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Abstract
Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) method has been established to discriminate genotypes for the human platelet antigen (HPA) systems HPA-1, HPA-2, HPA-3, HPA-4, and HPA-5. Gene fragments which contain polymorphic sequences corresponding to the HPA-1, HPA-2, HPA-3, HPA-4, and HPA-5 systems were PCR-amplified with specific primers. The amplified DNA was denatured and subjected to non-denaturing polyacrylamide gel electrophoresis followed by silver staining. The results obtained by the PCR-SSCP method were in good agreement with those of the allootypes determined by serological typing. Furthermore, the results agreed with those obtained by other DNA-based typing methods such as PCR-allele-specific restriction enzyme analysis and PCR-sequence-specific primer. These results indicate that PCR-SSCP is a simple and sensitive method for determining HPA genotypes and identifying unknown polymorphisms.

Introduction
Human platelet antigens (HPAs) play an important role in provoking neonatal alloimmune thrombocytopenic purpura (NAITP), post-transfusion purpura (PTP), and refractoriness to platelet transfusion therapy. In Caucasians, more than 90\% of the reported cases of NAITP \cite{1} and PTP \cite{2} were caused by antibodies to the HPA-la (Pl, Zw) antigen, whereas the HPA-4b (Yuk\textsuperscript{a}) antigen may play an important role in many cases of NAITP and PTP in Japanese \cite{3}. Moreover, most cases of refractoriness to HLA-matched platelet transfusion therapy were caused by antibodies to the HPA-2b (Ko, Sib\textsuperscript{b}) antigen in Japanese \cite{4}.

Thus HPA typing and the detection of platelet antibodies are essential in diagnosis of NAITP, PTP, and refractoriness to platelet transfusion therapy. In addition, HPA typing is important in the selection of donors for patients refractory to HLA-matched platelet transfusions. To date, five distinct diallelic HPA systems HPA-1 (Pl, Zw) HPA-2 (Ko, Sib), HPA-3 (Bak), HPA-4 (Yuk), and HPA-5 (Br) have been well established by means of serological analysis \cite{5}. Although these HPA types are usually determined by serological typing, occasionally some problems may occur due to the following: (1) it is difficult to obtain specific antisera to HPA la, 1b, 2a, and 3b antigens, and (2) few platelets can be collected from some patients with thrombocytopenia.
Table 1. Nucleotide sequences of primers used in this study

<table>
<thead>
<tr>
<th>System</th>
<th>Name</th>
<th>Primer sequences</th>
<th>Base position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CP1-A</td>
<td>5’-CATAGTTCTGATTGCTGGACTTC-3’</td>
<td>12493–12515</td>
</tr>
<tr>
<td></td>
<td>CP1-C</td>
<td>5’-CTCTAGTACTCGGGGCCCTCACT-3’</td>
<td>12625–12645</td>
</tr>
<tr>
<td>HPA-2</td>
<td>KY2-A</td>
<td>5’-CGTCCTGGACGTCTCCTTTCAAC-3’</td>
<td>399–420</td>
</tr>
<tr>
<td></td>
<td>CP2-D</td>
<td>5’-GTGTCGAGATTCTCCAGCCCATTC-3’</td>
<td>595–618</td>
</tr>
<tr>
<td>HPA-3</td>
<td>HPA-3L</td>
<td>5’-TGGAGAAGAACCTGGGAAAGG-3’</td>
<td>13834–13854</td>
</tr>
<tr>
<td></td>
<td>HPA-3R</td>
<td>5’-CTCCTTAACGTACTGGGAAAGC-3’</td>
<td>14261–14281</td>
</tr>
<tr>
<td>HPA-4</td>
<td>PL2-1</td>
<td>5’-GAATTTCCTCCATCCCAAGTGGC-3’</td>
<td>13790–13810</td>
</tr>
<tr>
<td></td>
<td>CP4-B</td>
<td>5’-CATGTATGTTGACACAGGGTC-3’</td>
<td>13970–13991</td>
</tr>
<tr>
<td>HPA-5</td>
<td>CP5-A</td>
<td>5’-GTGACCTAAAGACCTGGGAAGG-3’</td>
<td>1601–1623</td>
</tr>
<tr>
<td></td>
<td>CP5-B</td>
<td>5’-GATGAATGTAAACCACAATCTACTG-3’</td>
<td></td>
</tr>
</tbody>
</table>

1 Base positions are according to previous papers (see text).

Studies on the molecular structures underlying these HPAs have elucidated that each diallelic antigen is associated with a single amino acid substitution resulting from a point mutation [6–10]. Based on nucleotide sequence polymorphisms, several DNA-based typing methods such as polymerase chain reaction (PCR) allele-specific oligonucleotide probe (ASO) [11], PCR-allele-specific restriction enzyme analysis (ASRA) [12–14], and PCR-sequence-specific primer (SSP) [15] have been reported.

