for 2 min, and a final extension at 70 °C for 7 min. The PCR products were purified using the PCR clean-up purification kit (Viogene, Sunnyvale, CA), in preparation for the *in vitro* transcription/translation and automatic sequencing reaction.

In vitro transcription and translation

Purified RT–PCR product was mixed with T7 RNA polymerase and [³⁵S]L-methionine (100 µCi/µl)

Table 1. Primers used for the reverse transcription–polymerase chain reaction (RT–PCR) of *hMSH2* and *hMLH1*

Genes	Names	Primer sequences (5'→3')
<i>hMSH2</i>	Fragment A T7-1	GGATCCTAATACGACTCACTATAGGGAGACCACCATGCAGCCGAAGGAGACGC
	Fragment A SP6-1	AAGACTTGTTAACCAGTGGAT
	Fragment B T7-2	GGATCCTAATACGACTCACTATAGGGAGACCACCATGGGACAGTTTGAAGTACTAC
	Fragment B SP6-2	GTCAGCTTTGCTCACGTGTC
	Fragment C T7-3	GGATCCTAATACGACTCACTATAGGGAGACCACCATGGAAGCCCAGGATGCCATTG
	Fragment C SP6-3	CCCAGTAATGGAAATGAAGTAATATTGATAAGC
<i>hMLH1</i>	Fragment A T7-1	TAATACGACTCACTATAGGGAGACCACCATGCGGCGGCTGGACGAGACAGT
	Fragment A SP6-1	CACACCCATTCTGTACCTCAG
	Fragment B T7-2	TAATACGACTCACTATAGGGAGACCACCATGAACTACTCAGTGAAGAAGTGC
	Fragment B SP6-2	GTGGGCCTTGGCACAGCATC
	Fragment C T7-3	TAATACGACTCACTATAGGGAGACCACCATGTGTACCCCCGGAGAAGGAT
	Fragment C SP6-3	GCTTGCTAACCTGCCTGATC

(NEN Life Science Products, Inc., Boston, MA) in a rabbit reticulocyte lysate-based transcription/translation system (Promega, Madison, WI) and incubated at 30 °C for 2 h. Protein electrophoresis was performed in 4%/12% stacking/separating sodium dodecyl sulphate (SDS) polyacrylamide gels. The gels were fixed and dried under a vacuum at 80 °C before autoradiography.

Direct sequencing

A sequencing reaction was performed in all PCR samples from cDNA using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Inc., Foster City, CA). Electrophoresis was carried out using a Genetic Analyzer 310 (PE Applied Biosystems, Inc.) equipped with long-read sequencing capillary and POP-6 sequencing polymer (PE Applied Biosystems, Inc.). Table 2 lists the primers used for direct sequencing reactions.

DNA-based analysis

PCR conditions

PCR amplifications of *hMLH1* and *hMSH2* exons were performed in a 50-µl volume containing 10-mmol/l Tris-HCl (pH 8.3), 50-mmol/l KCl, 1.5–4.5-mmol/l MgCl₂, 50-mmol/l dNTPs, 0.25 mmol/l of each primer, 100 ng of genomic DNA, and 1 U of BioThermStar™ DNA polymerase (Gene Craft GmbH, Munster, Germany). The PCR cycling regime comprised an initial 10-min denaturation step at 95 °C to activate the BioThermStar™ DNA polymerase. Subsequent denaturing steps were performed at 94 °C for 20 s and extension steps at 72 °C for 45 s. Primer pairs and the adjusted temperatures for individual amplicons are given in Tables 3 and 4.

