Plenary paper

Simple PCR detection of haptoglobin gene deletion in anhaptoglobinemic patients with antihaptoglobin antibody that causes anaphylactic transfusion reactions

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Two anhaptoglobinemic patients showing anaphylactic transfusion reactions by antihaptoglobin antibody were found. Southern blot analysis indicated that 2 patients were homozygous for the deleted allele of the haptoglobin gene (Hpdel) as reported previously. We have identified the junction region of the deletion from genomic DNA of 1 patient using cassette-mediated polymerase chain reaction (PCR). Then, the deleted region from the 5' breakpoint to the promoter region of the Hp was amplified from genomic DNA of a control individual using PCR. DNA sequence analysis of these regions indicated that the 5' breakpoint of the Hpdel allele was located 5.2 kilobase (kb) upstream of exon 1 of the Hp and the 3' breakpoint was positioned between 52 and 53 base pair (bp) upstream of exon 5 of the haptoglobin-related gene. There was no significant homology between the DNA sequences flanking the 5' and 3' breakpoints, except for a 2-bp (TG) identity. To examine the gene frequency, we have developed a simple PCR method to detect the gene deletion. We found 8, 16, and 17 Hpdel alleles in 157 Koreans, 523 Japanese, and in 284 Chinese, respectively, but did not find the Hpdel in 101 Africans or in 100 European-Africans. The incidence of individuals homozygous for the Hpdel allele was therefore expected to be 1/4000 in Japanese, 1/1500 in Koreans, and 1/1000 in Chinese. This incidence is higher than that of IgA deficiency in Japanese. More attention should be paid on haptoglobin deficiency and antihaptoglobin antibody as the cause of transfusion-related anaphylactic reactions in Asian populations. (Blood. 2000; 95:1138-1143)

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Haptoglobin (Hp) is a hemoglobin-binding polymorphic plasma glycoprotein present in all vertebrates.1 Heritable normal variations in humans were first reported by Smithies and Walker,2 and there are 3 common genetic haptoglobin phenotypes, Hp1, Hp2, and Hp2-1, which are determined by a pair of codominant alleles—Hpα and Hpβ. Although the physiologic function of the protein is not completely understood, it may limit iron loss during normal erythrocyte turnover and during hemolysis. While certain pathologic states such as severe hemolysis and liver dysfunction are known to lead to secondary anhaptoglobinemia,3 previous reports have suggested that some individuals with hypohaptogloblinemia or anhaptogloblinemia (Hp0 phenotype) have a genetic origin.4-7 A silent allele Hpβ at the haptoglobin locus (Hp) has been postulated by studies on the anomalous inheritance of haptoglobin phenotypes, and the homozygosity of Hpβ has been suggested as a cause of anhaptogloblinemia.8,9 However, the genetic mechanisms of Hpβ allele(s) have not been understood for a long time. Recently, we have identified 1 of Hpβ allele(s) that was an allelic deletion in the haptoglobin gene cluster (Hpβdel).10 The gene deletion was larger than 20 kilobase (kb) and extended from upstream of the promoter region of the Hp to the 3' region of intron 4 of the downstream haptoglobin-related gene (Hpα), which shares a high degree of nucleotide sequence homology with the Hp.11-13

We have encountered 2 anhaptoglobinemic patients with anti-Hp antibody, who suffered from severe anaphylactic shock after infusion of blood products.14,15 Briefly, the first was a 33-year-old woman (NK) in the 31st week of her first pregnancy. She was hospitalized for threatened premature delivery due to chronic polyhydramnios. When she developed edema in her extremities with increased amniotic fluid and decreased serum albumin, 25% albumin solution was infused. After receiving several drops, she exhibited severe anaphylactic reactions, and immediate intravenous administration of corticosteroids improved her symptoms. The second case was a 94-year-old woman (SN) with myelodysplastic syndrome. She was transfused red blood cells 3 times and platelet-concentrate once during a 7-month period without any symptoms. However, when she was transfused platelet-concentrate 1 month later, she suffered from anaphylactic reactions, which responded to corticosteroids. She had borne 2 children without any trouble when she was young. Both these patients showed anti-Hp antibody and no detectable Hp in their sera.14,15

In the current study, we have determined that these 2 patients

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were homozygous for the \( Hp^{del} \) allele by Southern blot analysis. The junction region of the \( Hp^{del} \) allele was amplified by cassette-mediated polymerase chain reaction (PCR) and sequenced. We then developed a simple method to detect this deletion by PCR amplification for screening anhaptoglobinemic individuals to examine the gene frequency in various populations.

