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High-resolution melting analysis for genotyping Duffy, Kidd and Diego blood group antigens

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ABSTRACT

High-resolution melting (HRM) analysis is a simpler genotyping method than allele-specific PCR, PCR-restriction fragment length polymorphism and multiplex PCR. Duffy, Kidd and Diego are clinically important blood group antigens. We used a novel method to genotype these three blood group antigens.

Purified genomic DNA extracts of blood samples (354 Duffy, 347 Kidd and 457 Diego) were amplified using specific amplification primers. HRM curves were obtained by HRM analysis.

Results were in complete concordance with those obtained for previous phenotypes and genotypes. Nucleotide substitutions for these blood group antigens were differentiated by the HRM curves. HRM analysis is a simple genotyping method and is an alternative to serological typing.

Our results suggest that HRM analysis can also be used for genotyping blood group antigens whose allotype specificity is determined by single nucleotide substitutions.

1. Introduction

Traditionally, haemagglutination-based methods have been used as standard tests for antibody identification and blood group typing of patients and donors. These methods are simple and quick and have been used as the gold standard for blood group typing. However, they have a critical limitation in that they require specific antibodies such as human polyclonal antibodies or murine/human monoclonal antibodies. These antibodies have limited availability and are extremely expensive. In addition, some antibodies react weakly, making it difficult to type patients or donors by haemagglutination-based methods.

Recently, DNA-based assays were used for blood group typing [1–3]. Genes of most blood group systems have been cloned and sequenced, and molecular bases of blood group antigens have been defined, including the clinically important blood group antigens Duffy, Kidd and Diego [4–11].

The Duffy antigen is the erythrocyte chemokine receptor (DARC), encoded by the *DARC* gene, and has Fy^a and Fy^b polymorphisms. A single G to A substitution at nucleotide position 125 results in a glycine to aspartic acid substitution at amino acid position 42 [12–15]. The Fy(a−b−) phenotype is common in African blacks but rare in Caucasians. This phenotype is associated with a T to C substitution at nucleotide position −33. This mutation impairs the promoter activity in erythroid cells by disrupting a binding site for the GATA-1 erythroid transcription factor [16]. The Kidd antigen is the urea transporter (SLC14A1), encoded by the *SLC14A1* gene [17–19], and has Jk^a and Jk^b polymorphisms. A single G to A substitution at nucleotide position 838 results in an aspartic acid to asparagine substitution at amino acid position 280 [20]. The Diego antigen is the human erythrocyte membrane anion-transport protein (SLC4A1), encoded by the *SLC4A1* gene [21–23], and has Di^a and Di^b polymorphisms. A single T to C substitution at nucleotide position 2561 results in a proline to leucine substitution at amino acid position 854 [24]. Thus, the phenotypes of these blood group antigens result from a single nucleotide change in the gene.

PCR amplification is a fundamental DNA-based assay for analysis of nucleotide substitutions. PCR amplification using sequence-specific primers can be employed to predict the presence or absence of blood group antigens on red blood cells. The amplified DNA sequences of blood group antigens can be analysed by different methods such as allele-specific PCR (PCR-SSP), PCR-restriction fragment length polymorphism (PCR-RFLP) and multiplex PCR. These DNA-based assays have been used for genotyping several blood group antigens, including Duffy (*FYA* and *FYB*) [25–28], Kidd (*JKA* and *JKB*) [29] and Diego (*DI1* and *DI2*) [30].

In the field of DNA-based genotyping, high-resolution melting (HRM) analysis has been developed as a novel method for detecting a single nucleotide change in a gene [31]. Before performing HRM analysis, the target sequence is amplified in the presence of a double-stranded DNA-binding fluorescent dye, and the melting temperature (T_m) is increased from a lower to a higher temperature

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for HRM analysis. The differences in gene sequences between heterozygous and homozygous genotypes lead to differences in T_m. Heterozygous genotypes tend to have lower T_m than homozygous genotypes. The DNA-binding fluorescent dye is released during melting of double-stranded DNA and is measured continuously as the temperature is increased. HRM curves are used for differentiating between heterozygous and homozygous genotypes. HRM analysis is an innovative DNA melting analysis-based technique for genotyping [32].

