

# Genetic detection of Chinese hereditary nonpolyposis colorectal cancer

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## Abstract

**AIM:** To explore the germline mutations of the two main DNA mismatch repair genes (*hMSH2* and *hMLH1*) between patients with hereditary non-polyposis colorectal cancer (HNPCC) and suspected (atypical) HNPCC.

**METHODS:** Genomic DNA was extracted from the peripheral blood of the index patient of each family, and germline mutations of *hMSH2* and *hMLH1* genes were detected by PCR-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing techniques.

**RESULTS:** For PCR-SSCP analysis, 67% (4/6) abnormal exons mobility in typical group and 33% (2/6) abnormal exons mobility in atypical group were recognized. In direct DNA sequencing, 50% (3/6) mutation of MMR genes in typical group and 33% (2/6) mutation of MMR genes in atypical group were found, and 4/6 (66.67%) and 1/6 (16.67%) mutations of *hMSH2* and *hMLH1* were identified in typical HNPCC and atypical HNPCC, respectively.

**CONCLUSION:** Mutation detection of the patients is of benefit to the analysis of HNPCC and, PCR-SSCP is an effective strategy to detect the mutations of HNPCC equivalent to direct DNA sequence. It seems that there exist more complicated genetic alterations in Chinese HNPCC patients than in Western countries.

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## INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) is characterized by early onset of colorectal cancer, location of tumors in the proximal colon, and an increased risk of neoplasms of extracolonic organs, including endometrium, stomach, urothelium, small intestine, and ovary<sup>[1-6]</sup>. The International Collaborative Group on HNPCC (ICG-HNPCC) proposed a set of clinical diagnostic criteria (Amsterdam Criteria I) for HNPCC in 1990 and has revised them recently

(Amsterdam Criteria II) to provide a uniformity in collaborative studies<sup>[7,8]</sup>. According to these criteria, there should have at least three patients in two consecutive generations who had colorectal cancer or the other extracolonic malignancies including endometrial cancer, small bowel cancer, cancer of the ureter and cancer of the renal pelvis. One of them should be a first-degree relative of the other two, one cancer should be diagnosed before age 50, and familial adenomatous polyposis (FAP) should be excluded. In Asia, on the other hand, the Japan Research Society for Cancer of the Colon and Rectum developed the clinical criteria (Japanese criteria) for HNPCC in 1991<sup>[9]</sup>. According to these criteria, at least two relatives in at least two successive generations should have colorectal cancer, and one of them should be a first-degree relative of the other, also, we called them atypical HNPCC.

Nevertheless, according to those criteria above, all families that are diagnosed as having HNPCC are identified on the basis of a family history of colorectal or other certain malignancies. Sometimes the accuracy and reliability of the family history provided by patients themselves and their relatives or recorded by physicians are questionable, as there is much variability for the family history. Therefore, the requirements for establishing objective strategies to identify this disease are imperative. In the last decade, the progress in genetic study that DNA mismatch repair genes have been identified as being mutated in HNPCC including *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, and *hMSH6* makes it possible to identify the cohort of patients through genetic test. Totally, these genes are now believed to account for about 50%-70% of all families with HNPCC and over 90% of the identified mutations focused on the two genes, *hMSH2* and *hMLH1*<sup>[10-18]</sup>. There are many studies about the procedures of genetic testing of HNPCC, such as microsatellite instability (MSI), PCR-SSCP, immunohistochemistry (IHC) and direct DNA sequencing<sup>[19-24]</sup>. However, much knowledge and a higher grade of technical development are required, before this strategy becomes applicable to the general population.

It has no doubt that there is a large population of HNPCC in China<sup>[25,26]</sup>. Unfortunately, there have been few reports about clinical and genetic characteristics of HNPCC<sup>[27]</sup>. In this study, six HNPCC families and six atypical HNPCC families were enrolled and, the germline mutations of *hMSH2* and *hMLH1* in the index patients from each family were investigated.

## MATERIALS AND METHODS

### Subjects

The project was approved by the Institutional Review Board and informed consent was obtained from each participant before the procedures were carried out. Personal and family cancer history was obtained from the proband and participating relatives, and cancer diagnosis and deaths were confirmed by review of medical records, pathological reports or death certificates. Families were identified by the Amsterdam or Japanese criteria for HNPCC. Patients and families were classified as the HNPCC group according to the Amsterdam criteria, suspected HNPCC group according to the Japanese criteria and control group without any family history.

