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Molecular Genetic Analysis of a $B^3$ Allele in a Rare Subtype A,B, Blood-type Individual

Kazuko Doi$^{1,2}$, Kaoru Nishiyama$^2$

Molecular genetic analysis of a rare subtype, A,B$, is reported. The targets of nucleotide alterations were within exons 6 and 7. After sequencing analysis, two typical SNPs of 547G>A and 467C>T were observed. Adenine nucleotide at nt 547 was identified as $B^\vartheta$ (ref. Cho et al., 2004) on the $B$ allele in an $A,B$ by ASP-PCR-RFLP without a cloning procedure. This variant could be rapidly identified by the ASP-PCR with a variant-specific primer. The patterns of PCR-SSCP near nt 547 and 467 were altered. Finally this $A,B$ individual was identified as $A^\vartheta B^3$.

Key words
ABO gene, A,B$,$ blood group, $B^\vartheta$ allele, Genetic polymorphism, Mini-gel SSCP, ASP-PCR-RFLP

Introduction

In 1990, the $A'$ allele of human blood group typing (histo-blood group A transferase) was successfully cloned and the consensus sequence of the $A'$, $B$ and $O'$ allele was established$^1$. In 1993, molecular genetic alterations at the ABO locus in individuals in subgroups were first reported$^2$. Each variant was identified by the sequencing of exons 6 and 7. Usually the $B_3$ variant is characterized by mixed field hemagglutination of red blood cells with anti-$B$ and with anti-$A$, $B$ (O sera) antibodies$^3$. Yamamoto reported on his molecular genetic analysis of three $B_3$ cases consisting of one case of $A,B_3$ as the $B^\vartheta$ allele with a 1054C>T substitution near the 3'-terminal of the $B$ allele and two cases of a $B_3$ with a normal sequence around exons 6 and 7$^3$. Ogasawara indicated that $B109$ as a $B_3$ species might be generated by a gene conversion-like event between $B101$ and $O201$, and that, around the nt 646 area, a 646T>A substitution and lack of B specific polymorphism at nt 657 might indicate a hot spot for mutation$^4$. Recently 14 cases of $B_3$ were clarified$^5$. Of these, 13 had a mutation at the +5 nucleotide of intron 3 (intervening sequence 3 [IVS3] + 5 G>A mutation) and one had a 247 G>T mutation in exon 6. There have been reports of novel $B$ weak subtypes$^6$ (eight cases) with missense mutations in exon 7, of a pedigree analysis of $B$ weak subtypes$^7$ with a substitution at nt 556A>G, and of a novel $B^{\vartheta}$ (B$^{\vartheta}$) allele with a 547G>A mutation found in $A,B_3$ donors in two pedigrees$^8$. In this study, the authors explored a case of $B_3$ phenotype ($A,B_3$) and compared it with known $B_3$ variants.
Materials and Methods

Preparation of DNA specimens and PCR amplification

Blood specimens of the weak subtypes such as A,B; and A,B; classified by their serological testing (data not shown) were offered by the kindness of Hyogo Red Cross Blood Center. Common blood samples were collected from healthy volunteers (A; 22, AB; 6, O; 5 and B; 5 individuals, respectively) after obtaining their consent. Common ABO blood typing was based on a method in Japanese Association of Medical Technologists (JAMT) Library XII with a test tube. The following reagents were used: anti-A and anti-B murine monoclonal antibody (Ortho Clinical Diagnostic, Japan), and anti A; lectin from Dolichos biflorus (Ortho Clinical Diagnostic, Japan). A, B and O red cells were prepared from healthy ABO blood cells after checking A, B and O antigens for reverse testing.

The genomic DNAs were prepared using a DNA extraction kit (Dona Quick II, Dainihon Co. Ltd, Japan). 1 μl of DNA (100 ng) was added to 10 μl of PCR buffer containing 0.2 mM of dNTPs, 0.2 μM of each primer and 0.2 U of AmpliTaq Gold (Applied Biosystems, USA). The PCR steps were an initial heating at 95 °C for 10 min, followed by 30 cycles of 94°C for 30 sec, 62 °C for 30 sec and 72 °C for 90 sec, and a final extension at 72 °C for 5 min. All of the primers for the PCR, PCR-SSCP, PCR-RFLP and the Amplification Refractory Mutation System (ARMS) test are listed in Table 1.

