Genetic Characterization of Chinese Hereditary Non-polyposis Colorectal Cancer by DHPLC and Multiplex PCR

Ying Yuan, Yan-qing Huang, Shan-rong Cai, Yong-mao Song, Shu Zheng and Su-zhan Zhang

Cancer Research Institute, 2nd Hospital, Zhejiang University School of Medicine, Hangzhou, PR China

Received July 4, 2004; accepted August 26, 2004

Background: Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant disease due to germline mutations of human mismatch repair genes, mainly hMLH1 and hMSH2. The aim of the present study was to identify the point mutations and large genomic deletions of hMLH1 and hMSH2 genes in 14 Chinese HNPCC families.

Methods: Fourteen families fulfilling the Chinese HNPCC criteria were involved in this study. Genomic DNA isolated from peripheral blood samples was analyzed. Point mutations were detected by denaturing high performance liquid chromatography (DHPLC) followed by DNA sequencing. Multiplex polymerase chain reaction and GeneScan analysis were employed to detect the large genomic deletions of these two genes.

Results: Four of the 14 probands (29%) had sequence abnormalities that probably affect the protein function in the exonic regions of hMLH1 and hMSH2 genes. Included were one complete deletion of exons 1–7 and one missense mutation of the hMSH2 gene, and one nonsense mutation and one missense mutation of the hMLH1 gene. The large genomic deletion accounted for 25% (one out of four) of all mutations. Half (two out of four, 50%) of the mutations were missense mutation. In addition, one silent mutation, four polymorphisms in the exonic regions and four polymorphisms in the intronic regions were also discovered.

Conclusions: Point mutations and large genomic deletions of the hMLH1 and hMSH2 genes were responsible for nearly one-third of Chinese HNPCC families. Detection of large genomic deletions should be involved in the routine screening manual for HNPCC families.

Key words: hereditary non-polyposis colorectal cancer (HNPCC) – hMLH1 gene – hMSH2 gene point mutation – large genomic deletions

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) is the most common hereditary condition and predisposing factor for the development of cancer of multiple organs. It is a distinct autosomal dominant syndrome accounting for 5–10% of total colorectal cancer (1). HNPCC is characterized by early-onset colorectal cancer (mean age at diagnosis 45 years), right-sided predominance, excess synchronous and metachronous colorectal neoplasm, and an increased incidence of extracolonic malignancies, including endometrial, small bowel, gastric, renal pelvis and ureter, ovarian and skin neoplasm (1). Susceptibility to HNPCC is caused by germline mutations in one of six genes with the mismatch repair function (hMSH2, hMLH1, hPMS1, hPMS2, hMSH6 and hMLH3) (1,2). Among these mismatch repair genes, germline mutations in the coding regions of hMSH2 and hMLH1 are known to be responsible for up to 45–64% of HNPCCs (1–3). Some mutations in the hMSH6 gene were also reported (ICG-HNPCC Mutation Database, http://www.nfdnt.nl), while the role of the other three genes is still uncertain (4,5).

Up to now, the maximum mutation detection rate reported in the literature was 70% (3). There are still one-third of HNPCCs in which no mutation of the known genes above could be identified. Three possibilities should be considered. First, an epigenetic defect of mismatch repair genes might exist (6,7). Hypermethylation of the promoter region of the hMLH1 gene, for example, was reported in some colorectal cancers. Secondly, due to the limitation of present molecular techniques, some mutations cannot be detected by the familiar and routine screening methods. The third explanation is the existence of mutations in other, as yet unknown genes.

