High-resolution melting analysis as an alternative method for human neutrophil antigen genotyping

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Human neutrophil antigen (HNA)-typed granulocyte panels are widely used to screen for the presence of HNA antibodies and to determine antibody specificity. Many laboratories screen donors for HNA genotypes using low-throughput methods such as allele-specific polymerase chain reaction (PCR), PCR–restriction fragment–length polymorphism, and multiplex PCR. In the present study, we used a high-resolution melting (HRM) analysis to determine HNA genotypes. For the HRM analysis, purified genomic DNA samples were amplified via PCR with HNA-specific primers. Nucleotide substitutions in genes encoding HNAs were differentiated on the basis of the HRM curves, and the results of HRM and DNA sequencing analyses were determined to be in complete agreement. The gene frequency of HNA-1a, -1b, -1c, -3a, -3b, -4a, -4b, -5a, and -5b in the Japanese population was consistent with the previous reports. Our results suggest that HRM analysis can be used for genotyping HNA antigens determined by single nucleotide substitutions. *Immunohematology* 2015;31:7–13.

Key Words: neutrophil, HNA, antibody, genotyping, high-resolution melting analysis

Evidence indicates that leukocyte antibodies are one of the primary causes of nonhemolytic transfusion reactions, particularly in transfusion-related acute lung injury (TRALI).[1–4] Human leukocyte antigen (HLA) class I,[1,2,5] HLA class II,[6–9] and human neutrophil antigen (HNA)[10–15] antibodies have been associated with nonhemolytic transfusion reactions. Therefore, it is important to detect such antibodies in blood components used for transfusion.

HNAs have been classified into five systems (HNA-1, HNA-2, HNA-3, HNA-4, and HNA-5), and differences between these HNA polymorphisms can result in several alloimmunization responses. HNA-1 comprises the following antigens: HNA-1a, HNA-1b, HNA-1c, and HNA-1d; these are specifically expressed on neutrophils. Antibodies against HNA-1 are frequently thought to be the cause of alloimmune neutropenia (ANN), autoimmune neutropenia, and TRALI.[16–18] HNA-2 is represented by a single antigen and is expressed on neutrophils in Caucasian (97%), African American (95%), and Japanese (88%) populations.[19,20] Polymorphism of this antigen has not been reported. Antibodies against this antigen are associated with ANN, autoimmune neutropenia, febrile transfusion reactions, and TRALI.[12,21–23] HNA-3, comprising HNA-3a and HNA-3b, is expressed on granulocytes, lymphocytes, platelets, endothelial cells, kidney, spleen, and placental cells.[24] Alloantibodies to HNA-3a are associated with occasional cases of febrile transfusion reactions,[25] ANN,[26] and serious cases of TRALI.[14,27,28] The HNA-4 and HNA-5 antigens reside on the subunits of the β-2 integrin family (CD11a and CD11b, respectively). HNA-4 is expressed on granulocytes, monocytes, and NK cells, whereas HNA-5 is expressed on all leukocytes.[17] Moreover, alloantibodies against HNA-4a can cause ANN, but those specific for HNA-5a have not been clinically associated with neutropenia.[29]

The detection of antibodies against HNAs primarily relies on cell-based assays, the granulocyte immunofluorescence test (GIFT), and granulocyte agglutination test (GAT). The International Society of Blood Transfusion Working Party on Granulocyte Immunobiology recommends GIFT and GAT as reference methods for detecting HNA antibodies.[30] Although cells could be stored for a week following fixation for use with GIFT, they were difficult to use in high-sensitivity flow cytometry analysis because normal human sera revealed high background reactivity to neutrophils.

Genotyping via high-resolution melting (HRM) analysis can be used to rapidly predict the HNA antigen status of cells that could be used as panel cells in GIFT and GAT. In the field of genotyping, HRM analysis sensitively and specifically detects a single nucleotide change in a gene.[31] Further, HRM analysis is a simpler and more rapid genotyping method compared with allele-specific polymerase chain reaction (PCR), PCR–restriction fragment–length polymorphism, and multiplex PCR.[32] In the present study, we used HRM analysis to genotype for HNAs.

Materials and Methods

Blood Samples and DNA Preparation

Whole blood anticoagulated with ethylenediamine-tetraacetic acid was collected from healthy blood donors and used as leukocyte samples for genotyping HNAs. DNA was prepared using the QIAsymphony instrument and QIAsymphony DNA Mini kit (Qiagen, Hilden, Germany). This research project was approved by the ethics committee of the Japanese Red Cross Society Blood Service Headquarters.