**Materials and methods**

**DNA preparations from patients and healthy volunteers**

B lymphocytes from 2 patients were transformed by Epstein-Barr (EB) virus, and genomic DNA was extracted from EB virus-transformed lymphocytes using an organic solvent method. In addition, genomic DNA from peripheral leukocytes of family members of the patients, and randomly selected Japanese, Koreans, Chinese, Africans (Xhosa), and European-Africans of South Africa was isolated as described previously.\(^{16-20}\)

**Southern blot analysis**

The \( Hp^{del} \) allele was detected by Southern blot analysis as described previously.\(^{16}\) Briefly, genomic DNA (7 µg) was separated in a 0.8% agarose gel after digestion by an appropriate restriction endonuclease, transferred onto a nylon membrane and hybridized with the digoxigenin-labeled cDNA probe coding for the \( \alpha \) chain or \( \beta \) chain of haptoglobin (a kind gift from Dr. R. Lippen, Université Libre de Bruxelles, Service de Génétique Appliquée).\(^{21}\) The digoxigenin-labeled DNA probe was prepared using a DIG DNA labeling kit (Boehringer Mannheim, Mannheim, Germany).

**PCR amplification of the junction region of \( Hp^{del} \) allele**

A Marathon cDNA amplification kit (Clontech Japan, Tokyo, Japan) was used for isolation of the junction region of the \( Hp^{del} \). Genomic DNA (5 µg) from EB virus-transformed lymphocytes of the patient (NK) homozygous for the \( Hp^{del} \) allele was digested with 1 of several endonucleases (50 U) (\( DraI, EcoRV, PvuII, RsaI, \) and \( HaeIII) and ligated with a Marathon cDNA adapter (Clontech). Then, the junction region of the deletion was amplified using cassette-mediated PCR. Because there are AP1 and AP2 sequences within the adapter, we used the gene-specific primers (Hpr-first and Hpr-nest) as downstream primers and the AP1 and AP2 primers (Clontech) as upstream primers. The Hpr-first primer 5'-GGG CTT CCC ACA TAC TGT CAA GGA G-3', within intron 4 and exon 5 of the Hpr, and the AP1 primer were used for the first PCR, and then nested PCR was performed using the Hpr-nest primer 5'-CCA CAT ACT GTC AAG GAG AGG AAG A-3', and the AP2 primer. The temperature profile of PCR for amplification of the junction region of the \( Hp^{del} \) was at 94°C for 1 minute, followed by 25 cycles of denaturing at 98°C for 10 seconds, and annealing and extension at 68°C for 4 minutes. PCR amplifications were performed using 2.5 units of \( LA Taq \) polymerase (Takara, Shiga, Japan) in a 25 µL \( LA Taq \) buffer containing 5 pmol of each primer, 2.5 mmol/L MgCl\(_2\), and 400 µmol/L dNTP.

**PCR amplification of the deleted region**

The region between an upstream region of the \( S' \) breakpoint of the deletion of the \( Hp^{del} \) and the promoter region of the haptoglobin gene was amplified from genomic DNA of a control individual using primers \( Hp\text{-del-U} \) (5'-CTT TAT GGC ACT GGG CAA GGA CAA GCA TTT TG-3') (Figure 2) and \( Hp\text{-promoter-L} \) (5'-CCC ATC AAC AGG AGG AAG GGT CAA TAC TGC-3'), within a promoter region of the \( Hp\),\(^{13}\) The terms “\( U \)” and “\( L \)” indicate upper and lower primers, respectively. The temperature profile was at 94°C for 1 minute, followed by 30 cycles of denaturing at 98°C for 10 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 5 minutes. PCR amplification was performed using 2.5 units of \( LA Taq \) polymerase (Takara) in a 25 µL \( LA Taq \) buffer containing 5 pmol of each primer, 2.5 mmol/L MgCl\(_2\), and 400 µmol/L dNTP.