### Genetic testing

**Preparation of peripheral blood samples and DNA amplification** Genetic analysis was performed on a blood specimen from the proband in each family. Specimens were collected and immediately frozen in liquid nitrogen. DNA was extracted from blood specimens using the Wizard genomic purification kit (Qiagen, Shanghai) according to the manufacturer's instructions. Each of the exons from hMSH2 and hMLH1 genes was amplified by polymerase chain reaction (PCR). Next, the samples were heat-treated at 95 °C for 5 minutes to inactivate the enzyme, and used as the template DNA. All DNA amplification was performed in a 50 µl volume containing 100 ng template DNA, 10<sup>-3</sup> M Tris-HCl (pH 8.9), 50<sup>-3</sup>M KCl, 2.5 mM MgCl<sub>2</sub>, 0.2<sup>-3</sup>M of each dNTP, 10 pmol each primer, and 1U Taq polymerase was subjected to 35 PCR cycles (5 mins at 94 °C, 40 s at 94 °C, 60 s at 55 °C, and 40 s at 72 °C). Oligonucleotide sequences were designed from the sequences published in Genbank.

### PCR-SSCP analysis and direct sequencing

**Single-strand conformation polymorphism (SSCP)** The technique of single-strand conformational polymorphism (SSCP) was used to identify mutations in the mismatch repair genes<sup>[28,29]</sup>. Each exon of *hMSH2* and *hMLH1* was amplified specifically using PCR (details of oligonucleotide sequences and conditions are available from the authors). PCR products were electrophoresed on 10% non-denaturing polyacrylamide gels with an acrylamide: bisacrylamide ratio of 30:0.8 at 70volt for 12 h at room temperature. DNA was detected by silver staining according to the methods described by others. Those single stranded DNAs that took up an altered conformation appeared as aberrantly migrating bands on the electrophoresis gel.

### Direct sequencing

The nucleotide sequence of PCR products showing an abnormal mobility on SSCP was determined by direct sequencing. The PCR products were purified with 1.5% low melted point agarose, and then performed using automated DNA

sequencer. Sequence alterations with an allele frequency of at least 5% were considered as normal variants (polymorphisms) and not reported.

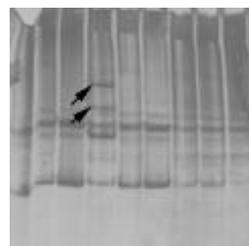
### Statistical analysis

The genetic differences of the typical HNPCC groups and the suspected HNPCC groups were analyzed for statistical significance using the chi square test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Results of genetic testing

Characterization of the variants of PCR-SSCP and DNA sequencing analysis found in the 12 families/individuals in the study is presented in Table 1. A total of seven abnormal mobilities were identified in PCR-SSCP including five in typical group and two in atypical group (part of PCR-SSCP analysis is shown in Figure 1). DNA sequencing found 6/7 (85.7%) abnormal mobilities of PCR-SSCP, which were proven to be pathogenic germline mutations of *hMSH2* and *hMLH1*. The other abnormal mobility, an "A" insertion in *hMLH1* intron 10 occurred in the C10-1 patient, was proven to be polymorphism.

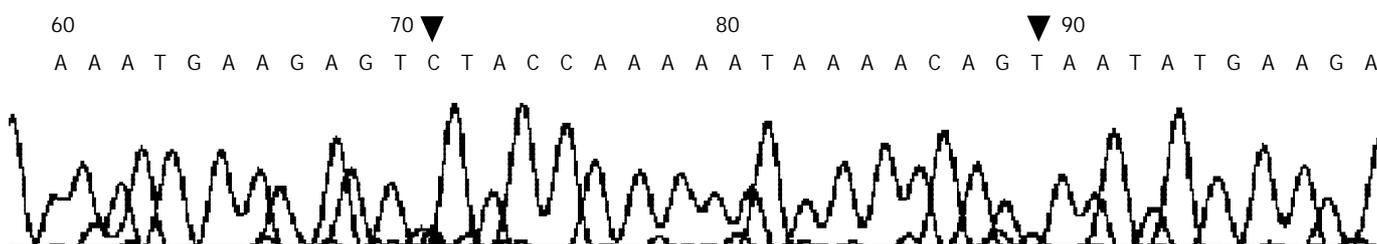


**Figure 1** Results of PCR-SSCP in C13 family (*hMSH2*-exon11). Arrow shows the abnormal mobility.

Totally, five patients were found to harbor MMR gene mutations in the direct DNA sequencing. Three of six (50%)