Single-strand conformation polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE),
heteroduplex analysis, direct sequencing analysis and the recent denaturing high performance liquid chromatography (DHPLC) method are the common and routine methods to detect gene mutations (8–11). They can detect various kinds of mutations, mostly missense mutations, nonsense mutations, small deletions and/or insertions, and splicing site alterations. None of these methods is effective for the detection of large genomic deletions encompassing one or more exons. However, the frequent occurrence of larger genomic deletions in \(hMLH1\) and \(hMSH2\) was reported recently by Wang et al. (12). Wijnen et al. (13) and Charbonnier et al. (14) proved that genomic deletions of \(hMLH1\) but not \(hMLH1\) were a frequent cause of HNPCC. In order to analyze fully the cause of mutations of \(hMLH1\) and \(hMSH2\) in our Chinese HNPCC families, here we simultaneously performed DHPLC followed by DNA sequencing to detect point mutations (missense mutations, nonsense mutations, small deletions and/or insertions, etc.), and modified multiplex polymerase chain reaction (PCR) together with Genescan analysis to discover large fragment deletions.

Clinically, HNPCC families were diagnosed based on the Amsterdam criteria established in 1991: (i) three or more relatives with histologically verified colorectal cancer, one of whom is a first-degree relative of the other two; (ii) colorectal cancer affecting at least two generations; and (iii) one or more colorectal cancer case diagnosed before the age of 50 years (15). Although the Amsterdam criteria unified the diagnosis of HNPCC worldwide, it was too rigid for small families and it excluded extracolonic cancers associated with HNPCC. A lot of new, modified or suggested criteria and guidelines were proposed, such as the Japanese criteria, suspected HNPCC criteria, Bethesda guidelines, Amsterdam criteria II, etc. (16–19). In April 2004, based on the suspected HNPCC criteria, considering the specific features of the tumor spectrum in China, the Chinese Hereditary Colorectal Cancer Collaboration established the criteria for Chinese HNPCC families (20). The details are as follows: two or more relatives with histologically verified colorectal cancer, two of whom should be first-degree relatives (such as siblings, parents and children); and also one of the following: (i) development of multiple colorectal tumors (including adenomas); (ii) at least one colorectal cancer diagnosed before the age of 50 years; or (iii) development of extracolonic cancer (stomach, endometrium, small intestine, ureter and pelvis, ovary or hepatobiliary system) in family members. Based on these newly established Chinese HNPCC criteria, we collected 32 families and 14 of them were available for genetic testing.

SUBJECTS AND METHODS

FAMILY INFORMATION

All 14 families involved in this study met the Chinese HNPCC criteria, three of them (H1, H4 and H17) also fulfilling the Amsterdam criteria. In total, 43 colorectal cancers developed in 38 patients. Among these 38 patients, 31 (82%) were diagnosed before the age of 50 years. There were two cases with synchronous colorectal cancer and three cases with metachronous colorectal cancer. In these 14 HNPCC families, five families had Lynch I syndrome in which colorectal cancer was the only type of malignancy in a family, while the other nine families had Lynch II syndrome in which extracolonic malignancies also developed. A total of 19 extracolonic cancers developed in the nine families with Lynch II syndrome, comprising eight gastric cancers, three esophageal cancers, two lung cancers, one endometrial cancer, one liver cancer, one thyroid cancer, one oral cancer, one leukemia and one infrequent malignant haematological disease. Gastric cancer was the most common type of extracolonic malignancy. The peripheral blood samples of probands and family members were collected if possible.

GENOMIC DNA PREPARATION AND PCR

Genomic DNA was extracted using the QIAamp DNA isolation kit (Qiagen, Valencia, CA), according to the manufacturer’s recommendations. PCR was performed using 100 ng of genomic DNA as template. The 25 \(\mu\)l reaction mixture consisted of 10–20 pmol of each primer, 1.5 U of Taq DNA polymerase (Transgenomics, UK) with a final concentration of 2 mmol/l Mg\(^{2+}\) and 0.2 mmol/l of dNTPs. PCR conditions were as follows: an initial denaturing cycle at 95\(^\circ\)C for 5 min was used, followed by 40 cycles of 95\(^\circ\)C for 30 s, 55–60\(^\circ\)C for 30 s, 72\(^\circ\)C for 40 s, and a final extension cycle at 72\(^\circ\)C for 10 min. All 35 sets of primers including 19 sets for \(hMLH1\) and 16 sets for \(hMSH2\) were reported as before (11,21).

