Human neutrophil alloantigen genotype frequencies in Thai blood donors

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Background. Antibodies to human neutrophil antigens (HNA) can cause transfusion reactions, as well as autoimmune and neonatal neutropenia. This study is the first to report the frequencies of human neutrophil antigen genotypes in the Thai population.

Materials and methods. Three hundred unrelated, healthy Thai blood donors at the National Blood Centre, Thai Red Cross Society, Bangkok, Thailand were typed for *HNA-1a*, *-1b*, *-1c*, *-3a, -3b* and *-4a* using polymerase chain reaction with sequence-specific primers. Moreover, *HNA-5a* genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism.

Results. The gene frequencies of *HNA-1a, -1b* and *-1c* were 0.470, 0.530 and 0.005, respectively. The frequencies of *HNA-3a* and *-3b* were 0.490 and 0.510, respectively. Additionally, the *HNA-4a*$^{+/+}$ and *HNA-4a*$^{+/-}$ genotype frequencies were 0.947 and 0.053, respectively. The frequencies of *HNA-5a*$^{+/+}$, *HNA-5a*$^{+/-}$ and *HNA-5a*$^{-/-}$ genotypes were 0.641, 0.297 and 0.062, respectively. Compared with other Asian populations, Thais have higher frequencies of *HNA-1b* ($P < 0.001$). On the other hand, the frequency of *HNA-5a* observed in Thais is lower than that reported among Koreans ($P < 0.001$).

Discussion. These findings suggest that Thais would be more susceptible to HNA-1b alloimmunisation. Furthermore, our results could establish a useful human neutrophil antigen donor file to provide more effective transfusion of blood and blood components.

Keywords: human neutrophil antigens, HNA, gene frequencies, Thais.

Introduction

Human neutrophil antigens (HNA) are found on white blood cell membrane glycoproteins and are the target of HNA antibodies which are involved in various clinical conditions including neonatal immune neutropenia, transfusion-related acute lung injury (TRALI), refractoriness to granulocyte transfusions, febrile transfusion reaction, immune neutropenia after stem cell transplantation, autoimmune neutropenia and drug-induced immune neutropenia[1]. The probability of developing HNA alloantibodies in each patient depends on the individual's existing antigens[2,3]. The Granulocyte Antigen Working Party of the International Society of Blood Transfusion (ISBT) agreed, in 1998, to establish a new nomenclature for well-defined neutrophil alloantigens based on the glycoprotein location of the antigens[4]. Currently, the HNA system includes eight antigens (HNA-1a, -1b, -1c, -2a, -3a, -3b, -4a and -5a) that are assigned to five antigen groups[5-7]. Commercial monoclonal antibodies specific to several HNA antigens are available and have been used to phenotype granulocytes; however, granulocyte viability is a test limitation[8]. Hence, to differentiate *HNA* alleles, polymerase chain reaction (PCR) assays such as PCR with sequence-specific primers (PCR-SSP) and PCR with restriction fragment length polymorphism (PCR-RFLP) have been implemented[9-11]. The frequencies of *HNA* have been characterized in different ethnic groups, and significant differences have been shown[6,9,11-18]. The frequency of *HNA-1a* is higher in most Asian populations, ranging between 0.300 and 0.680, whereas the frequency of *HNA-1b* is higher in Caucasians, ranging between 0.627 and 0.718[6]. The lower frequency of *HNA-1c* has also been reported[19]. The frequencies of *HNA-2a, HNA-4a* and *HNA-5a* in most populations are high[1,13,20,21]. The gene frequencies of *HNA-3a* and *HNA-3b* are 0.744 and 0.256, respectively, in the German population[11]. However, data about *HNA* gene frequencies in the Thai population are still unknown, and the commercial kit for *HNA-1a, -1b, -1c, -3a, -3b, -4a, -4b, -5a* and *-5bw* genotyping is expensive and not available in Thailand. The purpose of this study was to determine the genotype frequencies of the *HNA-1, -3, -4* and *-5* systems in the Thai population in order to estimate the potential risk for alloimmunisation.

Materials and methods

Subjects

Peripheral venous blood was collected into EDTA tubes from 300 unrelated, healthy Thai blood donors at the National Blood Centre, Thai Red Cross Society, Bangkok, Thailand. The donors were from central Thailand and their ages ranged from 19 to 58 years. Informed consent was obtained from each subject.

This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Thammasat University, Pathumtani, Thailand.

DNA standards

Samples with known *HNA-1a, -1b, -1c, -3a, -3b, -4a* and *-5a* DNA were provided by Dr. Núria Nogués, Laboratori d'Immunohematologia, Banc de Sang i Teixits, Passeig Taulat, Barcelona, Spain.