Primer Selection

We performed HNA-1, HNA-3, HNA-4, and HNA-5 genotyping using a PCR-HRM method.[31] HNA-1a, HNA-1b, and HNA-1c were encoded by *FCGR3B*1, FCGR3B*2,* and *FCGR3B*3* (GenBank accession number: NC_000001.10), respectively. Because *FCGR3B* highly resembles *FCGR3A*,[18] which encodes FcγR3a, we first amplified *FCGR3B*-specific DNA to avoid the contamination of *FCGR3A* DNA before performing PCR-HRM analysis for HNA-1 genotyping; we developed a pair of primers [5-GGCACATATGGGGACAAT-3, called "FCGR3B forward" (nucleotide position in *FCGR3B*, 6616–6633), and 3-GAGCTCACTGCAACTTCTG-5, called "FCGR3B reverse" (nucleotide position in *FCGR3B*, 7604–7622)] that were designed to amplify the *FCGR3B* fragment, including the five polymorphic sites described subsequently but not the *FCGR3A* fragment. *FCGR3B*1* differs from *FCGR3B*2* at five nucleotide positions, and a single nucleotide polymorphism (SNP) differentiates *FCGR3B*2* from *FCGR3B*3* (Table 1). HNA-1a differs from HNA-1b at five nucleotide positions (141, 147, 227, 277, and 349), which results in four amino acid residue changes at positions 36, 65, 82, and 106 in the membrane-distal domain of the glycoprotein. Furthermore, HNA-1b differs from HNA-1c at nucleotide position 266 alone, resulting in the substitution of Ala to Asp at position 78 (Table 1). Recently, HNA-1d was proposed as an additional allele of the HNA-1 system.[18] Reil et al. performed epitope mapping experiments using human embryonic kidney cells that express different recombinant variants of FcγRIIIb. We designed several primer sets to amplify the polymorphic sites of HNA-1, HNA-3, HNA-4, and HNA-5. The primer sequences are shown in Table 2. These primers were synthesized using standard phosphoramidite chemistry (Life Technologies, Carlsbad, CA).

PCR Amplification of *FCGR3B* Fragment Prior to HNA-1 Genotyping

Because nucleotide sequences of *FCGR3A* and *FCGR3B* are very similar, we developed a PCR pre-amplification system for HNA-1 to avoid the amplification of the *FCGR3A* fragment prior to HNA-1 genotyping. Each PCR contained 1 µL genomic DNA, 1 µL forward primer (5 µmol/L), 1 µL reverse primer (5 µmol/L), 8.5 µL RNase-free water (Qiagen), and 12.5 µL PrimeSTAR Max DNA polymerase premix (Takara, Seta, Japan) in a final volume of 25 µL. PCR amplification was performed with initial denaturation at 95ºC for 5 minutes, followed by 35 cycles of 10 seconds at 95ºC, at 55ºC for 5 seconds, and at 72ºC for 10 seconds. Amplicons were purified using Diffinity RapidTip2 (Sigma, Deisenhofen, Germany).

PCR Amplification for Genotyping of HNA-1 to -5

This assay used the Type-it HRM Kit (Qiagen). Each PCR contained 1 µL DNA, 1 µL forward primer (5 µmol/L), 1 µL reverse primer (5 µmol/L), 8.5 µL RNase-free water (Qiagen), and 12.5 µL of a 2× HRM PCR master mix (Qiagen). The purified amplicons described earlier were used as templates for the genotyping of HNA-1, and genomic DNA was used for the genotyping of the other HNAs. The final reaction volume was 25 µL. A Roter-Gene Q (Qiagen) instrument was used. PCR amplification was performed with initial denaturation at 95ºC for 5 minutes, followed by 40 cycles at 95ºC for 10 seconds, at 52ºC for 30 seconds, and at 72ºC for 10 seconds, with data acquisition during the 72ºC step.