DHPLC AND DNA SEQUENCING

DHPLC analyses were performed using the WAVE 3500HT system (Transgenomics, UK), according to the manufacturer’s recommendations. Samples of 5–15 \(\mu\)l were loaded for analysis. The temperature at which heteroduplex detection occurred was deduced from the melting profile of the specific DNA fragment using a newly developed algorithm. In cases of samples showing abnormal patterns on DHPLC, DNA sequencing was performed using the ABI 31000-Avant automatic DNA sequencer (Applied Biosystems, CA).

MULTIPLEX PCR AND GENESCAN ANALYSIS

Detection of genomic deletions was performed by a semi-quantitative multiplex PCR. PCR primers and conditions were as in Charbonnier et al. (14) and Wang et al. (12). All forward primers were labeled with the fluorescent dye 6-FAM at their 5’ end. Seven groups of multiplex PCRs were performed (Table 1). The final volume of each multiplex PCR was 25 \(\mu\)l, containing 50 ng of template DNA, 10–20 pmol of each primer, dNTPs with a final concentration of 0.2 mmol/l, and 1.5 U of Taq DNA polymerase. Unpurified products of multiplex PCR were then loaded onto 4.5% polyacrylamide
gels on an ABI 3100-Avant automatic DNA sequencer. Peak areas were calculated by the GeneScan Analysis software 2.1, exported to an Excel spreadsheet. The copy numbers were calculated as described (12).

RESULTS

SCREENING OF POINT MUTATIONS BY DHPLC

A total of 12 abnormal peak patterns were observed by DHPLC in 14 probands. The following DNA sequencing analysis proved that eight of them were base substitutions located in the exonic regions (Table 2), while the other four were located within intronic sequences (Table 3). Of the eight base substitutions in coding sequences, one (H20) was a nonsense mutation, two (H4 and H12) were missense mutations, one (H16) was a silent mutation, one (H11) was a novel polymorphism and the other three (H3, H7 and H16) were known polymorphisms.

The nonsense mutation discovered in family H20 was located at codon 749 in the last exon of the hMLH1 gene, resulting in a truncated protein without the C-terminal amino acids (Fig. 1A and B). The proband (H20) was a female patient with colon cancer diagnosed at the age of 46 years. There were another three cancer patients in her family. Her elder brother was diagnosed with rectal cancer at 40 years old. Her younger brother was first diagnosed with gastric cancer and later with colon cancer at the age of 40 years. One of her uncles died of gastric cancer at the age of 50 years. This family did not fulfill the classical Amsterdam criteria, but met our Chinese criteria (Fig. 1C).

The missense mutation located at codon 67 in exon 2 of the hMLH1 gene was identified in the proband of family H4. It has been reported as a pathological mutation in Swedish, Japanese and American families (ICG-HNPCC Mutation Database, http://www.nfdnt.nl). Also this base substitution was not detected in 100 normal Chinese controls. The G to A base substitution led to an amino acid change from glycine to arginine. The proband (H4) was a female patient with endometrial cancer at the age of 45 years, who later developed colon cancer at 56 years old. It was a typical family fulfilling the Amsterdam criteria. Her mother was diagnosed with rectal cancer at the age of 71 years. Her brother developed an ascending colon cancer at the age of 38 years, and later a descending colon cancer at the age of 52 years. Her sister was operated on for rectal cancer at the age of 45 years.

Family H12 had two colon cancer patients and only met the Chinese criteria. The proband was a 45-year-old male operated on for colon cancer and his father was diagnosed with descending colon cancer at the age of 50 years. A novel missense mutation at codon 839 (CAT → CGT) in exon 15 of the hMSH2 gene was discovered in this family. It was located in the Muts_V structural motif (amino acids 619–854) of the hMSH2 protein and led to an amino acid change from histidine to arginine. This A to G substitution was not detected in 100 normal Chinese controls, and thus was thought to be pathological.