**Table 1** Characterization of variants in PCR-SSCP and sequence detection

Family	Abnormal mobility	DNA change	Mutation	Group
C4-1	<i>hMLH1</i> exon18 <i>hMSH2</i> exon15	T insert in 2 081 C insert in 2 469 G insert in 2 471	Frameshift Frameshift Frameshift	Typical HNPCC
C13-1	<i>hMSH2</i> exon11	T insert in 1 760 A→C missense in 1 688	Frameshift Tyr563Ser	Typical HNPCC
C11-1	<i>hMSH2</i> exon13	T to A missense in 2 091	Cys697	Typical HNPCC
C1-1	<i>hMLH1</i> exon11	A insert in 934	Terminator Condon Frameshift	Atypical HNPCC
C8-2	<i>hMLH1</i> exon12	C to G missense in 1 198 C to G missense in 1 261 C insert in 1 364 and 1 372	Leu400Val Val421Leu Frameshift	Atypical HNPCC
C10-1	<i>hMLH1</i> intron10	-	Polymorphism	Typical HNPCC



**Figure 2** Results of direct sequencing of exon 11 of *hMSH2* in C13 family (A to C substitution in 1 688 and T insertion in 1 760).

patients with HNPCC and 2/6 (33%) patients with suspected HNPCC were found to harbor mutations. However, we found the mutations were complicated as the total number of mutations was more than five and all the mutations we found were novel and of unknown pathogenicity (part of sequence analysis is shown in Figure 2).

In typical HNPCC, the germline mutation of *hMLH1* and *hMSH2* was detected in 3 of 6 HNPCC families. Seven novel mutations were found in 3 exons of *hMSH2* and 1 exon of *hMLH1*, two mutations were detected at 11 exons of *hMSH2* in C13 family, one was the frame shift mutation resulted from an insertion of "T" at 1 760 so that a terminate cordon appeared ahead, the other was "A to C" missense mutation at 1 688 leading to Tyr-Ser substitution. A "T to A" substitution at 2 091 of exon 13 of *hMSH2* in C11 family resulted in a terminate cordon at 697. The mutations in C4 family were worthy of specific comment, as the mutation occurred in *hMLH1* as well as *hMSH2*, there was a frame shift mutation resulted from an insertion of T at 2 081 of exon 18 of *hMLH1*, there were "C, G" inserts at 2 469, 2 471 respectively. However, all families without germline *hMSH2* and *hMLH1* mutations were combined with extracolonic tumors.

In atypical HNPCC, only the germline mutation of *hMLH1* was detected in 2 of 6 HNPCC families, one was the frame shift mutation in exon 11 resulted from an insertion of "A" at 934 in C13 family. In C8 family, the mutation occurred at four locations, one was "C to G" missense mutation at 1 198 leading to Leu400Val substitution, one was "C to G" missense mutation at 1 261 leading to Val421Leu substitution and the other two shift mutations occurred at 1 364 and 1 372 resulted from the A insertion at the two locations respectively. The detailed results of sequencing are shown in Table 2.

**Table 2** Comparison of two kinds of HNPCC with PCR-SSCP and sequencing

	Typical HNPCC (n=6)	Atypical HNPCC (n=6)	P
PCR-SSCP	4 (67%)	2 (33%)	0.269
Sequencing	3 (50%)	2 (33%)	0.575

## DISCUSSION

To date, HNPCC is defined either by the so-called Amsterdam I+II criteria or by detection of a mutation in one of the mismatch repair genes. Once the positive mutation is identified, predictive testing of family members at risk is available. Screening recommendations for clinically identified families, mutation carriers, and their unaffected relatives at risk must be defined for clinical management<sup>[30]</sup>.

Recently, Watson *et al.*<sup>[31]</sup> published their results about that carrier risk status changes resulted from mutation testing in hereditary non-polyposis colorectal cancer and hereditary breast-ovarian cancer. They concluded that mutation testing could raise the accuracy of carrier risk assessments, and lower the number of persons at high carrier risk. The most common risk assessment change resulted from DNA testing was a change from those at risk to non-carriers. To the extent that these persons were aware of their carrier risk and were obtaining heightened cancer surveillance test, this could be expected to lead to a reduced emotional toll and a reduced pressure on limited medical resources.