In this study, we used HRM analysis for genotyping Duffy, Kidd and Diego blood group antigens.

2. Materials and methods

2.1. Blood samples and DNA preparation

Normal blood samples were obtained from blood donors in Japan after obtaining informed consent. Fy(a−b−) samples were obtained from the Serum, Cells, and Rare Fluids (SCARF) exchange group. DNA was prepared using the BioRobot MDx workstation and QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

2.2. Primer selection for PCR amplification

FYA and *FYB* alleles (G to A substitution at nucleotide position 125) of the *DARC* gene were amplified using the following primers: 5′-CTGAGAACTCAAGTCAGCTG-3′ and 3′-AGGATGAAGAAGGGCAGTGC-5′. *FYB-33* allele (T to C substitution at nucleotide position −33 in the promoter region) of the *DARC* gene was amplified using the following primers: 5′-CGTGGGGTAAGGCTTCCTGA-3′ and 3′-CTGTGCAGACAGTTCCCCAT-5′ (Fig. 1A). *JKA* and *JKB* alleles (G to A substitution at nucleotide position 838 in exon 9) of the *SLC14A1* gene were amplified using the following primers: 5′-GACTCAGTCTTTCAGCCCCAT-3′ and 3′-GAGGTGGGTTTGCCAGGTGA-5′ (Fig. 1B).

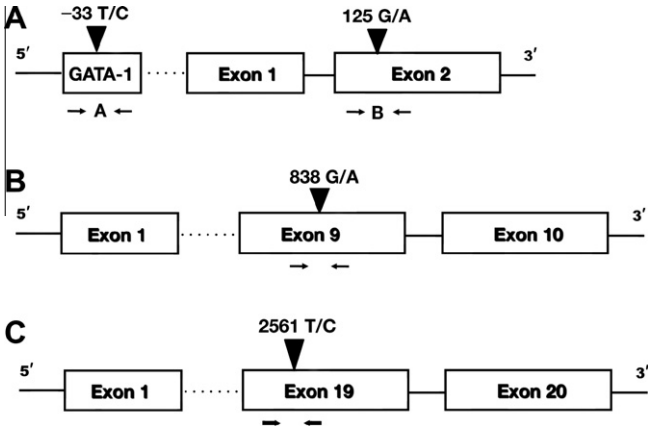

Fig. 1. Primers for performing PCR of *DARC*, *SLC14A1* and *SLC4A1* genes. (A) Duffy blood group antigens (Fy^a and Fy^b) are encoded by the *DARC* gene. The *DARC* gene consists of two exons and a promoter region, and has two alleles. Fy^a and Fy^b, which differ by a single base (125 G to A) in exon 2 of the *DARC* gene. Two primers (arrows B: 5′-CTGAGAACTCAAGTCAGCTG-3′ and 3′-AGGATGAAGAAGGGCAGTGC-5′) were used for PCR amplification. Fy(a−b−) phenotype, containing a T to C substitution at nucleotide position −33 in the GATA-1, was amplified using the two primers (arrows A: 5′-CGTGGGGTAAGGCTTCCTGA-3′ and 3′-CTGTGCAGACAGTTCCCCAT-5′). (B) Kidd blood group antigens (Jk^a and Jk^b) are encoded by the *SLC14A1* gene. The *SLC14A1* gene consists of 10 exons and has two alleles, Jk^a and Jk^b, which differ by a single base (838 G to A) in exon 9 of the *SLC14A1* gene. Two primers (arrows: 5′-GACTCAGTCTTTCAGCCCCAT-3′ and 3′-GAGGTGGGTTTGCCAGGTGA-5′) were used for PCR amplification. (C) Diego blood group antigens (Di^a and Di^b) are encoded by the *SLC4A1* gene. The *SLC4A1* gene consists of 20 exons and has two alleles, Di^a and Di^b, which differ by a single base (2561 T to C) in exon 19 of the *SLC4A1* gene. Two primers (arrows: 5′-CTGGCGCATGCACTTATTC-3′ and 3′-CCTGAAGATGAGCGGCAG-5′) were used for PCR amplification.