In order to clarify whether these three pathological mutations were segregating in their families, samples from other family members were collected if possible. Unfortunately, due to various reasons, none of the individuals of family H4 and

<table>
<thead>
<tr>
<th>Family</th>
<th>Gene</th>
<th>Nucleotide</th>
<th>Sequence change</th>
</tr>
</thead>
<tbody>
<tr>
<td>H11 hMLH1</td>
<td>1648</td>
<td>A→G</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>H15 hMSH2</td>
<td>222</td>
<td>C→G</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>H6 hMSH2</td>
<td>1323</td>
<td>T→A</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>H16 hMSH2</td>
<td>2000</td>
<td>T→C</td>
<td>Polymorphism</td>
</tr>
</tbody>
</table>
family H20 agreed to further DNA testing. In family H12, blood samples were collected from six healthy relatives of the proband, his own daughter, his elder sister and her son, and his younger sister and her two daughters. Except for the younger daughter of his younger sister, all other five individuals were diagnosed as carriers harboring the same missense mutation as the proband.

In addition to these three pathological mutations (one nonsense and two missense mutations), there was also one silent mutation and four polymorphisms in the exonic regions identified in these families. Three polymorphisms detected in the probands of family H3, H7 and H16 have been reported and documented either in the ICG-HNPCC Mutation Database (http://www.nfndnt.nl) or in the NCBI Database (http://www.ncbi.nlm.nih.gov/), while the other (H11) was newly discovered. The polymorphism detected in the proband H11 was an A to G change at codon 629 (CAA→CGA) in exon 12 of the hMSH2 gene. It led to an amino acid change from glutamine to arginine. It could be detected in seven out of 100 normal Chinese controls. Thus the frequency in the normal populations was 7%.

Of the four base substitutions in the intronic sequences, the two located at nucleotide 222 (C→G) and nucleotide 2000 (T→C), respectively, of the hMSH2 gene were known polymorphisms (NCBI Database, http://www.ncbi.nlm.nih.gov/), while the other two located at nucleotides 1648 (A→G) of the hMLH1 gene and at nucleotide 1323 (T→A) of the hMSH2 gene, respectively, had not been described up to now. The frequency of these two novel variants in normal populations was 9.7 (seven out of 72) and 6.9% (five out of 72), respectively.

ANALYSIS OF LARGE GENOMIC FRAGMENT DELETIONS

Only one large genomic fragment deletion was detected in the proband (numbered as III-2) of family H1. As shown in the GeneScan analysis, one copy of exons 1–7 of the hMSH2 gene was completely lost (Fig. 2A). This family fulfilled both the Chinese HNPCC criteria and the classical Amsterdam criteria (Fig. 2B). The family included three colorectal cancer patients affecting two successive generations. One cancer was located in the rectum, while the other two were located in the colon. All three cases were diagnosed before the age of 50 years. In order to clarify whether this deletion was genetically segregated, the blood samples of his father (II-4), another colorectal cancer patient in the family and healthy relatives II-1, III-5 and IV-1 were also collected. DNA analysis showed that patient II-4 and individuals II-1, III-5 and IV-1 harbored the same deletions as the proband III-2. Individual III-1 was negative for the mutation analysis. Therefore, three individuals (II-1, III-5 and IV-1) were diagnosed as mutation carriers and colonoscopy was suggested.

DISCUSSION

In this study, we screened the point mutations and large genomic deletions of the hMLH1 and hMSH2 genes with DHPLC and multiplex PCR in 14 Chinese HNPCC families. Four of the 14 probands (29%) had sequence abnormalities that probably affect the protein function in the exonic regions of the hMLH1 and hMSH2 genes. Included were one complete deletion of exon 1–7 of the hMSH2 gene, one nonsense mutation in exon 19 of the hMLH1 gene, one missense mutation in exon 2 of the hMLH1 gene and one missense mutation in exon 15 of the hMSH2 gene. The large genomic deletion accounted for 25% of all mutations. Half of the mutations were missense mutation. One silent mutation in exon 7 of the hMSH2 gene, four polymorphisms in the exons and four polymorphisms in the introns were also discovered.

