Allele-Specific, Inverse-PCR Amplification for Genotyping MN Blood Group

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The MN blood group system, usually typed serologically, is based on polymorphic red cell antigens. Glycophorin A (GPA), a sialoglycoprotein on the erythrocyte membrane, carries M and N antigens, based on two changes of the amino acid sequence at positions 1 and 5 (M, serine and glycine; N, leucine and glutamic acid). M and N alleles of the *GPA* gene are attributed to three nucleotide substitutions in exon 2 (Figure 1) (10,18) and are determined by restriction fragment-length polymorphism (RFLP) analysis (1,6,12), single-strand conformation polymorphism (SSCP) analysis of the polymerase chain reaction (PCR) products (1) or allele-specific PCR amplification (ASPA) (4,8,13). MN genotyping by DNA analysis is informative for forensic identification and in several clinical situations where serological phenotyping is difficult or impossible (8), such as (i) in a patient transfused with large amounts of blood from various donors, (ii) a fetus at risk for hemolytic disease and (iii) a patient with autoimmune hemolytic anemia. A G/T substitution in intron 1 of the *GPA* gene between M and N alleles (10) was found among conventional (serological) M alleles, being divided into M^G and M^T alleles (Figure 1) (1). All N alleles have the nucleotide T in that position. This G/T substitution may not affect the antigenicity. The frequencies of M^G and M^T alleles in the Japanese population were 0.547 and 0.040. The heterozygosity, the polymorphism information content and the probability of paternity exclusion of this improved MN system were 0.529, 0.425 and 0.232, respectively; whereas, those of the conventional MN system were 0.485, 0.367 and 0.184, respectively (1). Therefore, subtyping of M alleles may be informative for forensic and clinical purposes. M^G, M^T and N alleles can be typed by RFLP or SSCP analysis but not by the single ASPA technique. Because M^T

and N-specific sequences are also observed in glycophorin E (*GPE*) and glycophorin B (*GPB*) genes, respectively (Figure 1), which are more than 95% homologous in exons and introns with the *GPA* gene, one primer for ASPA should be GPA-specific to avoid co-amplification of GPB and GPE segments. Although primers specific to M^G and N alleles can be designed, the M^T- (and N-) specific nucleotide T in intron 1 is located too far (30–43 bp upstream) from the three M- (M^G and M^T) specific bases in exon 2 to design a single M^T-specific primer. Therefore, RFLP or SSCP analysis was performed to differentiate M^T from M^G and N alleles (1). Here, a novel method for genotyping the MN blood group was performed using allele-specific, inverse-PCR (ASIP) following GPA-specific PCR amplification.

GPA-Specific PCR

The GPA fragment (357 bp) was amplified using two GPA-specific primers: MN-FF (5'-GAG GGA ATT TGT CTT TTG CA-3') and MN-CR (5'-AGA GGC AAG AAT TCC TCC-3') (Reference 10; Figure 1a). The PCR mixture (25 μ L) consisted of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 μ M of each primer, 0.01 U/ μ L of AmpliTaq Gold™ DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) and 15 ng of template DNA. PCR consisted of preheating at 95°C for 9 min; 40 cycles of 95°C for 40 s, 60°C for 40 s and 72°C for 40 s; followed by a post-incubation step at 72°C for 2 min.

Ligation of the Amplified Fragment

The PCR products (0.5 μ L each) were added to 100 μ L of ligation mixture consisting of 66 mM Tris-HCl, pH 7.6, 6.6 mM MgCl₂, 10 mM dithiothreitol, 66 μ M ATP and 0.02 U/ μ L of T4 DNA Ligase (Toyobo, Osaka, Japan), followed by incubation at 16°C overnight.

Allele-Specific PCR

The ligation mixture (0.5 μ L) was added into the PCR mixture (25 μ L) containing 0.01 U/ μ L of *Taq* DNA

Polymerase (Boehringer Mannheim, Mannheim, Germany) and one of allele-specific primer sets: MN-MF (5'-GCA TCA AGT ACC ACT GGT-3') and MN-GR (5'-GCT CAC AAT TGC TGT ATA AC-3') for amplification of 351-bp M^G, or MN-MF, MN-NF (5'-att gtg agc ATA TCA GCA TT-3') and MN-TR (5'-gct cac aat TGC TGT ATA AA-3') for 351-bp M^T and 366-bp N sequences, respectively (Figure 1b). The annealing sites of MN-NF and MN-TR complementarily overlapped for 9 bp (shown by small letters in primer sequences). PCR consisted of preheating at 95°C for 2 min; 30 cycles of 94°C for 40 s, 63°C for 40 s and 72°C for 40 s; followed by a post-incubation step at 72°C for 2 min. The PCR products were electrophoresed on a 6% native polyacrylamide gel, followed by staining with ethidium bromide.

