Large genomic aberrations in MSH2 and MLH1 genes are frequent in Chinese colorectal cancer

Ming Zhu\(^b\), Jintian Li\(^b\), Xiaomei Zhang\(^b\), Xiaorong Liu\(^a\), Waltraut Friedl\(^c\), Yuanying Zhang\(^b\), Xiaoliu Wu\(^b\), Peter Propping\(^c\), Yaping Wang\(^a,\ast\)

\(^a\)Department of Medical Genetics, Medical School, Nanjing University, 22 Hankou Road, Nanjing 210093, People’s Republic of China
\(^b\)Department of Molecular Biology, Jiangsu Institute of Cancer Research, Nanjing 210009, People’s Republic of China
\(^c\)Institute of Human Genetics, University Hospital Bonn, Bonn, Germany

Received 18 August 2004; received in revised form 25 November 2004; accepted 8 December 2004

Abstract

Hereditary nonpolyposis colorectal cancer is caused by inactivating mutations in the genes of the DNA mismatch repair (MMR) system. Studies have shown that large-fragment aberrations in MMR genes are responsible for a considerable proportion of hereditary colorectal cancer (CRC), but it has been rarely reported in Chinese patients. Here we used multiplex ligation-dependent probe amplification to analyze the genomic rearrangements of 45 Chinese hereditary CRC families, 20 young-age CRC patients (onset of CRC at younger than 50 years and no family history), and 13 patients with sporadic CRC diagnosed at age 50 years or older. Overall, we found 9 (13.8%) large genomic deletions or duplications: 7 out of 45 CRC patients with family history and 2 out of 20 young CRC patients. In all alterations, five genomic deletions were uncovered in the MSH2 gene, as well as one deletion and three duplications in the MLH1 gene. Furthermore, two of the duplications unveiled in this study may have more than a four-copy increase of the exon showing duplication in MLH1. The results indicate that genomic aberrations, large-fragment deletions and duplications, in both MSH2 and MLH1 genes play a role in the pathogenesis of Chinese CRC patients with a family history, as reported in western populations. Moreover, the genomic aberrations in these genes might also be a frequent cause of CRC at a young age in China. © 2005 Elsevier Inc. All rights reserved.

1. Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC; Mendelian Inheritance in Man [MIM] no. 114500) is an autosomal dominant disease characterized clinically by early onset of colorectal cancer (CRC) and other associated tumors [1,2]. HNPCC is caused by inactivating mutations in the genes of the DNA mismatch repair system (MMR genes), at least four of which have been identified — MSH2, MLH1, PMS2, and MSH6 [3]. So far, most of the germline mutations in HNPCC families have been found in the MSH2 (MIM no. 120435) and MLH1 (MIM no. 120436) genes [4].

A large number of germline mutations, mainly small deletions or insertions leading to frameshifts, splice site alterations, and nonsense and missense mutations have been uncovered by conventional methods such as denaturing high-performance liquid chromatography (DHPLC), heteroduplex analysis, denaturing gradient gel electrophoresis (DGGE), single-strand conformational polymorphism (SSCP), and direct DNA sequencing [5–8]. Large genomic aberrations, deletions, or duplications of exons, however, cannot be detected by these techniques. Nevertheless, detection of large genomic variants is important in genetic testing in HNPCC because they account for up to 30% of all pathogenic mutations in MSH2 and MLH1 genes [9,10].

There are several methods for detecting large genomic deletions or duplications, such as Southern blotting, real-time polymerase chain reaction (PCR), protein truncation test (PTT), and semiquantitative multiplex PCR assay [11–15]. Nevertheless, it is difficult to use them conventionally in a laboratory because they are time consuming and have a low sample throughput. Schouten et al. [16] recently proposed a novel method, multiplex ligation-dependent probe amplification (MLPA), for relative quantification of DNA
sequences in high throughput. With this method, it is possible to check all the exons of the \textit{MSH2} and \textit{MLH1} genes for deletions or duplications in one reaction. Because only one pair of PCR primers is used, MLPA reactions result in a reproducible gel pattern with fragments ranging from 130 to 490 base pairs. Comparison of the gel patterns from suspected HNPCC patients with those from control samples will uncover an aberrant copy number in the screening sequences [10,17].