Primers

The primers used for *HNA* genotyping in this study are shown in Table I. The specific primers were similar to those previously described, with some modifications[10,13,14,22].

***HNA-1a, -1b* and *-1c* genotyping by polymerase chain reaction - sequence-specific primers**

HNA-1a, -1b and *-1c* genotyping was performed by a previously described PCR-SSP technique with some modifications[14]. Briefly, 2 µL of genomic DNA (100 ng/µL) were amplified in a total volume of 25 µL using 0.5 µM of HNA-1a forward and reverse primers for *HNA-1a* genotyping, 0.5 µM of HNA-1b forward and reverse primers for *HNA-1b* genotyping and 0.5 µM of HNA-1c forward and reverse primers for *HNA-1c* genotyping. Co-amplification of the human growth hormone gene (*HGH*) using 0.125 µM HGH (F) primer and 0.125 µM HGH (R) primer was run as an internal control. The PCR was performed with 12.5 µL of DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc., Glen Burnie, MD, USA) consisting of 2X Dream Taq green buffer, 0.4 mM of each dNTP, and 0.4 mM $MgCl_2$ in a G-STORM GS1 thermal cycler (Gene Technologies Ltd., Essex, UK). The cycle parameters of the PCR programme began with a first step of one cycle of 300 s at 95 °C, followed by 30 cycles of 30 s at 95 °C, 60 s at 57 °C and 30 s at 72 °C. The final extension step lasted 5 min at 72 °C and then the sample was kept at 4 °C. After amplification, PCR products were analysed on a 1.5% agarose gel using 1X Tris borate ethylenediaminetetraacetate (TBE) buffer containing SYBR® Green I nucleic acid gel stain (Invitrogen, Grand Island, NY, USA) and were visualised under UV illumination. The PCR product sizes of *HNA-1a, HNA-1b* and *HNA-1c* were 141 bp, 219 bp and 191 bp, respectively, whereas the PCR product size of the *HGH* gene, used as an internal control, was 434 bp.

***HNA-3* genotyping by polymerase chain reaction - sequence-specific primers**

The PCR-SSP technique was used for *HNA-3* genotyping with minor modifications as previously described[22]. Briefly, 2 µL of genomic DNA (100 ng/µL) were amplified in a total volume of 25 µL using 0.5 µM of HNA-3a (forward), HNA-3b (forward) and HNA-3 (reverse) primers. Co-amplification of the *HGH* gene using 0.125 µM HGH (F) primer and 0.125 µM HGH (R) primer was run as an internal control. PCR was performed with 12.5 µL of DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc.) consisting of 2X Dream Taq green buffer, 0.4 mM of each dNTP, and 0.4 mM $MgCl_2$ in a G-STORM GS1 thermal cycler (Gene Technologies Ltd.). The cycle parameters of the PCR programme began with a first step of one cycle of

Table I - Sequence of the primers for *HNA-1a, -1b, -1c, -3a, -4a, -5a* and the *HGH* internal control.

Primers	Sequence (5'→3')	Product size (bp)
HNA-1a (F)[14]	CAGTGGTTTCACAATGTGAA	141
HNA-1a (R)[14]	ATGGACTTCTAGCTGCAC	
HNA-1b (F)[14]	CAATGGTACAGCGTGCTT	219
HNA-1b (R)[14]	ATGGACTTCTAGCTGCAC	
HNA-1c (F)[14]	AAGATCTCCCAAAGGCTGTG	191
HNA-1c (R)[14]	ACTGTCGTTGACTGTGTCAT	
HNA-3a (F)[22]	AGTGGCTGAGGTGCTTCG	601
HNA-3b (F)[22]	GAGTGGCTGAGGTGCTTCA	
HNA-3 (R)*	ATCGCCATGGCAATGACCA	
HNA-4a (F)[10, 21]	CTCCCCACAGGGTGGTG	124
HNA-4a-positive (R)[10, 13]	AGTGACTCACCCTGCATGC	
HNA-4a -negative (R)[10, 13]	AGTGACTCACCCTGCATGT	
HNA-5a (F)[10,13]	CTTCAGCATCTCCACCTTGC	201
HNA-5a (R)[10]**	TTCTGATATTCCCCACCCTGA	
HGH (F)[9]	TGCCTTCCCAACCATTCCCTTA	434
HGH (R)[9]	CCACTCACGGATTTCTGTTGTGTTTC	

*Newly designed primer, **Primer with modification

180 s at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C and 60 s at 72 °C. The final extension step lasted for 10 min at 72 °C and then the sample was kept at 4 °C. After amplification, PCR products were analysed on a 1.5% agarose gel using 1X TBE buffer containing SYBR® Green I nucleic acid gel stain (Invitrogen) and were visualized under UV illumination. The PCR product size of both *HNA-3a* and *-3b* alleles was 601 bp, whereas that of the *HGH* gene, used as the internal control, was 434 bp.