Table 1. Alleles of FcγIIIb with location of single nucleotide polymorphisms and resulting amino acid changes

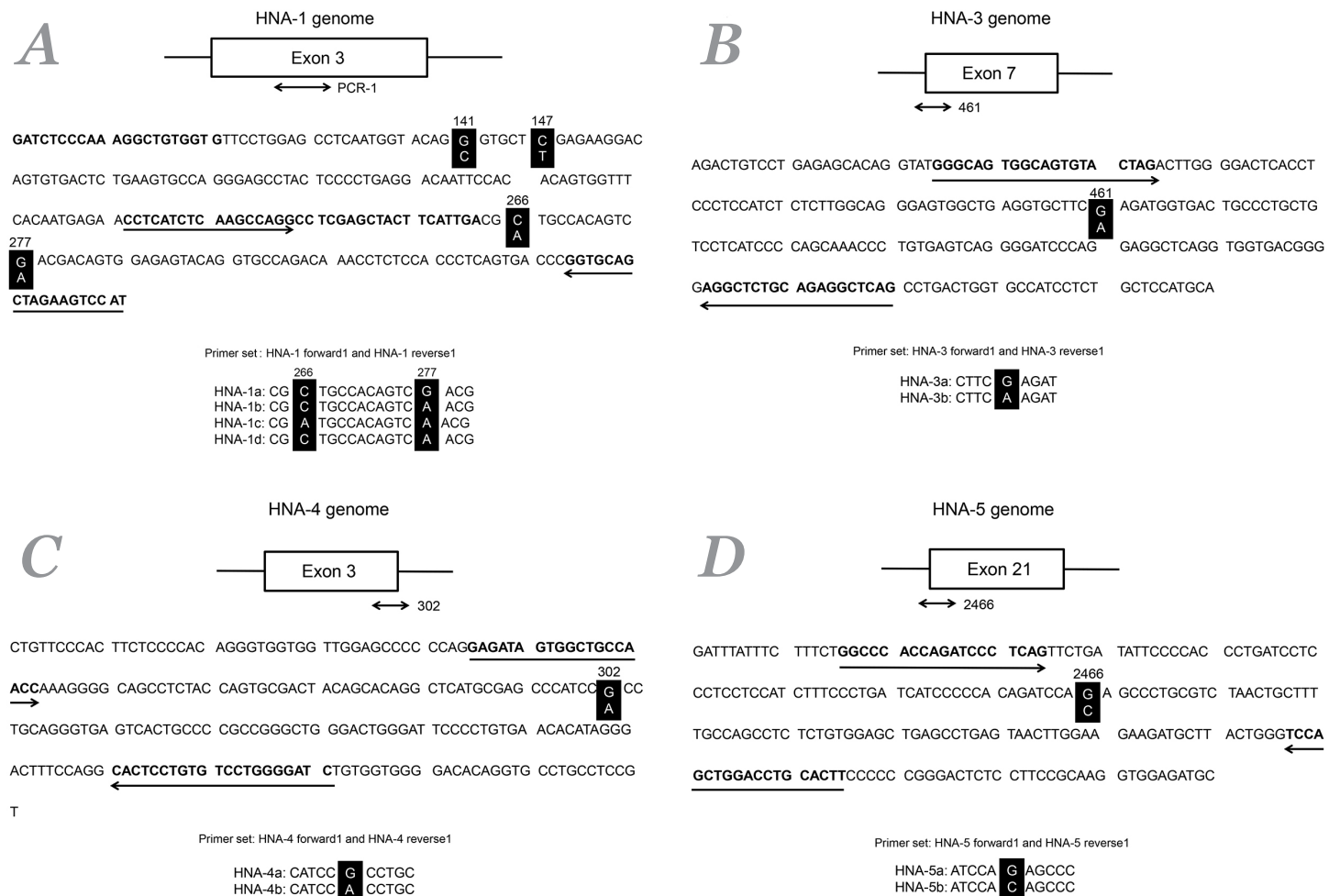| Antigen | Allele | Nucleotide position | | | | | |
		141*	147*	227*	266*	277*	349*
HNA-1a	*FCRG3B*01*	AGG (p.Arg36)	CTC (p.Leu38)	AAC (p.Asn65)	GCT (p.Ala78)	GAC (p.Asp82)	GTC (p.Val106)
HNA-1b	*FCRG3B*02*	AGC (p.Ser36)	CTT (p.Leu38)	AGC (p.Ser65)	GCT (p.Ala78)	AAC (p.Asn82)	ATC (p.Ile106)
HNA-1c	*FCRG3B*03*	AGC (p.Ser36)	CTT (p.Leu38)	AGC (p.Ser65)	GAT (p.Asp78)	AAC (p.Asn82)	ATC (p.Ile106)
HNA-1d	*FCRG3B*02*	AGC(p.Ser36)	CTC (p.Leu38)	AGC (p.Ser65)	GCT (p.Ala78)	AAC (p.Asn82)	ATC (p.Ile106)

*The underlined letters correspond to the position of the single nucleotide polymorphisms.