Table 2. Primers used for direct sequencing of reverse transcription-polymerase chain reaction (RT-PCR) products, *hMSH2* and *hMLH1*

Genes	Names	Primer sequences (5'→3')
hMSH2	SP1	TTGGAGAGCGCGGCCGA
	SP4	CAGGTTGGAGTTGGG
	SP7	CAGAGCCCTTAACC
	SP10	GATCCTAATCTCATCAGTG
	SP12	GCTCAGCTAGATGCT
	SP15	CTGCCTTGGCCAATCAG
hMLH1	SP1	TGAACCGCATCGCGGCGG
	SP4	GGAAGTTGTTGGCAG
	SP7	TTCTCCAGGATGT
	SP9	CAAAGGGGACTTCAG
	SP10	GAGTCTCCAGGAAG
	SP12	CTGAAGAAGAAGGCTG

Denaturing high-performance liquid chromatography (DHPLC)

DHPLC was carried out on automated HPLC instrument equipped with a DNASep column (Transgenomic Inc., San Jose, CA). The column was packed with proprietary 2-mm non-porous alkylated poly(styrene-divinylbenzene) particles (20). The mobile phase consisted of 0.1-mol/l triethylammonium acetate buffer (TEAA; Catalog no. 400613; PE Biosystems) (pH 7.0) containing 0.1-mmol/l tetrasodium ethylenediamine-tetraacetic acid (ED4SS; Sigma, St Louis, MO). Crude PCR products were subjected to an additional 3-min denaturing step at 95 °C, followed by gradual reannealing from 95 to 65 °C over a period of 30 min prior to analysis. The products were eluted with a linear acetonitrile (9017-03; J. T. Baker, Phillipsburg, NJ) gradient at a flow rate of 0.9 ml/min. The start- and end-points of the gradient were adjusted, according to the size of the PCR products, using an algorithm provided by the WAVEMAKER system control software (Transgenomic Inc.). Eight microliters of PCR product was injected for analysis in each run. Generally, analysis took about 10 min per injection, including column regeneration and re-equilibration to the starting conditions. The temperature required for successful resolution of heteroduplex molecules was determined using the DHPLC melting algorithm. Details about the temperature conditions and the algorithm itself were obtained using WAVEMAKER software. The appropriate experimental conditions for DHPLC are listed in Tables 3 and 4. Heterozygous profiles were identified by visual inspection of the chromatograms on the basis of the appearance of additional earlier eluting peaks. Corresponding homozygous profiles showed only one peak.

Direct sequencing

Sequencing reactions were performed only in the samples that showed heterozygous profiles during DHPLC analysis. The methods used for DNA-based direct sequencing were the same as those previously described for RNA-based analysis.

Results

A total of 266 individuals from 15 Taiwanese families, including 72 registered HNPCC patients (37 men and 35 women) who met the Amsterdam criteria, were recruited for this study. Most of the patients (59/72) had colorectal cancer. Other types of cancers included endometrial (six of 72), ovarian (two of 72), gastric (six of 72), pancreatic (one of 72), lung (two of 72), breast (two of 72), thyroid (one of 72), prostate (one of 72)

Table 3. Primers, amplicon sizes, annealing temperatures and denaturing high-performance liquid chromatography (DHPLC) conditions for each exon of *hMSH2*