Until now, most of the mutational data on HNPCC are based on western subjects. The frequency of large genomic rearrangements of MMR genes in Chinese HNPCC is still unknown. Although the incidence of colorectal cancer ranks fifth among all cancers in China, which is much lower than that in western populations, Chinese CRC is characterized by onset of disease at an earlier age (mean age about 10 years younger than that in western cases) [18]. The mechanism behind this is still unknown.

In this work, we checked the germline genomic deletion or duplication in \textit{MSH2} and \textit{MLH1} genes to investigate the frequency of large genomic rearrangements of MMR genes in different groups of Chinese patients suspected of having HNPCC.

2. Materials and methods

2.1. Patients

Screening for deletions and duplications in \textit{MSH2} and \textit{MLH1} genes was performed in three groups of Chinese patients. The first group contained 45 unrelated familial CRC patients, of whom 21 fulfill the Amsterdam criteria (AC$^+$ for HNPCC), and 24 (family history [FH]) have a familial clustering of CRC but fail to meet the AC (AC$^-$). The second group consisted of 20 young-age CRC patients (age at diagnosis younger than 50 years and without a family history of HNPCC-related cancers). The third group consisted of 13 sporadic CRC patients diagnosed at ages older than 50 years. All the three groups of patients had not been fully screened for point mutations and small insertions or deletions. In addition, four patients with known genomic deletions in the \textit{MSH2} gene or the \textit{MLH1} gene were used as positive controls, and 22 healthy individuals as normal controls. Peripheral blood was taken from all index patients and controls to extract genomic DNA for the detection of large genomic aberrations. Informed consent was obtained from all patients involved in the study.

2.2. Materials

MLPA P003 mix (presented by Dr. J.P. Schouten, MRC-Holland Corp, Amsterdam, The Netherlands) was used to check DNA for large deletions or duplications in \textit{MSH2} and \textit{MLH1} genes. The probe mixture in the MLPA kit contained 16 exon probe pairs for \textit{MSH2}, 19 exon probe pairs for \textit{MLH1}, and seven control probe pairs. The information of the MLPA probes and primers can be found in the website (http://www.mlpa.com/p003.htm). Genomic DNA was isolated from peripheral blood with the QIAamp DNA Blood Mini Kit (Qiagen Corp, Chatsworth, CA).

2.3. MLPA analysis

MLPA was performed according to the protocol supplied with the kit, with a few modifications according to the conditions in our laboratory. Briefly, 100 ng of template DNA was denatured at 98°C for 5 minutes and hybridized with probe mix at 60°C overnight. Ligation between long and short probes hybridizing with the same sequence was done with heat-stable Ligase-65 at 54°C for 15 minutes. The probes ligated in the mixture were amplified using a pair of universal primers. The upstream one was labeled with fluorescent dye 6-FAM at the 5’ end. PCR conditions were as follows: 95°C 30 seconds, 60°C 30 seconds, and 72°C 60 seconds for 30 cycles. Fragment analysis of PCR products was performed in a Genescan model on an ABI 3100 Avant sequencer (Applied Biosystems, Foster City, CA).

The peaks given by Genescan correspond to the PCR products of the exons identified according to their relative migration, based on their sizes. Peak areas were calculated by the Genescan analysis software 3.1 (Applied Biosystems), exported to an Excel spread sheet, and copy numbers of target sequences were calculated as described [16]. Briefly, all peak areas were normalized by dividing each peak area by the combined peak area of all peaks in that lane. The normalized peak area was divided by the average relative normalized peak areas of this probe obtained in all control samples. The value multiplied by two represents the copy numbers of the exon checked. In normal individuals, this calculation will result in a value of around 1.0, representing two copies of the target sequence in the sample. A value reduced to 0.5 was interpreted as a heterozygous deletion of the target sequence. On the other hand, a value above 1.5 indicated the existence of a duplication.

2.4. Confirmation of genomic deletion or duplication

Confirmation of genomic aberrations was performed by a semiquantitative multiplex PCR. We applied essentially the primer pairs, PCR conditions, and the fragment analysis described by Wang et al. [15]. We sequenced all the relative exons of the patients with suspected genomic aberrations to exclude the interference of point mutations and small insertions or deletions in the sequences of probe hybridization.

2.5. Statistical analysis

Fisher’s exact test was used to test the difference in frequency of large genomic aberrations between different CRC groups.
Fig. 1. Peak pattern of exons from $MSH2$ and $MLH1$ genes in MLPA. The x-axis shows the size of PCR products, and the y-axis reflects the relative quantity of PCR products. The arrows indicate a deletion of exon 1–7 in the $MSH2$ gene discovered in patient N01 (A). The amount of PCR products of exons 1–7 in the $MSH2$ gene was only half of a normal control (B).