The PCR-single-strand conformation polymorphism (SSCP) method has been developed as a simple and rapid method for detecting the presence of mutations in a DNA fragment [16]. It is based on the principle that the electrophoretic mobility of a single-strand DNA fragment in a non-denaturing polyacrylamide gel is altered by the conformational change resulting from certain nucleotide substitution(s), and it can detect sequence polymorphisms at a variety of positions in DNA fragments, although the exact sites of the mutations must be defined by other methods. The PCR-SSCP method has some advantages over the PCR-ASRA or the PCR-SSP method: (1) it can detect polymorphisms which cannot be identified by restriction enzymes or specific primers, and (2) it may detect unknown or ‘new’ polymorphisms [17]. Furthermore, in HPA dimorphic systems, the PCR-SSCP method is applicable to relatively large numbers of samples compared to PCR-ASRA and PCR-SSP methods.

In the present study, we established DNA-based typing systems for HPA-1, HPA-2, HPA-3, HPA-4, and HPA-5 by means of PCR-SSCP and compared the results with those of conventional serological typing as well as PCR-ASRA and PCR-SSP typing.

**Materials and Methods**

**Samples**

Blood samples were obtained from 100 healthy Japanese donors. Ten blood samples which were distributed at the 7th Japanese Workshop on Platelet Serology (1994) and five genomic DNA samples distributed at the 7th International Society of Blood Transfusion Platelet Serology Workshop (1994) were also examined.

**Serological Analysis**

The HPA allotypes were determined by the mixed passive hemagglutination (MPHA) method [18]. In all 110 blood samples, allotypes of HPA-1a, HPA-1b, HPA-2b, HPA-3a, HPA-4a, HPA-4b, HPA-Sa, and HPA-Sb were determined with this method. Because the available volume of anti-HPA-2a and anti-HPA-3b sera were limited, only 20 samples were examined for HPA-2a and HPA-3b antigens.

**Preparation of Genomic DNA**

Ten milliliters of peripheral blood was collected with 10% citratephosphate-dextrose. Lymphocytes were isolated from blood samples by immunomagnetic beads (Dynabeads HLA class I, Dynal, Oslo, Norway). Lymphocytes (1.5 x 10^7) were suspended in 500 μl of 50 mM Tris-HCl (pH 8.8 at 25°C), 10 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.5% Tween 20, and 500 μg/ml proteinase K, and incubated at 56°C for 1 h, followed by incubation at 94°C for 10 min to inactivate proteinase K. Supernatant was collected and used for PCR without further purification.

**Specific PCR Amplification**

Gene fragments which contain polymorphic sequences corresponding to HPA-1, HPA-2, HPA-3, HPA-4, and HPA-5 systems were specifically amplified by the PCR method [19]. Sequences and genomic base positions of the oligonucleotide primers are shown in Table 1. These primers were newly designed based on the previously published sequences [14, 20–22], except for the primers for HPA-2 (KY2-A and CP2-D by Saji et al., manuscript in preparation) and HPA-3 [12].

PCRs were performed in 20 μl of reaction mixture containing 50 mM Tris-HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 50 μM each of dATP, dGTP, dCTP, and dTTP, 10 pmol of each primer, 1 μl of...
genomic DNA solution, and 0.5 U of Taq DNA polymerase (Takara, Kyoto, Japan), using a thermal cycler (9600, Perkin Elmer, Norwalk, Conn., USA). After preheating at 95°C for 4 min, 30 cycles of amplification were performed (denaturation at 95°C for 30 s, annealing at 65°C for 1 min, and extension at 72°C for 1 min), followed by incubation at 72°C for 5 min. Amplification of DNAs was checked by 5% polyacrylamide gel electrophoresis and ethidium bromide staining.

SSCP Analysis
SSCP analysis was performed basically according to the method described previously for the HLA-DRBI gene [23]. One microliter of the amplified DNA solution was mixed with 7 μl of denaturing solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF). The mixture was denatured at 95°C for 5 min and quickly chilled on ice, and then 1 μl of the mixture was applied to a polyacrylamide gel (acrylamide:bisacrylamide 49:1). A minigel electrophoresis apparatus with a constant temperature control system (AE-6410, Atto, Tokyo, Japan) was used. Electrophoresis was carried out in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at a constant current (20 mA/gel). In order to determine optimal conditions for each HPA system, various conditions were examined. We first tried the gel with 10% acrylamide and 5% glycerol, and 22°C electrophoretic temperature, then compared the results from different conditions as follows. Electrophoresis was performed at various temperatures: 6, 10, 22, 25, 30, 35, or 37°C. Various concentrations of acrylamide (10% or 12.5%) and glycerol (5% or 10%) were also tested. Single-strand DNA fragments in a gel were detected by silver staining (Daiichi Pure Chemicals, Tokyo, Japan).