Genotyping by the SSCP analysis

We have previously reported on genotyping using SSCP analysis10. PCR products were diluted 9 - 20 times with a denature-reagent, 95 % formamide-25 mM EDTA-0.05 % xylene cyanol pH 8.0, heated at 98°C for 10 min and then chilled on ice water immediately. Each

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<th>Primer names</th>
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<th>Sequence (5' &gt; 3')</th>
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<td>SSCP(exon 6), ARMS test 4</td>
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<td>1st PCR for exon 7, sequencing, ARMS test</td>
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<td>772-792</td>
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<td>547A</td>
<td>s</td>
<td>cgecactacaagcagtggcacagA</td>
<td>528-547</td>
<td>ARMS test for B&lt;sup&gt;B&lt;/sup&gt; , ASP-PCR-RFLP(&lt;i&gt;Alu&lt;/i&gt; I)</td>
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<tr>
<td>547G</td>
<td>s</td>
<td>gcctacaagcagctggcagG</td>
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treated aliquot was applied to a polyacrylamide gel (PAG) mini-slab without glycerol, 120 (W) × 90 (H) × 0.75 (D) mm, and electrophoresed with a 0.5 × TBE buffer. The buffer temperature was maintained at 15 - 25 °C with a heat exchanger (Tempcon, AE-6370, Atto Co., Japan) equipped with an external thermostatically controlled circulator (Superstat MINI, AB-1600, Atto Co., Japan). After electrophoresis, SSCP-patterns were visualized by silver staining. SSCP analysis of the IVS3+5G>A mutation was performed with the IVS2 and IVS3 as primers for the PCR, and Bsm I was applied in the same manner as Alu I for RFLP analysis (Bsm I and Alu I were purchased from Nippon Gene Co., Ltd., Japan).

**Sequence analysis**

Sequence analysis was developed by the dye-termination method with the Sequencer AB-310 (Applied Biosystems, USA). Ex710 and ex714 as the sequencing primers were applied to the forward major part and 3'-terminal part, respectively.

**Results**

**Direct sequencing analysis of exon 7 in the A.B3 individual**

Compared with ordinal AB sequence, this A.B3 had two points of single nucleotide polymorphisms (SNPs). The first SNP, C>T, could be detected at nt 467 (Fig. 1-A). This was assumed to be derived from the A1v allele. The second SNP was detected at nt 547 with a dominant A and a little of G (Fig. 1-B). Hence in the common AB type G at this site was considered as a single sharp spike. The A.B3 individual had two SNPs at nt 467 and 547 in exon 7 of the ordinal A and B alleles.

**SSCP analyses of exons 6 and 7**

Exon 6 exhibited differences at nt 261 and 297 among the common A, B, O' and O'' alleles. The corresponding nucleotides were
Fig. 1 Partial sequencing data of exon 7 from the A*B3 individual. The nucleotide sequences between nt 385 and 1079 of the ABO gene are shown. (A) near nt 467, (B) near nt 547.

Fig. 2 Mini-slab gel PCR-SSCP patterns for the PCR products of exon 6 in the ABO genes. Genomic DNA fragments of exon 6 were amplified, as described in Materials and Methods. The PCR-SSCP was performed with an 8 % PAG mini-slab and 0.5 × TBE buffer at 7 W, 15°C for 1 hr. (A) lanes 1-9; AA, AO', AO''', O''O', O'O', O''O', AB, BB and BO' alleles, respectively. (B) lanes 1-3; rare subtypes, A*B, A*B and AB.