Note that for HNA-1d, amino acid positions and nucleotide positions were estimated according to the reactivity of the antisera against HNA-1d as reported by Reil et al.[18]

Table 2. DNA sequences of primers used in polymerase chain reaction and high-resolution melting analyses

Gene name	Primer name	Primer sequence (5′ to 3′)
	FCGR3B forward	GGCACATATGGGGACAAT
	FCGR3B reverse	GAGCTCACTGCAACTTCTG
FCGR3B (NC_000001.10)*	HNA-1 forward1	CTCATCTCAAGCCAGG
	HNA-1 reverse1	ATGGACTTCTAGCTGCACC
SLC44A2 (NC_000019)*	HNA-3 forward1	GGGCAGTGGCAGTGTACTAG
	HNA-3 reverse1	CTGAGCCTCTGCAGAGCCT
ITGAM (NG_011719.1)*	HNA-4 forward1	GAGATAGTGGCTGCCAACC
	HNA-4 reverse1	GATCCCCAGGACACAGGAGTG
ITGAL (NC_000016.9)*	HNA-5 forward1	GGCCCACCAGATCCCTCAG
	HNA-5 reverse1	AAGTGCAGGTCCAGCTGGA

*GenBank accession numbers.

Creation of Positive Control Plasmid for Genotyping

Synthetic DNA fragments (Fig. 1A–D) were cloned into the pCR2.1 TOPO plasmid (Life Technologies, Carlsbad, CA) and served as positive controls.

HRM Analysis

For HRM analysis, amplified samples bound to the fluorescent dye were heated from 65°C to 95°C. The temperature was increased by 0.1°C/second at each step using the Roter-Gene Q and covered the full range of expected melting points. HRM data were analyzed using the Roter-Gene Q software. Fluorescence intensity values were normalized between 0 percent and 100 percent by defining linear baselines before and after the melting transition of each sample. The fluorescence of each acquisition was obtained from HRM curves and was

Fig. 1. Outlines of high-resolution melting analysis for HNA-1 to HNA-5 genotyping. The structures of responsible exons of each human neutrophil antigen (HNA), the sequences of synthetic DNA fragments used in high-resolution melting (HRM) analysis as positive controls, and the location of each single nucleotide polymorphism (SNP) of HNA alleles are presented. Arrows indicate the primers were used. The regions with the SNP are highlighted in each DNA sequence. The nucleotide position for each SNP is shown above the highlight. The regions with the SNPs in different alleles are shown below each synthetic DNA fragment. Polymerase chain reaction (PCR) amplification regions of HNA-1 (A), HNA-3 (B), HNA-4 (C), and HNA-5 (D) are shown.

calculated as the percentage of fluorescence between the top and bottom baselines of each acquisition temperature, with a confidence threshold of 80 percent of the controls.

Statistical Analysis

Genotype and allele frequencies were calculated by the counting method. The validity of the Hardy-Weinberg equilibrium was tested by calculating the expected number of subjects for each genotype. Agreement of the observed and expected genotypes, based on the Hardy-Weinberg equilibrium, was determined using the χ^2 test. The level of statistical significance was set at $p < 0.05$.

Results

HRM Analysis for Genotyping of HNAs

In the field of DNA-based genotyping, HRM analysis was 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 (Tm) is increased from a lower to a higher temperature for the HRM analysis. Differences in the gene sequences between the heterozygous and homozygous genotypes lead to differences in Tm. Heterozygous genotypes tend to have lower Tm than homozygous genotypes, and, consequently, the overall HRM curves will shift to the left. The differences in the HRM curves were determined using the Roter-Gene Q software.