HNA-4a genotyping by polymerase chain reaction - sequence-specific primers

HNA-4a genotyping was performed by a PCR-SSP technique, as previously described with some modifications[10,13]. Briefly, 2 µL of genomic DNA (100 ng/µL) were amplified in a total volume of 25 µL using 0.5 µM of HNA-4a (forward), HNA-4a-positive (reverse) and HNA-4a-negative (reverse) primers. Co-amplification of the *HGH* gene using 0.125 µM HGH (F) primer and 0.125 µM HGH (R) primer was run as an internal control. The PCR was performed with 12.5 µL of DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc.) consisting of 2X Dream Taq green buffer, 0.4 mM of each dNTP, and 0.4 mM $MgCl_2$ in a G-STORM GS1 thermal cycler (Gene Technologies Ltd.). The cycle parameters of the PCR programme began with a first step of one cycle of 180 s at 95 °C, followed by 30 cycles of 60 s at 95 °C and 60 s at 58 °C and 60 s at 72 °C. The last step was final extension for 5 min at 72 °C and the sample was kept at 4 °C. After amplification, PCR products were analysed on a 1.5% agarose gel using 1X TBE buffer containing SYBR® Green I nucleic acid gel stain (Invitrogen) and were visualised under UV illumination. The PCR product size of both *HNA-4a*-positive and *-4a*-negative alleles was 124 bp, whereas that of the internal control, the *HGH* gene, was 434 bp.

HNA-5a genotyping by polymerase chain reaction - restriction fragment length polymorphism

HNA-5a genotyping was performed using a previously described PCR-RFLP technique with some modifications[10,13]. Briefly, the PCR was performed in a total volume of 25 µL made up of 0.5 µL of genomic DNA (50 ng); 12.5 µL of DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc.) consisting of 2X Dream Taq green buffer, 0.4 mM of each dNTP and 0.4 mM $MgCl_2$; 0.5 µL of each HNA-5a primer (10 pmol/µL); and 11 µL of sterile-filtered H_2O. The reaction was performed in a G-STORM GS1 thermal cycler (Gene Technologies Ltd.), under the following conditions: 95 °C for 180 s (initial denaturation); 35 cycles of 95 °C for 30 s (denaturation), 57 °C for 30 s (annealing), and 72 °C for 60 s (extension); and 72 °C for 5 min (final extension). The PCR product, without further purification, was digested with *Bsp1286*I (FastDigest®, Fermentas, CA, USA) for 15 min in at 37 °C water bath. PCR-RFLP products were electrophoresed at 100 V for 45 min with a 2% agarose-TBE gel containing SYBR® Green I nucleic acid gel stain (Invitrogen) and were visualised under UV illumination. The typical results of RFLP analysis for the *HNA-5a*-positive allele showed 136-bp and 65-bp fragments after *Bsp1286*I digestion, while the PCR products of the *HNA-5a*-negative allele were not split (201 bp) by the same treatment.

To validate the *HNA* genotyping in the Thai population, the PCR-SSP and PCR-RFLP were performed using known *HNA-1a, -1b, -1c, -3a, -3b, -4a* and *-5a* genotype panels.

Statistical analysis

Gene frequencies were calculated by gene counting as described by De La Vega *et al.* and Steffensen *et al*[9,15]. The chi-square test was used to test for Hardy-Weinberg equilibrium for the *HNA*-system and to compare the published *HNA* genotype frequencies among different populations. The analysis was performed using Excel software (Microsoft Office, 2007, version 12.0). P values of 0.001 or less were considered statistically significant, as previously described by Hauck *et al*[11].

Results

In this study, the determined *HNA* genotypes are consistent with the Hardy-Weinberg equilibrium and the chi-square test results for observed and expected frequencies in the Thai population showed no significant difference for any *HNA* allele.

HNA-1a, 1b and _-1c_ genotyping

HNA-1a, -1b and *-1c* gene frequencies were determined in 300 Thai blood donors. The *HNA-1b* allele was most frequent, 0.53 (273/300), followed by *HNA-1a*, 0.47 (255/300), with *HNA-1c* being the least frequent, 0.005 (3/300), in the Thai population.