261G and 297A for the A allele, 261G and 297G for the B allele, 261G-del and 297A for the O' allele and 261G-del and 297G for the O'' allele. Thus, 10 patterns AA, AO', AO''', O''O', O'O', O''O', AB, BB, BO' and BO''', arose in these common alleles. Nine SSCP patterns are shown in Fig. 2-A. Specifically, the A, B, O' and O'' homozygous alleles had their own electrophoretic patterns. Their combinations were observed as heterozygous alleles. The SSCP patterns of the variant blood groups A*B and A*B proved to be similar to the ordinary AB blood groups, respectively, as shown in Fig. 2-B.

In exon 7, SSCP analysis was carried out in two parts of the PCR product, a 5'-terminal area, 272 bp (nt 430-701) and 257 bp (nt 445-701), and a 3'-terminal area, 233 bp (nt 847-1079). The A' allele and the O' allele had the same sequence in the 5'-terminal area (nt 430-701). Compared with the A' allele, the B allele had two points of substitutions at nt 526C>G and 657C>T, and O' allele had two points of substitutions at nt 646T>A and 681G>A, respectively, in this area. Significant bands of 272 bp in SSCP analysis migrated into two groups (Fig. 3-A). In the first group, the migrated bands of the A' and O' alleles were distinguishable from those of the B allele, but not from those of the A'' allele under this condition. The pattern of the A*B genome obviously differed from those of the common A'I, B, A''O' and O'O' alleles. Another SSCP analysis of a 5'-terminal area PCR product (257 bp) was performed with a 9 % PAG mini-slab at 5 W, 140 min, 25°C. The A allele of A*B individual was confirmed to be the same as the control A*I allele but distinct from the A'I(O') allele (Fig. 3B). The migration pattern of the B allele (Fig. 3B lane 2, duplet arrows) was a little different from that of the ordinal B allele (Fig. 3B lane 3, arrows). Thus, this A*B individual was determined as the heterozygote of an A'' allele and a variant of B' allele. For SSCP analysis of the 3'-terminal area, another nested PCR between nt 847 and 1079 (233 bp) was performed. Among the ordinal A', B, O' and O'' alleles, only the B allele had a substitution at nt 930G<A. The SSCP pattern of A*B (Fig. 3-C lane 1) with an 11 % PAG mini-slab at 7 W, 2 hrs, 25°C was identical to that of the ordinal AB blood group (Fig. 3-C lane 4), but differed from that of the A'B blood group with A' allele as 1054C>T (Fig. 3-C lane 2).
Fig. 3 The close-up mini-slab gel PCR-SSCP patterns for exon 7 in the ABO gene.

Genomic DNA fragments of exon 7 were amplified as described in Materials and Methods. Electrophoresis for PCR-SSCP was performed on 9 and 11% PAG mini-slabs and with 0.5 × TBE buffer at 5-7 W, 25 °C for 2-2.5 hrs. (A) 11% PAG at 7 W, 25 °C for 2.5 hrs. Lane 2; product (nt 430-701) from A1B3, as a subtype. Arrow are used to distinguish the variant from other types. Lanes 1 and 3-5; products from four common A1B, BB, A1vA and Ov alleles as the control. (B) 9% PAG at 5 W, 25 °C for 140 min. Lane 2; product (nt 445-701) from A1vB as a subtype. Lanes 1 and 3-8; products from common A1vB, BB, A1vA, Ov, OvO, A1vO, OvO alleles. Arrows and doublet arrows showed the B and the E (547G>T) alleles, respectively. (C) 11% PAG at 7 W, 25 °C for 2 hrs. Lanes 1-6; products (nt 847-1079; 3’-terminus) from the A1vE, A2B, A1vA, A1B, A1vA and BB alleles. The arrow showed the variant.

Simple confirmation analysis of nucleotide substitutions of G>A at nt 547 and C>T at nt 467 by the ARMS test

The products of two paired V- and W-characteristic bands (174/173 bp with 547A/G primers and 256/255 bp with 467T/C primers, respectively) and a control band (317 bp) from the A1B3 individual are shown in Fig. 4. However, 174 bp product was not observed in the left lane with 547A of the AB individual (Fig. 4-Test 2-V). The results of the ARMS tests confirmed the existence of the A1v allele and the B' allele in this A1B3 genome (Fig. 4-Test 1).