**Results**

**Characterization of the haptoglobin gene cluster of patients with anti-Hp antibody**

The haptoglobin gene cluster of 2 patients with anti-Hp antibody was analyzed by Southern blot analysis with cDNA probes coding for \( \alpha \) or \( \beta \) chain of Hp. Figure 1 shows typical hybridization results. When genomic DNA was digested by SacI, a 3.4-kb DNA fragment corresponding to the \( Hp \) and a 2.4-kb signal corresponding to the \( Hpr \) were identified by an \( Hp \) \( \beta \) cDNA probe in a control individual (lane N). In addition to these 2 bands, an unexpected band of 7.5 kb was found in a hypohaptoglobinemic individual who was previously shown to be heterozygous for the \( Hp^{del} \) allele (lane H). Only the 7.5-kb band was found in 2 anhaptoglobinemic patients who had anti-Hp antibody (lane A). When genomic DNAs of the 2 patients were digested by XhoI, and by \( HindIII \), only unexpected band of 6.5 kb and of 4.4 kb, corresponding to the \( Hp^{del} \), was detected by the \( Hp \) \( \beta \) cDNA probe, respectively, and no signal was detected by the \( Hp \) \( \beta \) cDNA probe.
detected by the \( Hp \alpha \) cDNA probe (not shown). In addition, we failed to amplify exons 1 ~ 4 of the \( Hp \) using PCR from genomic DNA of the patients. These results indicated that the 2 patients with anti-\( Hp \) antibody were homozygous for the \( Hp^{\text{del}} \) allele, which is the sole null allele of the \( Hp \) reported to date.\(^{10} \)

**Identification of the junction region of the \( Hp^{\text{del}} \)**

Previous mapping results suggested that the 3' end of the gene deletion occurred within a 284-bp stretch between the \( AatI \) site within intron 4 and the \( BamHI \) site in exon 5 of the \( Hpr \). To identify the junction region of the \( Hp^{\text{del}} \) allele, cassette-mediated PCR was performed using several endonuclease-digested libraries constructed from genomic DNA of the EB virus-transformed lymphocytes of 1 patient (NK). The longest PCR product (2.5 kb) was obtained from \( DraI \) library. DNA sequence analysis of the 2.5-kb PCR product indicated that the sequence of 2380 bp of 5' region of the product has not been identified previously, and that 60 bp of 3' region of the product was identical to the intron 4 and exon 5 of the \( Hpr \).\(^{13} \) The DNA sequence between 781-bp upstream of the 5' breakpoint and 228-bp downstream of the 3' breakpoint was positioned between 52- and 53-bp upstream of \( Hpr \) (Figure 3A). From these results, the size of the deletion of the \( Hp^{\text{del}} \) allele is estimated to be 28 kb. The DNA sequences flanking the 5' and 3' breakpoints showed no significant DNA sequence homology in the junction region of the deletion, except for 2 bases (TG) (Figure 3B). Computer analysis of the 6540-bp sequence obtained in this study by the Repeat Masker program through the Washington University Human Genome Center (http://ftp.genome.washington.edu/cgi-bin/) identified that about 70% of this sequence were repetitive.

**Screening and identification of the \( Hp^{\text{del}} \) allele using PCR**

For screening for the \( Hp^{\text{del}} \), we amplified the \( Hp^{\text{del}} \) allele using conventional PCR with primers (\( Hp\text{-del-U} \) and \( Hp\text{-del-L} \), encompassing the junction of the \( Hp^{\text{del}} \) (Figure 2). As shown in Figure 4, a 315-bp DNA fragment was amplified from genomic DNA of individuals homozygous or heterozygous for the \( Hp^{\text{del}} \), whereas no DNA fragment was amplified from genomic DNA of a control individual. Direct DNA sequencing of this 315-bp PCR product from the 2 patients indicated the specific amplification of the junction region of the breakpoints. Homozygosity for the \( Hp^{\text{del}} \) allele was confirmed by the absence of amplified product of exon 1 of the \( Hp \). These PCR results were consistent with those from Southern blot analysis in 3 individuals homozygous and 10 individuals heterozygous for the \( Hp^{\text{del}} \) allele.
PCR products underwent electrophoresis in a 1.2% agarose gel and were stained by ethidium bromide. The size of the PCR product is indicated. Msp I-digested pBlue-script (M) was used as a molecular size marker.