Amplicon	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)	Annealing temp.	DHPLC temp.	Gradient ^a
MSH2 e1	TCGCGCATTCTTCTCAACC	GTCCCTCCCCAGCACGC	312	62	65	55–64% B 4.5 min
MSH2 e2	GAAGTCCAGCTAATA CAGTGC	CTTCACATTTTTATT TTCTACTC	286	52	54	54–63% B 4.5 min
MSH2 e3	GCTTATAAAAATTTAA AGTATGTTT	GCCTTTCCTAGGCCT GGAATCTCC	414	52	57	57–66% B 4.5 min
MSH2 e4.1	TTCATTTTTGCTTTTCTTATTCC	ATATGACAGAAAATATCCTTC	316	52	56	53–62% B 4.5 min
MSH2 e4.2	TTCATTTTTGCTTTTCTTATTCC	ATATGACAGAAAATATCCTTC	316	52	52	53–62% B 4.5 min
MSH2 e5.1	CCAGTGGTATAGAAATCTTCG	CCAATCAACATTTTAAACCC	241	52	57	52–61% B 4.5 min
MSH2 e5.2	CCAGTGGTATAGAAATCTTCG	CCAATCAACATTTTAAACCC	241	52	52	52–61% B 4.5 min
MSH2 e6.1	GTTTTCACTAATGAGCTTGCC	GTGGTATAATCATGTGGC	251	52	58	50–59% B 4.5 min
MSH2 e6.2	GTTTTCACTAATGAGCTTGCC	GTGGTATAATCATGTGGG	251	52	55	53–62% B 4.5 min
MSH2 e7	GACTTACGTGCTTAGTTG	GTATATATTGTATGAGT TGAAGG	326	52	53	55–64% B 4.5 min
MSH2 e8.1	GATTTGTATTCTGTAA AATGAGATC	GGCCTTTGCTTTTTAA AAATAAC	222	52	56	51–60% B 4.5 min
MSH2 e8.2	GATTTGTATTCTGTAAA ATGAGATC	GGCCTTTGCTTTTTAA AAATAAC	222	52	52	51–60% B 4.5 min
MSH2 e9	GTCTTTACCCATTATTTATAGG	GTATAGACAAAAGA ATTATTATTCC	217	52	56	51–60% B 4.5 min
MSH2 e10.1	GGTAGTAGGTATTT ATGGAATAC	CATGTTAGAGCATTAGGG	259	52	56	53–62% B 4.5 min
MSH2 e10.2	GGTAGTAGGTATTTA TGAATAC	CATGTTAGAGCATTAGGG	259	52	52	53–62% B 4.5 min
MSH2 e11	CACATTGCTTCTAGTACA	CAGGTGACATTCAGAAC	198	52	54	50–59% B 4.5 min
MSH2 e12	ATTCAGTATTCCTGTGTAC	CGTTACCCCCACAAAGC	327	52	56	55–64% B 4.5 min
MSH2 e13.1	CGCGATTAATCATCAGTG	GGACAGAGACATACA TTTCTATC	353	52	58	53–62% B 4.5 min
MSH2 e13.2	CGCGATTAATCATCAGTG	GGACAGAGACATAC ATTCTATC	353	52	54	56–65% B 4.5 min
MSH2 e14	TACCACATTTTATGTGATGG	GGGGTAGTAAGTTTCCC	353	52	57	56–65% B 4.5 min
MSH2 e15	CTCTTCTCATGCTGTCCC	ATAGAGAAGCTAAGTTAAAC	261	52	58	53–62% B 4.5 min
MSH2 e16	TAATTACTCATGGGACATTC	TACCTTCATTCCATTACTGG	276	52	57	53–62% B 4.5 min

^aEluent B: 0.1-M triethylammonium acetate (TEAA) buffer, pH 7.0, containing 25% acetonitrile.

and liver (two of 72). Ten of the patients had two types of cancer. Age at cancer onset could be traced in 45 individuals and ranged from 19 to 65 years (mean age 43.2 years).

Three mutations were detected in *hMLH1*. One was found using RNA-based methods, another by DNA-based methods; the other was detected by both methods. The RNA-based analysis, which comprised *in vitro* transcription-translation (IVTT) and direct sequencing of all RT-PCR products, detected two constant bands of *hMLH1*, fragment C, in family K. These two bands were of similar density: one was of the expected size (769 bp) and the other was smaller (604 bp) (Fig. 1a). This pattern was found in all three affected members of the same family, but not in the other members. IVTT testing of the smaller band showed that this mutation resulted in a 6-kDa loss in the protein product (Fig. 1b). Further sequencing of the PCR product revealed an exon-16 deletion in the smaller band (Fig. 1c). The exon-16 deletion in the cDNA was caused by a 5,197-bp genomic deletion in *hMLH1*, as shown

in Fig. 1(d), according to amplification of genomic DNA with different sets of PCR primers followed by direct sequencing. The same approach was adapted for the mutational analysis of *hMSH2* in these HNPCC kindreds. No frameshift or point mutation was found in *hMSH2* using IVTT and automatic sequencing of the RT-PCR products.