DI1 and *DI2* alleles (T to C substitution at nucleotide position 2561 in exon 19) of the *SLC4A1* gene were amplified using the following primers: 5′-CTGGCGCATGCACTTATTC-3′ and 3′-CCTGAAGATGAGCGGCAG-5′ (Fig. 1C). These oligonucleotide primers were synthesised using standard phosphoramidite chemistry (Life Technologies, Carlsbad, CA).

2.3. PCR amplification for HRM analysis

A PCR mixture containing 10 ng template DNA and 0.7 μM forward and reverse oligonucleotide primers (final reaction volume of 25 μL) was prepared using a Type-it HRM Kit (Qiagen, Hilden, Germany). Rotor-Gene Q (Qiagen, Hilden, Germany), which has a unique centrifugal rotary design that makes it the most precise and versatile real-time PCR cycler currently available, was used for this study. A 36-well rotor and 0.2 mL PCR tubes were used for this assay. PCR amplification was performed with initial denaturing at 95 °C for 5 min followed by 45 cycles at 95 °C for 10 s, at 52 °C for 30 s and at 72 °C for 10 s, with data acquired during the 72 °C step.

2.4. HRM analysis

For HRM analysis, amplified samples bound to the fluorescence dye were heated from 65 to 95 °C. Temperature was increased by 0.1 °C at each step using Rotor-Gene Q, covering the full range of the expected melting points. HRM data were analysed using Rotor-Gene Q software. Fluorescence intensity values were normalised between 0% and 100% by defining linear baselines before and after the melting transition of each sample. The fluorescence of each acquisition was obtained by HRM curves and was calculated as the percentage of fluorescence at the top and bottom baseline of each acquisition temperature, the confidence threshold being 80%.

3. Results

3.1. HRM analysis for genotyping FYA and FYB alleles

Sixteen Fy(a+b+), 14 Fy(a−b+), 6 Fy(a+b−) and 2 Fy(a−b−) samples were amplified and analysed using Rotor-Gene Q. *FYA* and *FYB* alleles were genotyped by HRM analysis. Fig. 2a shows the representative HRM curves, indicating that the samples had either low-temperature (*FYA/FYA, FYB/FYB* and *FYA/FYB*) or high-temperature (*FYB-33T* and *FYB-33C*) melting curves. The blue lowest-temperature melting curve (A) represented the heterozygous *FYA/FYB* genotype (G/A). The green intermediate-temperature melting curve (B) represented the homozygous *FYB/FYB* genotype (A/A). The red highest-temperature melting curve (C) represented the homozygous *FYA/FYA* genotype (G/G). The brown melting curve (D) represented the homozygous *FYB-33T/FYB-33T* genotype (T/T). The purple melting curve (E) represented the homozygous *FYB-33C/FYB-33C* genotype (C/C). These curves were clearly separated. Normalised fluorescence percentages of alleles of the *DARC* gene at a T_m of 82.16 °C are shown in Table 1. Normalised fluorescence percentages of *FYA/FYA*, *FYA/FYB* and *FYB/FYB* were 69.86%, 58.01% and 64.40%, respectively. Normalised fluorescence percentages of *FYB-33T/FYB-33T* and *FYB-33C/FYB-33C* were 19.43% and 42.28%, respectively, at a T_m of 86.07 °C. In all, 354 samples were analysed by the HRM method, and the numbers of samples serologically phenotyped for Fy(a+b−), Fy(a+b+), Fy(a−b+) and Fy(a−b−) were 175, 101, 76 and 2, respectively (Table 2). All samples were genotyped by HRM analysis. The results obtained were in complete concordance with those obtained for serological phenotypes and genotypes by PCR-SSP.