To amplify the Hp del and the Hp (exon 1) in the same reaction tube using Hp-del-U and Hp-del-L primers, and Hp-Ex1-U and Hp-Ex1-L primers, PCR was performed from individuals with anhaptoglobinemia and hypohaptoglobinemia and from a control individual. A 315-bp band was amplified from an individual homozygous for the Hp del, whereas only a 476-bp band was amplified from a control individual. Both 315-bp and 476-bp bands were amplified from individuals heterozygous for the Hp del allele.

We then examined the Hp del in the members of the 2 families of the patients (SN and NK). Consistent with the results from Southern blot analysis, the 2 patients were homozygous for the Hp del allele (Figure 5, lanes 1 and 4). Two children of SN were heterozygous for the Hp del allele. The parents and a brother of NK were heterozygous for the Hp del allele.

To examine the gene frequency of the Hp del allele, PCR amplification was performed from genomic DNAs of 523 randomly selected Japanese in 4 different areas of Japan (Akita, northeastern part of Japan; Tokyo, central part of Japan; Fukuoka, west part of Japan; Okinawa, southwest island of Japan), 157 Koreans (Seoul), 284 Chinese (Shenyang, northeast part of China; Guangzhou, south part of China), 101 Africans (Xhosa) (Cape Town, South Africa), and 100 European-Africans (Cape Town). Sixteen individuals in 523 Japanese, 8 in Koreans, and 17 individuals in 284 Chinese were found to be heterozygous for the Hp del allele (Table 1). Because there was no significant difference between gene frequencies of the Hp del in 4 subpopulations of Japan and between those in 2 subpopulations of China, the allele frequency of the Hp del in Japanese, in Koreans, and in Chinese, was calculated to be 0.015, 0.025, and 0.030, respectively. On the other hand, we did not detect the Hp del allele in any of the 101 randomly selected Africans (Xhosa) or in any of the 100 European-Africans. However, these findings cannot exclude the absence of the Hp del in Africans and in European-Africans, because of the small number of samples examined.

Table 1. Allele frequencies of the Hp del in various populations

<table>
<thead>
<tr>
<th>Populations</th>
<th>Number of Individuals</th>
<th>Number of Allele</th>
<th>Allele Frequency</th>
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</thead>
<tbody>
<tr>
<td>Japanese (total)</td>
<td>523</td>
<td>16</td>
<td>0.015</td>
</tr>
<tr>
<td>Akita</td>
<td>120</td>
<td>5</td>
<td>0.021</td>
</tr>
<tr>
<td>Tokyo</td>
<td>126</td>
<td>4</td>
<td>0.016</td>
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<tr>
<td>Fukuoka</td>
<td>140</td>
<td>3</td>
<td>0.011</td>
</tr>
<tr>
<td>Okinawa</td>
<td>137</td>
<td>4</td>
<td>0.015</td>
</tr>
<tr>
<td>Korean</td>
<td>157</td>
<td>8</td>
<td>0.025</td>
</tr>
<tr>
<td>Chinese (total)</td>
<td>284</td>
<td>17</td>
<td>0.030</td>
</tr>
<tr>
<td>Shenyang</td>
<td>132</td>
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<tr>
<td>Guangzhou</td>
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<td>7</td>
<td>0.023</td>
</tr>
<tr>
<td>African</td>
<td>101</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>European-African</td>
<td>100</td>
<td>0</td>
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</tr>
</tbody>
</table>

Discussion

Southern blot analysis in this study indicated that the 2 patients with anti-Hp antibody were homozygous for the Hp del allele previously identified.10 The results indicated that the large haptoglobin gene deletion was the causal mutation for the anhaptoglobinemia, resulting in the production of antibody after blood transfusions. We have determined that the 5’ breakpoint of the gene deletion was near an LTR-like sequence that was located about 5.2-kb upstream of exon 1 of the Hp. From these results, the size of deletion was calculated to be 28 kb. In this study, we have determined the nucleotide sequence of the 5’ flanking region of the Hp del allele (Figure 3B). Thus, these sequences might play some role in this deletion occurred by nonhomologous recombination. Nonhomologous recombination has been characterized by the presence of short repeats (2 ~ 6 bp),22 whereas homologous recombination needs larger homologous sequence (more than 14 bp).23 The sequence features such as A-T rich elements, alternating purine/pyrimidine tracts, polypurine-pyrimidine tracts, palindromic sequences, and topoisomerase I and II cleavage sites are often found at or near the junction surrounding nonhomologous recombination breakpoints.24 Among these motifs, an A-T rich element was found in 5’ flanking region of the the 5’ breakpoint, and a polypurineimidine tract was detected in the 5’ flanking sequence of the 3’ breakpoint (Figure 3B). Thus, these sequences might play some role in this large haptoglobin gene deletion.