In the DNA-based analysis, we used DHPLC as a first screen to identify heterozygous profiles. After completing the sequencing results and comparing these data with the family pedigree, we found one exon-3 (family H) and one exon-4 (family T) mutation in *hMLH1*. The exon-3 mutation was a two-nucleotide loss at codons 84 and 85, causing a frameshift mutation at codon 84 from cysteine (TGT) to a stop codon (TGA) (Fig. 2a, 2b). The exon-4 mutation was a point mutation (C→T transition) in codon 117, which resulted in an amino acid change from threonine to methionine (Fig. 3a, 3b). This mutation had also been detected from the cDNA-based PCR product, but not from the IVTT, by RNA-based

Table 4. Primers, amplicon sizes, annealing temperatures and denaturing high-performance liquid chromatography (DHPLC) conditions for each exon of *hMLH1*

Amplicon	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)	Annealing temp.	DHPLC temp.	Gradient ^a
MLH1 e1	AGGCACTGAG GTGATTG	TCGTAGCCCTTAAGTGAGC	219	52	61	53–62% B 4.5 min
MLH1 e2.1	AATATGTACATT AGAGTAGTTG	CAGAGAAAGGTCCTGACTC	213	52	59	47–56% B 4.5 min
MLH1 e2.2	AATATGTACATT AGAGTAGTTG	CAGAGAAAGGTCCTGACTC	213	52	56	51–60% B 4.5 min
MLH1 e3	AGAGATTTGG AAAATGAGTAAC	ACAATGTCATCACAGGAGG	207	52	55	51–60% B 4.5 min
MLH1 e4.1	AACCTTCCCTTT GGTGAGG	GATTACTCTGAGACCTAGGC	226	52	58	50–59% B 4.5 min
MLH1 e4.2	AACCTTCCCTTT GGTGAGG	GATTACTCTGAGACCTAGGC	226	52	55	53–62% B 4.5 min
MLH1 e5	GATTTTCTCTTT CCCCTTGGG	CAAACAAAGCTTCAACAATTTAC	190	52	55	50–59% B 4.5 min
MLH1 e6	GGGTTTTATTTCAAGT ACTTCTATG	GCTCAGCAACTGTTC ATGTATGAGC	236	52	57	52–61% B 4.5 min
MLH1 e7	AAAAGGGGGCTCT GACATCT	TAAACAAAACCATCCCCCA	190	58	55	50–59% B 4.5 min
MLH1 e8.1	CTCAGCCATGAGA CAATAAATCC	GGTCCCAAATAATGTGATGG	217	52	57	49–58% B 4.5 min
MLH1 e8.2	CTCAGCCATGAGA CAATAAATCC	GGTCCCAAATAATGTGATGG	217	52	52	52–61% B 4.5 min
MLH1 e9.1	TGGGTGAACAGAC AAATGGA	GGGCTGAGCACAGACTTAGG	193	62	56	58–67% B 4.5 min
MLH1 e9.2	TGGGTGAACAGA CAAATGGA	GGGCTGAGCACAGACTTAGG	193	62	53	54–63% B 4.5 min
MLH1 e10	CATGACTTTGTGTG AATGTACACC	GAGGAGAGCCTGAT AGAACATCTG	239	52	58	53–62% B 4.5 min
MLH1 e11.1	GGGCTTTTTCTCC CCCTCCC	AAAATCTGGGCTCTCACG	289	62	61	50–59% B 4.5 min
MLH1 e11.2	GGGCTTTTTCTCC CCCTCCC	AAAATCTGGGCTCTCACG	289	62	56	56–65% B 4.5 min
MLH1 e12	AGTACTGCTCCAT TTGGGGA	GAAAAGCCAAAGTTAGAAGGCA	434	52	59	59–68% B 4.5 min
MLH1 e13	TGCAACCCACAAAA TTTGGC	CTTCTCCATTTCCAAAACC	292	52	57	55–64% B 4.5 min
MLH1 e14	TGGTGTCTCTAGTTCTGG	CATTGTTGTAGTAGCTCTGC	254	52	57	55–64% B 4.5 min
MLH1 e15	CCCATTTGTCCCAACTGG	CGGTCAAGTTGAAATGTCAG	181	52	57	49–58% B 4.5 min
MLH1 e16	CATTTGGATGCTCC GTTAAAGC	CACCCGGCTGGAAATTTATTTG	276	52	58	53–62% B 4.5 min
MLH1 e17	GGAAAGGCACTGGAGA AATGGG	CCCTCCAGCACACATGCATGTACCG	228	52	58	52–61% B 4.5 min
MLH1 e18	TAAGTAGTCTGTGATCTCCG	ATGTATGAGGTCCTGTCC	247	52	56	54–63% B 4.5 min
MLH1 e19	GACACCAGTGTATGTTGG	AGAGAAAGAAGAACACATCCC	449	52	56	57–66% B 4.5 min