To confirm validity of HRM analysis for genotyping HNA-1, we amplified the control plasmids of each HNA-1 allele (a/a, b/b, c/c, a/b, a/c, and b/c) and analyzed them using the Roter-Gene Q software. Because four nucleotide substitutions are involved in HNA-1 polymorphism, we set up a PCR system to categorize the "1a," "1b," and "1c" alleles based on the SNPs at positions 266 and 277. Figure 2A presents the representative HRM curves of synthesized DNA samples containing 1a, 1b, or 1c sequences. The melting curve shift for each of the synthetic DNA samples was estimated using Roter-Gene Q software, and these curves successfully defined each HNA-1 genotype. Subsequently, we used these curves as standard allotype-specific curves. With regard to HNA-1 genotyping, we first pre-amplified HNA-1–specific PCR amplicons and used these amplicons as templates for HNA-1 genotyping to avoid amplification of *FCGR3A,* whose DNA sequence highly resembles that of HNA-1. The absence of contamination of *FCGR3A* in the amplicons was confirmed using 15 representative genomic DNA samples derived from blood donors based on the following two points: (1) the
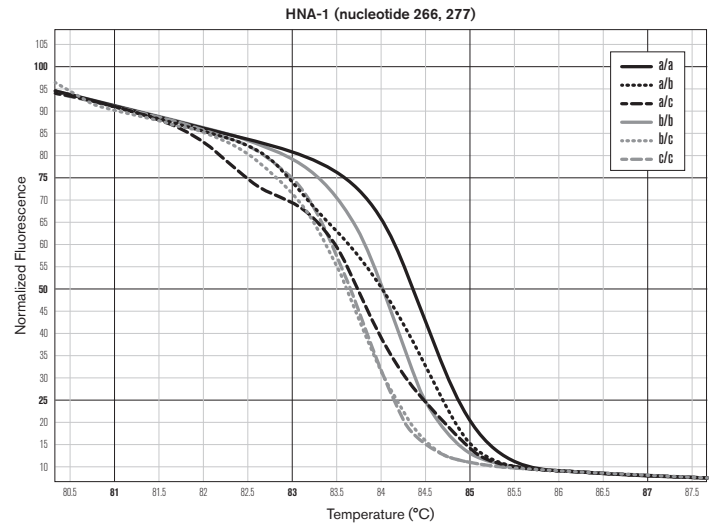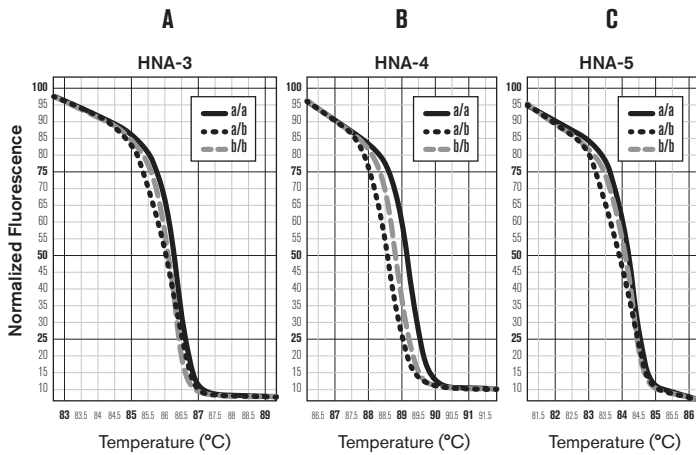

Fig. 2. High-resolution melting analysis for HNA-1d allele. The synthetic DNA samples representing each HNA-1 were amplified using the primer set, which distinguished the changes at nucleotides 266 and 277, and were analyzed using high-resolution melting (HRM). Six representative HRM curves (a/a, a/b, a/c, b/b, b/c, and c/c) are shown.

amplicons revealed only *FCGR3B*-specific bands on agarose gel electrophoresis, and (2) subsequent DNA sequence analysis revealed that the amplicons were derived from *FCGR3B*. After excluding the possibility of contamination of *FCGR3A* DNA, we genotyped these 15 genomic DNA samples using the PCR-HRM method. Following analysis of all samples, samples 6, 7, and 2 were determined as HNA-1a/1a, HNA-1b/1b, and HNA-1a/1b, respectively (data not shown). The results of HRM analysis were in complete agreement with the sequencing data. Using the two different sets of HRM analysis, we successfully determined the genotypes of the three HNA-1 alleles.

Then, we analyzed HNA-3 (a/a, b/b, and a/b), HNA-4 (a/a, b/b, and a/b), and HNA-5 (a/a, b/b, and a/b). The HRM curves of HNA-3 (Fig. 3A), HNA-4 (Fig. 3B), and HNA-5 (Fig. 3C) clearly identified the individual genotypes using the synthetic DNA samples, and the results for 15 blood donors completely coincided with the DNA sequence analyses (data not shown). Therefore, HRM analysis successfully detected all the HNA polymorphisms.