Although the relationship of mismatch repair genes and HNPCC has been studied intensively throughout the world, it is only in recent years that attention has been paid to this aspect by Chinese researchers. Here we would like to
summarize the studies of Chinese HNPCC families. Cai et al. characterized the clinical features of 30 families meeting the Amsterdam criteria in 2003 (22). The frequency of HNPCC was 2.6% in their series of patients. A propensity to involve the proximal colon, an excess of early onset and a high frequency of multiple foci of colorectal cancer were described, similar to the reports from other countries. Contrary to American and European reports, gastric cancer seemed to be more frequent than endometrial cancer in Chinese patients. Therefore, they suggested formulating new criteria for Chinese patients. Similar results were presented by Song and Zheng in their 12 HNPCC families and 42 suspected HNPCC families (23). For mutation analysis, Cai et al. identified three pathological germline mutations (two in the hMSH2 gene and one in the hMLH1 gene) in their four typical Chinese HNPCC families (24). Cui et al. described that 50% (three out of six) of typical HNPCC families and 33% (two out of six) of atypical or suspected HNPCC families had detectable mutations of hMLH1 or hMSH2 (25). Zhao et al. reported an hMLH1 germline mutation in one large HNPCC kindred (26). PCR-SSCP and DNA sequencing were the methods employed to detect mutations in all the above studies. This study, to our knowledge, is the first to report screening of mutations of mismatch repair genes with DHPLC in Chinese kindreds, and also the first to report large genomic deletions in the Chinese patients.

With the DHPLC and multiplex PCR methods, in total, one large fragment deletion, one nonsense mutation, one silent mutation, two missense mutations, four DNA polymorphisms in the exons and four polymorphisms in the introns were detected in our 14 Chinese HNPCC families. Of the 12 point mutations uncovered, three would lead to changes in the amino acid sequences that probably affect the protein function. The total mutation rate was 29% (four out of 14). The large genomic deletion accounted for 25% (one of four) of all mutations. As for the mutation type, missense mutations seemed to be the most common types in our study, while most of the published data showed that frameshift mutations due to deletions or additions of several base pairs were the most common (1). This mutation rate was lower than that reported by Western countries (USA, UK, Finland, etc.), but relatively close to those reported from Asian countries (Korea and Japan) (1,2,17,27,28). One possible explanation might therefore be a racial difference. Another reason based on the reported data was that most of the Western families tested for muta-

tion screening fulfilled the Amsterdam criteria, while our 14 families fulfilled the Chinese HNPCC criteria which were much less stringent than the Amsterdam criteria. In our three families meeting both the Amsterdam criteria and Chinese criteria, two mutations (one deletion of exons 1–7 of the hMSH2 gene and one missense mutation of the hMLH1 gene) were detected. The mutation rate was about two-thirds (67%), close to the 50–70% mutation rate of families fulfilling the Amsterdam criteria. However, more families should be involved to expand this study.

Our Chinese HNPCC criteria were established referring to the suspected HNPCC criteria proposed by Park et al. at the Annual Meeting of the American Society of Colon and Rectum Surgeons held in 1997 in Philadelphia, Pennsylvania. With the PCR-SSCP method, Yuan and Zheng reported that the mutation rates of hMLH1 and hMSH2 genes in Korean suspected HNPCC families and HNPCC families fulfilling the Amsterdam criteria were 29.4 and 31.0%, respectively (29). A further study demonstrated that these two groups of families had similar clinical features, similar mutation rates, types and distributions, indicating a similar genetic background. An International Collaborative study presided over by Park et al. affirmed the significance of the suspected HNPCC criteria (30). This collaborative study concluded that the suspected HNPCC criteria were particularly in favor of the families who did not fulfill the Amsterdam criteria but where a genetic basis of colon cancer was strongly suspected. Furthermore, the criteria had the advantages that they can be applied to nuclear families and also include extracolonic cancers. They suggested that families fulfilling the suspected HNPCC criteria should be offered genetic testing. Based on such data and the actuality of downsizing of Chinese families, therefore, the Chinese Hereditary Colorectal Cancer Collaboration selected the suspected HNPCC criteria as the basis for our new Chinese HNPCC criteria to unify the diagnosis of HNPCC in China. A total of
32 families were collected by our Cancer Institute, and blood samples from 14 families were available for this study.