Localization of an nt 547G>A alteration in the A1B3 genome

ASP-PCR 264 bp products with 547G and ex713 between nt 529 and 792 in the AB individuals could be digested into 174 and 90 bp fragments by Alu I, but fragmentation from the A1B3 individual could not be observed (Fig. 5, Analysis-1). Since the targeting site of Alu I in the B allele was at nt 703G, 547G should not exist on the B allele in the A1B3 individual. Another ASP-PCR-RFLP analysis with 547A and ex713 showed obvious fragmentation by Alu I in the A1B3 individual, but not in the AB individual (Fig. 5, Analysis-2). In this A1B3 genome, the guanine nucleotide was proven to be present at nt 547 in the A1v allele and the adenine nucleotide was found at nt 547 in the B' allele.

Other genetic information about the A1B3 individual

There have been several reports regarding genomic analyses of the B' allele; the substitution of nt1054 C>T from an A1B3 individual, a gene conversion-like event by the B and Ov alleles, two-point substitutions at 646T>A and 657C in the B109 (B') allele and IVS3+5G>A or 247T>G mutations. We checked whether these variable points were present in our A1B3 individual. The PCR-SSCP patterns of the PCR products from nt 847 to 1079 are shown in Fig. 3-C. The pattern of A1B3 was similar to that of AB but clearly differed from that of A1B with a 1054C>T change in the A1v allele. This fact was confirmed by direct-sequencing and the ARMS test with 1054T/C as the variant/wild-ASP. Clearly, the nt 1054 of this A1B3 individual was not T but C as in ordinary A and B alleles. Direct sequencing proved there were no abnormalities at nt 646 and 657. In addition, it was confirmed that there was no
IVS3+5G>A mutation in this A,B3 individual by the ARMS test (data not shown) with IVS3+5A or IVS3+5G as the specific primer and by RFLP analysis with Bsm I (data not shown). Regarding the 247T>G substitution checked by the ARMS test (data not shown) and the SSCP analysis of exon 6 (Fig. 2), it was shown to be a common B sequence at this point in these A,B3 individual.

Discussion

A blood group system usually consists of one or more antigens. In addition, weak B subgroups, such as B\textsubscript{3}, B\textsubscript{x}, B\textsubscript{m} and Bel, are very rare and are classified into subgroups as A variants. Generally, the \textit{B\textsuperscript{v}} allele is rare and may be heterogeneous. In fact, the major substitution site of the \textit{B\textsuperscript{v}} allele in the A,B\textsubscript{3} individual reported here was not nt 1054, nt 646 nt 657, nt 247 or IVS3+5, but nt 547. This 547A>G variant found in a Japanese individual is the second report of the \textit{B\textsuperscript{wv}} allele that was originally described by Cho et al.\textsuperscript{13}. The present study showed that a variation site could be pinpointed without the complicated cloning process. For primary genetic sorting of the \textit{ABO} alleles and their subtype alleles, the PCR and electrophoresis techniques were applied as in other genetic investigations. SSCP analysis was effective in recognizing the SNPs under selective conditions, such as gel concentration, temperature, the length of PCR products and electrophoresis time for

![Fig. 4 ARMS test for \textit{A\textsubscript{iv}} and \textit{B\textsuperscript{v}} alleles of 467C>T or 547G>A substitution.](image)

The PCR products of 256/255 bp with 467T/C (2.0 μM) and 174/173 bp with 547A/G (0.1 μM) as the V- and W-ASPs, and 317 bp with a pair of consensus primers (ex710, 0.2 μM and ex712, 0.2 μM) as the PCR control were separated on 2 % agarose gel, as described in Materials and Methods. The paired ARMS test was composed of the products in the left lanes derived from 467C>T or 547G>A mutation of the \textit{A\textsuperscript{iv}} or \textit{B\textsuperscript{v}} allele (arrow). The right lanes were derived from 467C or 547G of the common \textit{A\textsuperscript{v}}, \textit{B} and \textit{A\textsuperscript{w}} alleles. Test 1: A,B\textsubscript{3}, Test 2 : AB individuals, respectively. M4: molecular size marker.