Other DNA-Based Typing
The PCR-ASRA typing for HPA-1, HPA-2, and HPA-3 [12] was performed for comparison. Briefly, the amplified DNA fragments were digested with the restriction enzymes, MspI, SfaNI, and FokI for HPA-1, HPA-2, and HPA-3 systems, respectively. The PCR-SSP typing for HPA-1, HPA-2, HPA-3, and HPA-4 (Smitest HPA genotype, Sumitomo Metal, Tokyo, Japan) was also performed according to the manufacturer’s instructions.

Results and Discussion
Figure 1 shows the results of PCR-SSCP analysis of HPA systems. In order to distinguish HPA genotypes clearly, optimal gel composition and electrophoretic temperature were determined (fig. 1, legend). Figure 1a shows the representative electrophoretic patterns of the HPA-1 system by SSCP analysis. HPA-1a homozygotes (fig. 1a, lane 1) showed two major bands corresponding to coding and noncoding strands [23]. HPA-1b homozygotes (fig. 1a, lane 3) also showed two major bands, but the lower band was clearly distinguished from that of HPA-1a homozygotes. Heterozygotes for HPA-1a and HPA-1b (fig. 1a, lane 2) showed the combined feature of both patterns. A rare variant possessing a single base substitution from HPA-1b which was provisionally called HPA-1bvar [24, 25] could also be discriminated (fig. 1a, lane 4).

In the other HPA systems, all three phenotypes, i.e., one heterozygous and two homozygous ones were again clearly distinguished from each other. Figure 1b–1e shows representative patterns of HPA-2, HPA-3, HPA-4, and HPA-5 systems, respectively.

The results of PCR-SSCP typing for HPA-1, HPA-4, and HPA-5 systems were compared with allotypes determined by serological typing of 110 samples, and no discrepancy was observed between the results obtained by the two methods (data not shown). Moreover, the results of PCR-SSCP typing for HPA-2 and HPA-3 systems were identical to allootypes determined by serological typing of 20 samples. The other 90 samples were serologically typed with HPA-2b and HPA-3a antisera. HPA-2b-negative samples were all identified as homozygous for HPA-2a, and HPA-3a-negative samples were all also identified as HPA-3b homozygous.

In the present study, the results of PCR-SSCP typing of 115 DNA samples were compared with the genotypes determined by PCR-ASRA (for HPA-1, HPA-2, and HPA-3) and PCR-SSP (for HPA-1, HPA-2, HPA-3 and HPA-4). Although there was no discrepancy among the results obtained by these three methods, several problems of PCR-ASRA and PCR-SSP methods were identified. Some restriction endonucleases used in PCR-ASRA are expensive or cannot be obtained commercially, dispensing the restriction endonuclease into the reaction mixture may increase the risk of contamination, and the amplification condition must be optimized to ensure the amplification specificity especially in PCR-SSP.

Two groups have reported the PCR-SSCP analysis for the HPA system. Jin et al. [26] used the SSCP method to identify mutation(s) associated with Glanzmann thrombasthenia in the GP IIIa gene. They were able to distinguish HPA-1a and HPA-1b clearly, and also identified all three known silent polymorphisms in the GP IIIa gene. Walchshofer et al. [25] also applied the same method as Jin et al. [26] to confirm the rare variant of HPA-1 (HPA-1bvar). Although both groups clearly identified the HPA-1 alleles, their method is too complicated to be routinely performed: (1) they used a radioisotope for PCR amplification and detected the DNA fragments by autoradiography, and (2) they used a long gel and electrophoresed for at least 16 h.

In the present study, we established a fairly simple PCR-SSCP method which requires no radioisotope labeling and a less than 2-hour electrophoretic run with a minigel apparatus. Furthermore, we found that the electrophoretic temperature is most important to obtain high reproducibility. Varying other conditions such as gel concentration and glycerol concentration did not have much effect on the migration patterns.
HPA typing is essential in the selection of donors for patients refractory to HLA-matched platelet transfusions. Thus it is desirable that HPA and HLA are typed at the same time. In this study, we have established a HPA DNA-based typing method using genomic DNA isolated from lymphocytes, in which lymphocytes remaining from those collected for routine HLA typing can be used. In addition, genomic DNAs isolated by conventional methods could be used in our PCR-SSCP method.

In conclusion, our results showed that the typing results obtained by PCR-SSCP are consistent with those obtained by the conventional serological method and that this method is useful in discriminating HPA alleles as well as in screening ‘new’ variants. Our PCR-SSCP method may be adopted for routine typing of relatively large numbers of samples compared to the PCR-ASRA and PCR-SSP methods.

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References