Genomic deletions of mismatch repair genes were described in detail by Wijnen et al. in 1998 (13). Large genomic deletions in the hMSH2 gene have been considered to account for more than one-third of the reported pathological hMSH2 mutations among their Dutch HNPCC patients and account for 6.5% of HNPCC families meeting the Amsterdam criteria. Their results suggested that hMSH2 genomic deletions were a frequent cause of HNPCC. In 2000, Charbonnier et al. presented a new technique to detect exon deletions and duplications using multiplex PCR of short fluorescent fragments (31). A high proportion of exonic and promoter rearrangements in the hMSH2 gene, but not in hMLH1 and hMSH6, were reported in their French families (14). Genomic rearrangements in the hMSH2 gene were detected in 14 out of 61 patients from families meeting the Amsterdam criteria or with multiple primary cancers belonging to the HNPCC spectrum, in which no point mutations had been uncovered by conventional methods. Only a few hMLH1 deletions have ever been reported in the literature, including a founder mutation in Finland (32), and two others in French patients (31,33). More recently, in 2003, Wang et al. modified Charbonnier’s multiplex PCR method and reported 12 large deletions of the hMLH1 and hMSH2 genes in 95 families fulfilling the Amsterdam criteria (12). These 12 large deletions accounted for 21.8% of all pathological mutations detected in these 95 families. Unlike the results described by Wijnen et al. and Charbonnier et al. in Dutch and French patients, respectively, Wang et al. identified a considerable proportion (42%) of deletions in the hMLH1 gene in subjects of German descent (12–14). In this study, no large deletions of the hMLH1 gene were detected, and a large deletion of the hMSH2 gene accounted for 25% of all mutations detected in our 14 Chinese HNPCC families. Therefore, we suggest that detection of large genomic deletions should be involved in the routine screening manual for HNPCC families.

DHPLC was put forward as an elegant, new method for mutation detection in 1995 by Oefner and Underhill (34). In brief, the method detects heteroduplex formation in renatured PCR fragments possessing heterozygous sequence variations. Until now, it has been applied successfully to mutation screening of various genes. In the past few years, we had searched successfully for mutations in hMLH1 and hMSH2 genes in 29 HNPCC families and 34 suspected HNPCC families with PCR-SSCP and DNA sequencing methods (29). However, it was considered that SSCP was inefficient in detecting mutations and was expected to miss ~20% of point and frameshift mutations (2,35). Though DNA sequencing is supposed to be a screening method with high sensitivity, it was time-consuming and expensive at the same time. It was reported by Holinski-Feder et al. that the sensitivity of DHPLC for screening mutations of hMLH1 and hMSH2 genes could reach 97% (11), and no false negatives were observed by Kurzawski et al. (21). Furthermore, the system is highly automated; the running time per sample averaged only 7 min. Because of these advantages that DHPLC offers, we decided to establish this system in this study. Its high throughput and reduced costs should enable a broad screening of HNPCC families for human mismatch repair gene alterations in routine analysis.

CONCLUSION

Point mutations and large genomic deletions of hMLH1 and hMSH2 genes were responsible for the disease in some Chinese HNPCC families. Other mechanisms such as promoter hypermethylation should be studied further to clarify the cause in mutation-negative families. Detection of large genomic deletions should be involved in the routine screening manual for HNPCC families.

Acknowledgments

This work was supported by the Chinese National ‘863’ program (No. 2001AA227111) on collection and storage of Chinese hereditary colorectal cancer families.

References