![Fig. 5 ASP-PCR-RFLP patterns with \textit{Alu} I.](image)

The ASP-PCR amplification products for \textit{A\textsuperscript{v}}, \textit{A\textsuperscript{w}}, \textit{B} and \textit{B\textsuperscript{v}} alleles were obtained with 547G or 547A for a sense primer and ex713 for an anti-sense primer. After cleavage by \textit{Alu} I, the lysates were simultaneously separated by 2 % agarose gel electrophoresis. Analysis-1 with 547G, and Analysis-2 with 547A. M4: molecular size marker.
suitable conditions. As a result, the fine mini-slab gel SSCP-pattern was obtained. By SSCP analyses of the exon 6 and the 3'-terminal area between nt 847-1079 in exon 7, it was confirmed that the genome in this AIB individual consisted of an A\textsuperscript{iv} allele and a B allele (Fig. 2 and 3-C lane 4). And the corresponded allele to an A\textsuperscript{v} allele was neither a cisAB allele nor an O\textsuperscript{I} allele.

The B\textsuperscript{I} allele with nt 547G >A mutation was detected on a 9 % PAG mini-slab and distinguished from other A\textsuperscript{(O)}, A\textsuperscript{iv} and B alleles. If the presence of A\textsuperscript{iv} in a genome could be known before the SSCP analysis, the B\textsuperscript{I} allele could be found as variant allele. A variant could also be found with the 11 % PAG mini-slab electrophoresis, but under this condition, it was not clear enough to confirm the variant as the B\textsuperscript{I} allele, nor enough to distinguish them into A\textsuperscript{(O)} and A\textsuperscript{iv} alleles. With the ARMS test or ASP-PCR-RFLP analysis, the presence of known variants in a PCR product could also be verified by simple procedures. All the procedures used in this study were based on simple electrophoresis with PAG mini-slab gel or agarose gel, after the selection of primers for the PCR and a restriction enzyme for cleaving.

The clusters predominantly between nt 536-548, 641-721, 829-873 and 965-1060 are related to the active sites of the A and B glycosyltransferases\textsuperscript{12,16}. The amino acid substitutions in these regions can affect protein conformation. The surrounding region between aa 180 and 186, RWQDVSM, is a notable cluster which is highly preserved for its enzyme activity and has been suggested to be involved in the binding site for nucleotide sugars to the enzyme\textsuperscript{10} and in product release\textsuperscript{15}.

Among the other B weak specimens, two SNPs have been observed as nt 539G>A (Arg 180 His) and nt 548A>G (Asp 183 Gly) in the above cluster\textsuperscript{19}. In addition, a 556A>G variant observed in the B\textsuperscript{v} allele has been reported to cause a change in a Met by a Val at position 186\textsuperscript{12}. Owing to this amino acid change, it was concluded that its reactivity deriving from the B\textsuperscript{v} allele was poor. The exact nt 547 position in the B\textsuperscript{I} allele found here localized in this RWQDVSM cluster and corresponded to the substitution of G to A. Therefore, an amino acid change at 183 of an asparagine from an aspartic acid could be predicted. This change did not seem as drastic as the change of a valine from a methionine in the B\textsuperscript{v} allele.

Another substitution of the nt 467C>T substitution was observed in the A\textsuperscript{iv} (A102) allele. The ARMS test showed the allelic frequencies of A\textsuperscript{i} and A\textsuperscript{iv} to be 4 (11.8 %) and 30 (88.2 %), respectively, in A and AB blood types from healthy volunteers (29 individuals; AA :5, AO :18, AB :6, total 34 A alleles, unpublished data). Indeed, the A\textsuperscript{iv} allele is dominant in Japan\textsuperscript{19,20} and China\textsuperscript{21}. The AIB\textsubscript{3} individual in this study also had a 467C>T mutation (Fig. 4) and was found to be a heterozygote with the A\textsuperscript{iv} allele with 467C>T mutation and B\textsuperscript{I} allele with 547G>A mutation genetically.

**Acknowledgements**

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