3. Results

3.1. MLPA analysis of the negative and positive controls

Before screening the genomic DNA of suspected HNPCC, young CRC, and sporadic CRC patients, we tested the reproducibility of MLPA with DNA samples from healthy controls and from patients with known exon deletions in $MSH2$ and $MLH1$ genes. No genomic aberrations of the $MSH2$ and $MLH1$ genes were found in 22 normal individuals, and the known deletions of the 4 positive controls in $MSH2$ and $MLH1$ genes were reproducibly unveiled by MLPA assay (patient 281, $MSH2$ EX1_6del; patient 325, $MLH1$ EX7_10del; patient 372, $MSH2$ EX1_10del; patient 449, $MLH1$ EX1_10del; data not shown).

3.2. Detection and confirmation of large genomic deletions or duplications

MLPA was performed for screening genomic deletion or duplication in 21 patients of AC$^+$ HNPCC, 24 patients

Fig. 2. Peak pattern of MLPA performed in patient N04 and control. (A) Electropherogram of PCR products of patient N04, who carries a duplication of exon 1 in the $MLH1$ gene (arrow). (B) Electropherogram of a normal control.
Table 1
Aberrations identified in nine patients by MLPA

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Diagnostic criteria</th>
<th>MSH2</th>
<th>MLH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>N01</td>
<td>AC</td>
<td>Del exons 1–7</td>
<td></td>
</tr>
<tr>
<td>N04</td>
<td>FH</td>
<td>Dup exon 1</td>
<td></td>
</tr>
<tr>
<td>N10</td>
<td>AC</td>
<td>Del exon 1</td>
<td></td>
</tr>
<tr>
<td>N18</td>
<td>AC</td>
<td>Del exon 11</td>
<td></td>
</tr>
<tr>
<td>N37</td>
<td>Young age</td>
<td>Del exons 9–14</td>
<td></td>
</tr>
<tr>
<td>N45</td>
<td>FH</td>
<td>Del exons 2–6</td>
<td></td>
</tr>
<tr>
<td>N52</td>
<td>FH</td>
<td>Dup exon 3</td>
<td></td>
</tr>
<tr>
<td>N56</td>
<td>Young age</td>
<td>Del exon 7</td>
<td></td>
</tr>
<tr>
<td>N59</td>
<td>AC</td>
<td>Dup exon 19</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AC, Amsterdam criteria; FH, familial clustering of CRC (AC−); young age, CRC patients diagnosed at younger than 50 years and have no family history.

Overall, nine large genomic aberrations (six deletions and three duplications) were unveiled in the MSH2 and MLH1 genes of the 78 patients (Figs. 1 and 2). Five out of nine aberrations were uncovered in the MSH2 gene and four in the MLH1 gene (Table 1). All the discovered deletions and duplications had been confirmed by semiquantitative multiplex PCR (Fig. 3). Patients N37 and N45 had the deletion of exons 9–14 and exons 2–6 in MSH2, respectively. Both are novel deletions. Patient N52 had an extraordinarily high peak area of MLH1 exon 3, the calculated copy number of the exon was 8, and a similar copy number of exon 3 was calculated by semiquantitative multiplex PCR analysis (Fig. 4). Patient N59 had an extraordinarily high peak area of MLH1 exon 19, with 12 calculated copies (Fig. 5). There were no point mutations and small insertions or deletions found in the MLPA probe hybridization sequence of the patients with suspected genomic aberrations. The family members of patient N01, with a deletion of exons 1–7 in MSH2, were included in the deletion analysis. It was discovered that the deletion cosegregated with the occurrence of CRC in this family.

3.3. Frequencies of genomic aberrations uncovered in different CRC groups

The genomic aberration was uncovered in 7/45 (15.6%) suspected HNPCC patients, 4/21 (19.0%) AC+ patients, and 3/24 (12.5%) patients with family history of CRC but who were AC−. In addition, genomic aberrations were also discovered in 2/20 (10.0%) young sporadic CRC patients (diagnosed before the age of 50 years). No genomic deletion or duplication was found in older sporadic CRC (age 50 years or older; Table 2). The frequency of aberrations was compared between different patient groups. No significant differences were found between AC+ and AC− patients, and between young CRC and AC+ or AC− patients (P > 0.05).