^aEluent B: 0.1-M triethylammonium acetate (TEAA) buffer, pH 7.0, containing 25% acetonitrile.

methods. Among the 12 heterozygous profiles found during *hMLH1* screening, five had amino acid changes. Two were the pathogenic mutation, as mentioned above, and three were hypothesized to represent polymorphism after comparing the profiles with the family pedigree. There were four nucleotide changes in the intron but not in the splicing sites. Another three profiles were heterozygous, but no abnormal sequencing results could be defined. Similar methods applied during *hMSH2* screening in these HNPCC kindreds also did not reveal any mutations. Therefore, although 13 heterozygous profiles were found, only one polymorphism in exon 2, seven nucleo-

tide changes in the intron, but not the splicing sites, and five profiles without any detectable abnormalities, were identified with automatic sequencing.

Discussion

We detected three mutations (two of which were novel) in *hMLH1* using both RNA- and DNA-based genetic screening. The first mutation, a 5.2-kb genomic DNA loss that resulted in an in-frame deletion of exon 16, was detected using an RNA-based method. Previous reports found that the majority of the germline mutations in the

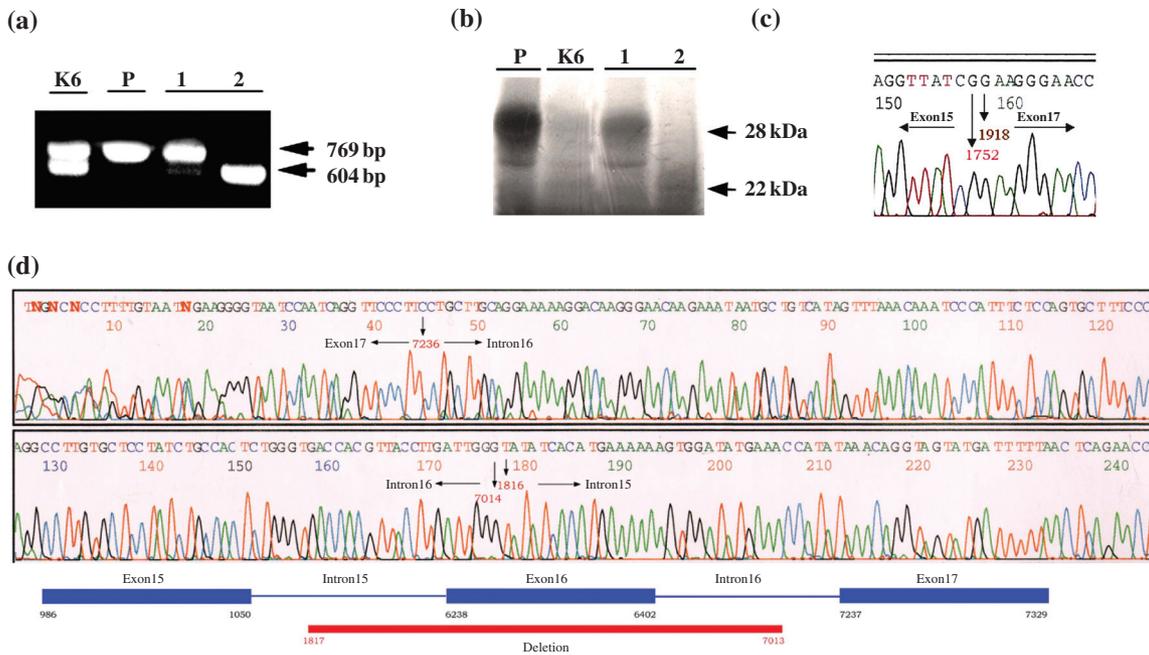


Fig. 1. Exon-16 deletion of *hMLH1*. (a) Two constant bands were detected in *MLH1*, fragment C, of family K (K6). The two bands were of similar density. One was of the expected size (769 bp), as in the positive control of plasmid (P), whereas the other was smaller (604 bp). The two bands were isolated and eluted for further analysis by *in vitro* transcription-translation (IVTT) (lanes 1 and 2). (b) IVTT of the purified polymerase chain reaction (PCR) products from the isolated bands shown in (a) revealed the truncated product with a loss of about 6 kDa. P, positive control from plasmid; K6, patient; 1, isolated from the 769-bp PCR product; 2, isolated from the 604-bp PCR product. (c) Automatic sequencing of the PCR product reveals exon-16 deletion of the smaller band (604-bp product). (d) Automatic sequencing of the PCR product from genomic DNA shows a long-segment deletion (5197 bp) from intron 15 to intron 16 (including exon 16).