In this study, we have developed a method to identify the Hp del allele using PCR. This method can detect the Hp del in all 13 individuals who were determined to be heterozygous or homo-
zygous for the Hp(del) using Southern blot analysis. We then examined the gene frequency of the deletion in Japanese, Koreans, Chinese, Africans, and European-Africans. We estimated that the gene frequency of the Hp(del) was 0.015 in Japanese, 0.025 in Koreans, and 0.030 in Chinese. Accordingly, we estimated that individuals with true anhaptoglobinemia, who are homozygous for the Hp(del), are 1 in every 4000 Japanese, 1 in every 1500 Koreans, and 1 in every 1000 Chinese. Recently, Yoshioka et al.25 found 1 individual with true anhaptoglobinemia out of 9711 unrelated Japanese. Therefore, it is likely that the Hp(del) allele is the most frequent Hp allele in Japanese. Our results also suggest that about 0.025% Japanese, 0.067% Koreans, and 0.1% Chinese are at risk to produce an antibody against haptoglobin after repeated blood transfusions. The first description of congenital absence of haptoglobin reported over 30 years ago in individuals of African descent.4 However, we did not find the Hp(del) in any of the 101 Africans or the 100 European-Africans. These results were consistent with previous reports that no detectable change was found in the haptoglobin gene cluster of African individuals with anhaptoglobinemia.26,27 Although we cannot rule out the possibility that the frequency of the Hp(del) allele in these populations was much lower than that in Asian populations, a recent study reported that the frequency of true congenital anhaptoglobinemia was at approximately 1 in every 1000 Europeans, and that anhaptoglobinemia was more frequent in Africans.7 Therefore, the genetic mechanisms for anhaptoglobinemia might be different between East Asian populations and other ethnic groups. It would be of interest to know whether Africans with congenital anhaptoglobinemia have ever been found to have antibodies against Hp or to have experienced transfusion reactions.

Recently, it has been reported that haptoglobin gene-knockout mice showed a higher susceptibility during acute hemolysis compared with control mice, although the clearances of free plasma hemoglobin in these mice and in control mice were not significantly different.28 These mice also had a small reduction in postnatal viability. These results suggest that individuals homozygous for the Hp(del) allele may be at a disadvantage during acute hemolysis.

Undesired reactions caused by blood transfusion have focused on the hemolytic immune reactions with red blood cells. However, less attention has been paid to nonhemolytic reactions after blood transfusion or infusion of human blood products. Anaphylactic reactions to blood and blood components in patients with IgA deficiency, or with fourth component of complement (C4) deficiency, have been reported to be associated with antibodies to IgA, and to C4, respectively.29-32 IgA anaphylactic reactions have occurred in persons who were IgA-deficient and in whom class-specific anti-IgA was detected in the serum. Determination of serum concentrations of Hp could be used for initial screening of patients at risk for anti-Hp transfusion reaction. However, the measurement of plasma Hp concentration is not sufficient for determination of true anhaptoglobinemia, since plasma Hp concentration is known to decrease to below detectable levels in certain pathologic states such as hemolysis and liver dysfunction.3 In addition, certainly the incidence of anhaptoglobinemia in many populations is so low that it would not be cost-effective to do such screening in the interest of preventing what would be a very rare incidence of transfusion reactions.

The incidence of IgA deficiency in Japanese was reported to be about 1/30 000, which is lower than those in Europeans (1/2 500).33,34 However, the incidence of anhaptoglobinemia appears to be much higher than that of IgA deficiency in Asian populations. More attention should be paid on haptoglobin deficiency and anti-haptoglobin antibody as the cause of transfusion-related anaphylactic reactions in East Asians. Accordingly, our simple PCR method to detect the Hp(del) is recommended as a reliable method to prevent anaphylactic transfusion reactions in individuals having a risk to produce anti-Hp antibody by blood transfusions. If patients who are shown to be homozygous for the haptoglobin deletion need to be transfused with red cells, it would be necessary to meticulously wash the red cells before transfusion to avoid an immunologic transfusion reaction.30

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References


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