4. Discussion

Several studies have shown that genomic deletions and duplications in MSH2 and MLH1 are a frequent cause of HNPCC [9,10,17]. It has been suggested that investigations for large genomic aberrations should be included in routine HNPCC mutation screening protocols. However, it is almost impossible to uncover exon deletions or duplications at the genomic DNA level with the traditional mutation detection methods such as heteroduplex analysis, DGGE, SSCP, or
Fig. 4. Semiquantitative multiplex PCR analysis of patient N52 with primers corresponding to exons 3, 4, and 11 in \textit{MLH1}, and exons 3 and 14 in \textit{MSH2}. An increase of PCR product of exon 3 (arrow) in \textit{MLH1} of patient N52 (A) reflects about three times duplication in checked sequence compared with a normal control (B).

DHPLC [5–8]. Southern blotting was first applied successfully for the detection of large genomic aberrations in mismatch repair genes. Nevertheless, it is difficult to use in routine genetic testing because of its time-consuming nature and its demand for large amounts of DNA [11]. Semiquantitative multiplex PCR assay could overcome the shortage of Southern blotting, but the pairs of primers working in the same multiplex PCR might interfere with each other, thus limiting the efficiency of the method [14,15]. PTT might be used to detect large genomic rearrangements at the RNA level, but the instability of mRNA molecules requires a high quality of tissue samples [13].

MLPA is a promising newcomer with the potential to detect dosage changes of the checked sequence. It could perform a multiplex PCR in which up to 45 specific sequences can be quantified simultaneously [16]. Based on only one pair of PCR primers used, MLPA reactions show a very reproducible chromatographic pattern. Moreover, MLPA has the advantage of high throughput and less consumption of DNA samples.

Fig. 5. MLPA analysis of patient N59 and control. (A) The arrow shows a very high peak reflecting a duplication of exon 19 in \textit{MLH1}. (B) Pattern of a normal control.
In comparison with data based on western cases of HNPPC, a similar proportion of large genomic aberrations was detected in Chinese patients with suspected HNPPC, including those who were AC\(^+\) (19.0\%) and those with family history who were AC\(^-\) (12.5\%). Gille et al. [10] found large deletions in 13.0\% (17/126 kindreds), and Wang et al. [9] found deletions in 13\% of patients with AC\(^+\) and in 11\% those with family history but AC\(^-\). Interestingly, a similar frequency (2/20) of large genomic aberrations was also discovered in young Chinese patients with sporadic CRC. However, it had not been found in 27 western cases of young CRC with MSI\(^+\)-H [9]. We have known that the pathogenesis of CRC among Chinese is characterized by a lower incidence and relatively young age of onset of CRC [18]. The mechanism behind this has not been discovered yet. Our results in this study suggest that young Chinese CRC patients might have similar genetic backgrounds as CRC patients with familial clustering, and that large genomic aberrations in MSH2 and MLH1 genes are frequent causes of young Chinese CRC.

The large genomic duplication, as one kind of gene mutation, could theoretically occur in MMR genes at a similar proportion, but so far has been reported rarely. It was found neither in Gille’s investigation of 126 suspected HNPPC patients, nor in Wang’s 180 patients [9,10]. Nevertheless, one duplication in the MSH2 gene was reported by Charbonnier et al. [19]. In our study, three (33\%) of the nine genomic aberrations unveiled were duplications in the MLH1 gene. Two of them may have more than a four-copy increase in the affected exons of MLH1. This phenomenon has been reported rarely. It indicates that duplication can also play an important role in the pathogenesis of HNPPC in Chinese populations, but its mechanism remains to be uncovered.

In summary, nine genomic aberrations were found in this study—five (56\%) are in MSH2 and four (44\%) in MLH1 genes. Genomic aberrations in both MSH2 and MLH1 genes are a frequent cause in Chinese suspicious HNPPC and in young CRC patients. However, a high rate of duplication in MLH1 was found in Chinese patients as well. There is an apparent difference between the genetic alterations of western and Chinese patients with suspected HNPPC.

Acknowledgments

Dr. J.P. Schouten, MRC-Holland corp, kindly provided the MLPA P003 kit. The study was supported by the Health Department, Jiangsu Province, P.R.China (H0207, RC 200207).

References