MMR genes in HNPCC patients affected either *hMSH2* or *hMLH1* and were quite unevenly distributed, with some clustering in *hMSH2* exon 12 and *hMLH1* exon 16 (14, 21). An HNPCC-related exon-16 deletion in *hMLH1* was reported in studies carried out in Sweden and Finland (22, 23), although the observed genomic loss (3.5 kb) was different from that reported in the present study (5.2 kb). The second mutation that we identified, a two-nucleotide deletion in *hMLH1* exon 3 with a frameshift mutation that resulted in a stop codon at codon 84, was detected using a DNA-based method. Although this mutation can be detected using the RNA-based method, we reviewed the sequencing profile from RT-PCR and the IVTT results and found that the respective mutation was masked by background in the sequencing profile (Fig. 2c), and that the truncated protein (predicted size 9.2 kDa) was too small to be detected. A comparison of the sequencing results from RT-PCR- and PCR-amplified genomic DNA (Fig. 2b, 2c) revealed that the mutation was more easily detected from genomic DNA in this particular case. Furthermore, as the mutated form may be less stable than the wild-type RNA, the level of expression would decrease, making detec-

tion by RT-PCR products more difficult using the RNA-based method. The third mutation that we identified in *hMLH1* exon-4 codon 117 had been reported previously (24–26) in western countries. This mutation was shown to be defective in the *in vitro* MMR system (26). Only a single nucleotide change was observed in the RNA, which was different from the second mutation we found, and hence might explain why this mutation could be detected using both DNA- and RNA-based methods.