A better understanding of the molecular basis of hereditary colorectal cancer syndromes such as hereditary nonpolyposis colorectal cancer syndrome (HNPCC) and familial adenomatous polyposis (FAP) would have profound consequences for both the diagnosis and (prophylactic) treatment of (pre)malignant neoplastic lesions<sup>[32]</sup> Clinically, when we see a patient with

colorectal cancer with a family history of suspected HNPCC, we need to work out our surgical strategies for this patient, the same as we do for the patients with HNPCC, in whom colonoscopy surveillance or prophylaxis surgeries such as subtotal colectomy are needed. One of the evidences of special surgical treatment has been found to be genetic detection<sup>[33,34]</sup>. Since HNPCC is resulted from the dysfunction of mismatch repair genes and some reports indicated that mutation of *hMSH2* and *hMLH1* accounted for 40%-70% HNPCC<sup>[35-39]</sup>, we decided to detect the germline mutation of *hMSH2* and *hMLH1* in this study. In PCR-SSCP analysis of genomic DNA of 12 probands, we identified seven abnormal mobilities, five in typical HNPCC and two in suspected HNPCC and six of them were testified to be mutations by DNA sequencing. When we compared the genetic investigation of two groups we studied, we also concluded that there were no statistical differences in the results of both PCR-SSCP and direct sequence.

We also found that the mutations occurred both in typical HNPCC group and in atypical HNPCC group. Beck *et al* found germline HNPCC mutations in six families in which none fulfilled the Amsterdam criteria. They highlighted that if germline mutations of the mismatch repair genes were common in families with features of HNPCC, but not fulfilling the Amsterdam criteria, then it was very important that all such families were also referred to cancer clinics for assessment and possible genetic testing<sup>[40]</sup>. It was suggested that many suspected HNPCC patients might not be recognized and might be excluded from genetic counseling<sup>[41,42]</sup>.

Upon reviewing the literature and checking the variants registried in the ICG-HNPCC mutation database (<http://www.nfdht.nl>), we found that all the mutations were novel. It is rarely reported that there exist multiple mutations in the same exons. However, it seemed to be common in our analysis, two mutations were found at 11 exons of *hMSH2* in C13 family and C8 family. The mutation occurred at four locations, one was "C to G" missense mutation at 1 198 leading to Leu400Val substitution, one was "C to G" missense mutation at 1 261 leading to Val421Leu substitution and the other two shift mutations occurred at 1 364 and 1 372 resulted from the A insertion at the two locations, respectively. Furthermore, it is the first report that the mutation occurred in both *hMLH1* and *hMSH2* in C4 family.

Several techniques for genetic detection of HNPCC have been developed including MSI, immunohistochemistry, SSCP, denaturing gradient gel electrophoresis (DGGE) and sequencing. SSCP was considered inefficient in detecting mutations<sup>[43,44]</sup> and was expected to miss approximately 20% of point and frameshift mutations. Furthermore, it was unable to detect the whole exon deletions, which occurred in some HNPCC families<sup>[45]</sup>. We chose SSCP first because it was relatively simple, quick and cheap, and did not require special apparatus. In our study, 12 abnormal bands found in SSCP were proven to represent 11 mutations (91.7%). We found it was a practical procedure for the genetic screening of HNPCC.

Given the limitations of current techniques, direct PCR product sequencing analysis is more efficient for detecting mutations, but it is laborious, more expensive in term of reagents and data analysis time and not 100% efficient. It is much more acceptable clinically to take the screening techniques, such as SSCP, first and use sequencing to analyze the details of the mutation. Recently, some studies demonstrated that large genomic rearrangements accounted for 10%-20% of all *hMSH2* mutations, and a lower proportion of all *hMLH1* mutations. Nakagawa<sup>[46]</sup> used the multiplex ligation-dependent probe amplification (MLPA) method to screen *hMSH2* and *hMLH1* deletions in 70 patients whose colorectal or endometrial tumors were MSI positive, yet no mutation was found by genomic exon-by-exon sequencing of *hMSH2*, *hMLH1*, and *hMSH6*. They

identified five candidates with four different hMSH2 deletions and one candidate with an hMLH1 deletion.

In summary, our study demonstrates that the Amsterdam criteria are important but inappropriate for the establishment of diagnosis, some atypical families not fulfilling all the Amsterdam criteria probably possess the similar genetic alterations. We conclude that the mutation detection of patients is of benefit to the analysis of HNPCC and, PCR-SSCP is as effective as direct DNA sequence in detecting the mutations of HNPCC. Furthermore, it seems that there exist more complicated genetic alterations in Chinese HNPCC patients than in Western countries.

However, there are also some cases without any mutation, further investigations should be carried out such as detection of other mismatch repair genes as well as detection of gene methylation of hMLH1.

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