Frequencies of HNA Genotypes Among Japanese Blood Donors

Having demonstrated that HRM analysis can be used to determine HNA genotypes, we subsequently genotyped 500 Japanese individuals for HNA-1, HNA-3, HNA-4, and HNA-5 using HRM analysis and calculated the genotype frequency (Table 3) and the allele frequency (Table 4) of the

Fig. 3. High-resolution melting analysis of HNA-3, HNA-4, and HNA-5. Synthetic DNA samples were amplified and analyzed using high-resolution melting (HRM). HRM curves for each human neutrophil antigen (HNA) and its corresponding genotypes are presented in the following panels: (A) heterozygous HNA-3a/3b, homozygous HNA-3b/3b, and homozygous HNA-3a/3a; (B) heterozygous HNA-4a/4b, homozygous HNA-4b/4b, and homozygous HNA-4a/4a; and (C) heterozygous HNA-5a/5b, homozygous HNA-5b/5b, and homozygous HNA-5a/5a.

HNA system in Japan. The deviation of the observed numbers of genotypes from the expected numbers on the basis of the Hardy-Weinberg equilibrium was not statistically significant (Table 3). The occurrence of HNA-1 to -5 was similar to that reported elsewhere.[33–35]

Discussion

HRM is a very attractive, advanced, fast, and cost-effective SNP genotyping technology based on the analysis of the melting profile of PCR products, using intercalating fluorescent dyes to monitor the transition from double-stranded to single-stranded (melted) DNA. This method was used to confirm HLA genotypic identity between unrelated individuals before allogeneic hematopoietic stem-cell transplantation.[32,36] Subsequently, several blood group antigens, including some in the Duffy, Kidd, and Diego blood group systems, were also genotyped using HRM analysis.[37] Further, SNPs in the genes encoding human platelet antigens 1–6 and 15 were analyzed using HRM analysis.[38] Thus, HRM analysis is a useful method for genotyping SNPs. In the present study, we applied HRM analysis to HNA genotyping and clearly genotyped and distinguished homozygous from heterozygous HNA alleles (Fig. 3).

Knowledge of HNA frequency is important for predicting the risk of alloimmunization to HNA. Previous studies had reported the frequency of HNA-1 to HNA-5 (except

HNA-1d) among the Japanese population.[18,33,34] The results reported here, using a different method, are consistent with these findings (Tables 3 and 4). The frequency of HNA-1d has not been reported previously. The HNA-1 system has three *FCGR3B* alleles. *FCGR3B*01* encodes only one antigen (HNA-1a), while *FCGR3B*02* and *FCGR3B*03* encode two antigens each (HNA-1b and HNA-1d, and HNA-1b and HNA-1c, respectively). Because the occurrence of HNA-1c was not observed in this study, the frequency of HNA-1d was similar to that of HNA-1b.

Occasionally, the contamination of PCR products interferes with genotype testing. HRM analysis determines genotypes of individual test samples using a real-time PCR instrument with a sample cap piercing feature, which eliminates a potential source of contamination.

The frequency of HNA-1 among the Japanese population considerably differs from that of Caucasians, who express HNA-1b more frequently than HNA-1a.[34] Additionally, the

Table 3. HNA genotypes among Japanese blood donors

	Genotypes	Observed number	Observed prevalence (%)	Expected number	Remarks
HNA-1	a/a	175	35.0	194.3	$\chi^2 = 3.89$
	a/b	256	51.2	234.8	$p < 0.05$
	a/c	0	0	0	
	b/b	69	13.8	71.0	
	b/c	0	0	0	
	c/c	0	0	0	
HNA-3	a/a	210	42.0	213.8	$\chi^2 = 0.591$
	a/b	234	46.8	226	$p < 0.05$
	b/b	56	11.2	59.8	—
HNA-4	a/a	500	100.0	—	—
	a/b	0	0	—	
	b/b	0	0	—	
HNA-5	a/a	378	75.6	384.6	$\chi^2 = 5.52$
	a/b	120	24.0	107.8	$p < 0.05$
	b/b	2	0.4	7.5	

HNA = human neutrophil antigen.

Table 4. HNA alleles among Japanese blood donors

HNA	Number	Allele prevalence (%)
HNA-1a	500	60.6
HNA-1b		39.4
HNA-1c		0
HNA-3a	500	65.4
HNA-3b		34.6
HNA-4a	500	100
HNA-4b		0
HNA-5a	500	87.6
HNA-5b		12.4

HNA = human neutrophil antigen.

frequency of HNA-3b seems to be higher in the Japanese population than in Caucasians.[34,39,40] This observation suggests a higher risk of alloimmunization for individuals homozygous for HNA-3b by exposure to the HNA-3a antigen during transfusion or pregnancy. Detection of antibodies against HNA-3a was infrequent in Japanese patients with TRALI, however. Therefore, additional factors specific for the Japanese population may elicit antibodies against HNA-3a.

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