Although the clinical features (age of cancer onset, right-side colon predominant, cancer types) of patients in our series were, overall, similar to those of previous reports (19, 27), the results of genetic analysis were quite different. In our study, the mutation rates for *hMSH2* and *hMLH1* were 0% and 20%, respectively, and were lower than those in previous reports (15, 16). These differences could be related to sampling methods, methods used to detect the mutations, and ethnic differences. For example, we selected patients based on clinical history, and all the selected families met the Amsterdam criteria. Other studies included some mutation-screening results that did not meet Amsterdam criteria or that only met modified criteria

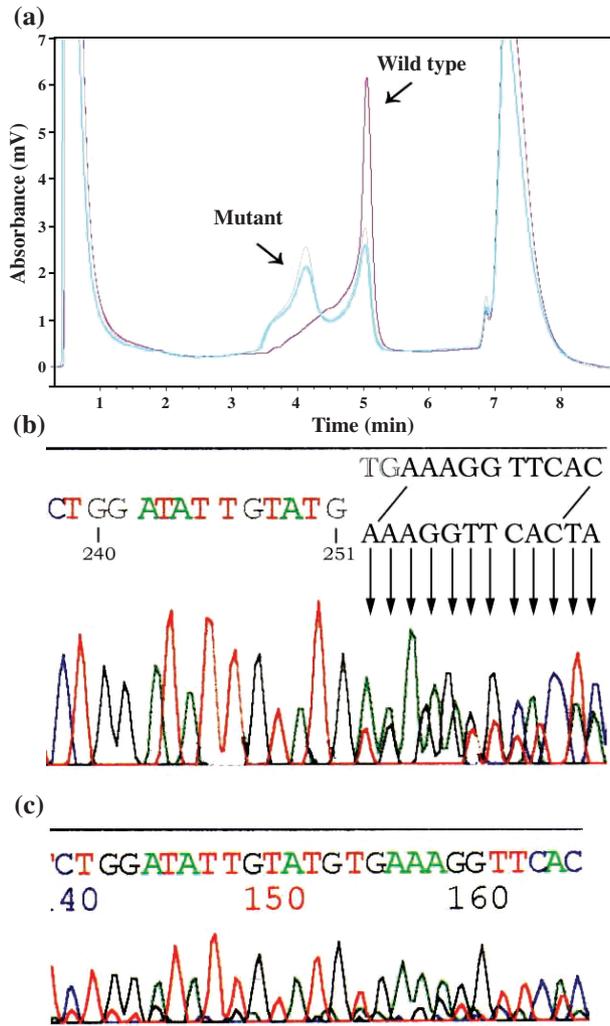


Fig.2. Mutation of *hMLH1* exon 3. (a) Heterozygous profile, as detected by denaturing high-performance liquid chromatography (DHPLC), in *hMLH1*, exon 3, of two index cases. (b) Further sequencing showed an allele with a TG-loss in codon 84, *hMLH1*, exon 3. (c) The same section as (b), sequenced by reverse transcription–polymerase chain reaction (RT–PCR) product. The TG loss was masked by background.

(19, 28–32), which might have increased or decreased the mutation rate. In addition, prior selection factors that included screening proband tumors for the microsatellite phenomenon (25), linkage and/or mutation analysis (17, 21, 25, 33), might also have affected the estimated mutation rate.

Methods previously used to detect mutations in the *hMSH2* and *hMLH1* genes include degenerative gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), IVTT, heteroduplex analysis, and direct sequencing (28, 34–36). Recently, DHPLC was confirmed to be highly sensitive (97%), specific (100%), and cost-effective, with a particularly high potential to detect *hMSH2* and *hMLH1*