Genotypes M^GM^G, M^GM^T, M^GN, M^TN and NN typed by ASIP (Figure 2) completely corresponded to those typed by the PCR-RFLP method (1). This result suggested that intermolecular ligation producing wrong alleles (for example, M^T-specific, 351-bp fragment amplified following intermolecular ligation between 3' end of M^G and 5' end of N fragments) did not occur, but that intramolecular ligation of each allele occurred under the experimental conditions described above. The annealing sites of the primers MN-NF and MN-TR overlapped for 9 bp, which meant that the annealing site for the reverse primer in the PCR product from the 357-bp circular template annealed with the forward primer was 9 bp shorter than the reverse primer. However, fragments of the expected length (366 bp) were amplified from N alleles. This was likely because the first PCR products contained little nontarget sequences. Consequently, the reverse primer annealed to the 9-bp shorter annealing site of the 357-bp target sequence, resulting in the duplication of the 366-bp fragment. Therefore, the fragment acted as a complete template.

The second inverse-PCR using a short circular template was performed with the *Taq* DNA polymerase (Boehringer Mannheim), which is a mutant *Taq* polymerase lacking 5' to 3' exonuclease activity (16) [as does the Stoffel fragment (11)] to avoid cleaving

Benchmarks

the annealed primer and its extension after duplication of the whole sequence. The polymerase lacking 5' to 3' exonuclease activity was suggested to permit efficient amplification of long fragments (7).

For ASIP analysis, intramolecular ligation is absolutely required. If intermolecular ligation between different alleles occurred dominantly, the wrong alleles or haplotypes would be detected, and polymorphism analysis would end in failure. Linear or circular products of intermolecular or intramolecular ligation, respectively, are predicted according to the DNA concentration (5); intramolecular ligation will occur dominantly when the $[51.1/(\text{concentration})]^2/(\text{molecular weight})$ value is >2 . For intramolecular ligation of a 357-bp fragment, the ligation mixture should include $<6.8 \mu\text{g}$ per 100 μL of the frag-

ment. PCR usually generates a product $<1 \mu\text{g}$ per 5 μL reaction volume, because the amount of product reaches a plateau during multiple cycles of amplification (9). Therefore, intramolecular ligation was expected by adding 0.5 μL of the first PCR product to 100 μL of the ligation mixture, and the results shown in Figure 2 suggest that predominantly intramolecular ligation occurred under these experimental conditions.

However, when one-tenth of the amount (equivalent to 0.05 μL) of the first PCR product was added to 100 μL of the ligation mixture, nonspecific fragments were detected by ASIP, which interfered with the genotyping (data not shown). *Taq* DNA polymerase is known to catalyze non-templated addition of a nucleotide (principally adenosine) to 15%–90% of the 3' ends of PCR products, according to the se-

quence on the 5' end of reverse primers (2,3,15). Adenylation of blunt ends of PCR products resulting in noncomplementary 3'-stalk termini inhibits intramolecular ligation. This indicates that only a portion of the first PCR products acted as templates for the second PCR, and the addition of 0.05 μL of the first PCR products into the ligation mixture likely resulted in a lack of templates for the second PCR, inducing nonspecific amplification. In this study, the first PCR was performed using AmpliTaq Gold, so that the amount of product would reach a plateau. Because this enzyme is activated by heat, nonspecific amplification occurring at low temperatures can be suppressed, and more cycles can be added to the typical PCR (consisting of 25–30 cycles) to increase the yield of specific products (according to the manufacturer's instruction). The efficiency of ligation may be increased following restriction digestion of both ends of the first PCR products to generate complementary termini. For this purpose, the restriction sites can be inserted into the 5' end of primers for the first PCR. However, this ASIP method did not include the restriction step to simplify the procedure.

Inverse PCR, or inside-out PCR, was explored to analyze unknown sequences that flank a region of known sequence (14,17). This technique, which involves ligation of separated regions at the ends of a sequence (a restriction fragment or a PCR product), can also be applied to closely linked polymorphisms. Haplotypes consisting of heterozygous polymorphisms have been determined by pedigree analysis. However, using inverse PCR, the linked

Figure 1. Strategy for ASIP analysis of MN blood group. (a) The 357-bp sequences including nucleotide substitutions specific to M^G , M^T and N alleles are amplified using GPA gene-specific primers MN-FF and MN-CR. (b) Following intramolecular ligation of the amplified fragments, PCR amplification is carried out using an allele-specific primer set, MN-MF and MN-GR for M^G , or MN-MF, MN-NF and MN-TR for M^T and N alleles. The annealing sites of 5' ends of MN-NF and MN-TR overlapped complementarily for 9 bp, being predicted to produce 366-bp N fragment from 357-bp circular template.

Figure 2. ASIP analysis of MN genotypes $M^G M^G$, $M^G M^T$, $M^G N$, $M^T N$ and NN. Lanes marked G show amplification of 351-bp M^G allele using primers MN-MF and MN-GR. T lanes show amplification of 351-bp M^T and/or 366-bp N allele(s) using primers MN-MF, MN-NF and MN-TR.

Benchmarks

polymorphisms can be analyzed by a single procedure using allele-specific primers. In this study, M^G, M^T and N alleles were regarded as haplotype alleles and analyzed by ASIP. Although these alleles can be typed by allele-specific nested PCR following GPA-specific PCR, ASIP, rather than nested PCR, can be applied to haplotyping of polymorphisms separated by a distance that is too long to be amplified by PCR.

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