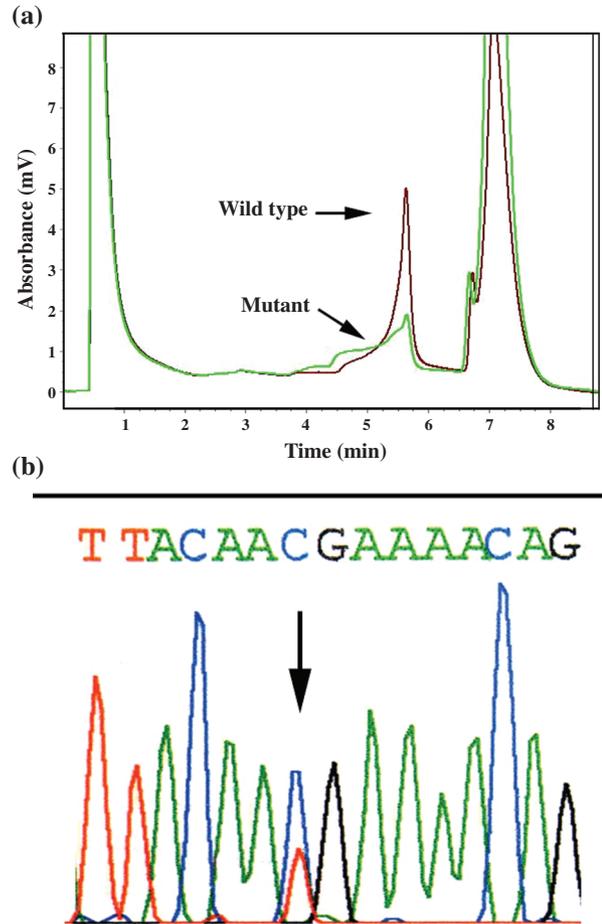


Fig.3. Mutation of *hMLH1* exon 4. (a) Heterozygous profile, as detected by denaturing high-performance liquid chromatography (DHPLC), in *hMLH1*, exon 4, of four index cases. (b) Further sequencing showed a C→T transition at nucleotide 350 in codon 117.

gene mutations in the diagnostic setting (37, 38). DHPLC analysis is less expensive than other sensitive modalities, such as sequencing and DGGE, and less-sensitive methods, such as SSCP. Similarly to SSCP, DGGE, and sequence analysis, the DHPLC method is limited by the lack of detection of complete deletions of exons. However, it is useful as a complementary test to rapidly screen for point mutations and small deletions or duplications (37). These methods can be divided into RNA-based and genomic DNA-based analyses. Optimal mutation detection methods remain controversial (39). Several authors have raised concerns about RNA-based MMR-gene analysis, citing significant numbers of false-positive results (40). Kohonen-Corish et al. suggested that the results should be confirmed in at least two patients per family or from genomic DNA. In our series, we confirmed mutation-detection results using both RNA- and genomic

DNA-based methods. We also validated our methods by detecting the mutations in colon cancer cell lines of known mutations (data not shown). The results indicate that the methods we used are reliable in detecting mutations in LoVo and HCT 116 cell lines. However, the first mutation we detected using the RNA-based method was not detected by the DNA-based method. Moreover, the second mutation we detected using DNA-based method also was not detected by the RNA-based method. Therefore, both RNA- and DNA-based methods should be used as a more complete genetic screen. From our study results, we suggest using the DNA-based method for detecting point mutations and microdeletions, and the RNA-based method for detecting, in addition, large-fragment deletion.

Finally, ethnic differences may be responsible for the variation observed in the mutation rate in different countries and populations. Weber et al. and Lewis et al., who also detected a relatively low rate of *hMSH2* and *hMLH1* mutations, suggested that other genes may be involved in HNPCC (35, 41). Thus, the genetic basis of HNPCC still needs further exploration. Further studies should examine mutations in other MMR genes, especially *hMSH6*, and the expression level of *hMSH2* and *hMLH1* genes.

In summary, although the clinical characteristics of Taiwanese HNPCC kindreds were similar to those reported in other countries, RNA- and DNA-based genetic scanning for *hMSH2* and *hMLH1* of 15 Taiwanese HNPCC kindreds found a lower (0% and 20%) mutation rate than previously reported. The genetic basis of different ethnic groups is unique. For a more complete genetic screen, both RNA- and DNA-based detection should be performed.

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