HNA-3a and _-3b_ genotyping

The gene frequencies of *HNA-3a* and *HNA-3b* were also determined in the 300 Thai blood donors. The gene frequency of *HNA-3a* was 0.493 (282/300) while that of *HNA-3b* was 0.507 (286/300). *HNA-3ab* heterozygous individuals were the most common (268/300).

HNA-4a and _HNA-5a_ genotyping

The *HNA-4a* and *HNA-5a* genotype frequencies and gene frequencies are shown in Table II.

Table II - *HNA-4a* and *HNA-5a* genotype frequencies and gene frequencies in Thais.

		HNA-4a	*HNA-5a*
Genotype frequencies	+/+	0.947 (284/300)	0.641 (186/300)
	+/−	0.053 (16/300)	0.297 (86/300)
	−/−	0.000 (0/300)	0.062 (18/300)
Gene frequencies	+	0.973	0.790
	−	0.027	0.210

HNA gene frequencies among different populations

A comparison of *HNA* gene frequencies reported from studies among Asian and Caucasians[11,13,16-18], is presented in Table III. The frequencies of *HNA-1a* and *-1b* were similar to those found in a previous study in German and Turkish populations[11], but were significantly different (P <0.001) from those in studies in Asian populations[13,16-18]. *HNA-1c* is the genotype with the lowest incidence among different populations. The frequency of *HNA-3a* was lower than that of *HNA-3b* in Thais, whereas the frequency of *HNA-3a* was higher in other populations[11,13,16-18]. The frequency of *HNA-4a* in Thais was similar to that in Koreans[13,16], but was significantly higher than in German and Turkish populations[11]. In addition, the frequency of *HNA-5a* in the Thai population was significantly lower than in Korean populations[13,16], while the frequency was similar to those in German and Turkish populations[11].

Discussion

Because the molecular basis of *HNA* has been previously elucidated, *HNA* genotyping using different PCR techniques was preferred over serological techniques. A disadvantage of serological techniques for HNA phenotyping is that it is time-consuming to isolate granulocytes, which may affect test results due to low viability. Generally, *HNA-1, -3, -4* and *-5* genotyping can be performed by PCR-SSP and PCR-RFLP; however, HNA-2a is only defined by serological techniques[1,5,8].

This study is the first to report the gene frequencies of *HNA-1a, 1b, -1c, -3a, -3b, -4a* and *-5a* in the Thai population. Three hundred unrelated Thai blood donors were genotyped for *HNA-1a, -1b, -1c, -3a, -3b* and *-4a* by PCR-SSP and *HNA-5a* was genotyped by PCR-RFLP. For the *HNA-1* system, the frequency of *HNA-1a* in most Asian populations ranges between 0.300 and 0.680[6]; however, a lower frequency of *HNA-1a* and a higher frequency of *HNA-1b* were found in Thais compared with other Asian populations, suggesting that Thais would be more susceptible to HNA-1b alloimmunisation[13,16-18]. Moreover, the lower frequency of *HNA-1c* was observed in Thais, as in previous studies[10,19,21].

Unlike in Han Chinese and German populations, it was found that *HNA-3a* and *HNA-3b* frequencies in Thais were significantly different[11,18], which may be due to the high frequency of heterozygous *HNA-3ab* in this study. Moreover, regarding a previous Thai Stem Cell Donor Registry study, it is proven that the population of central Thailand is a mixture of original Thais and Southern Chinese[23]. The results of this present study indicate that alloimmunisation against HNA-3 antibodies could be less frequent in Thais. Concerning the technique used for *HNA-3a* genotyping, a previous study showed that the PCR-SSP technique can produce misleading results in *HNA-3ab* heterozygotes with the additional CTL2-537T variation of the HNA-3a antigen[24], suggesting the need for further study.

The gene frequency of *HNA-4a* in the Thai population is similar to that in Korean populations[13,16]; therefore, antibodies to HNA-4a may be involved in neonatal alloimmune neutropenia and autoimmune neutropenia as previously found in other studies[20,25]. Interestingly, the frequency of the *HNA-5a* gene in Thais is comparable to the frequencies reported in

Table III - *HNA* gene frequencies in different populations.

Populations	***HNA* gene frequencies**						
	HNA-1a	***HNA-1b***	***HNA-1c***	***HNA-3a***	***HNA-3b***	***HNA-4a***	***HNA-5a***
Thai: this report* (n=300)	0.47	0.53	0.005	0.49	0.51	0.97	0.79
Korean[13,16] (n=200), (n=110)	0.58∂ NA	0.42∂ NA	NA NA	NA NA	NA NA	NA 0.99	NA 0.96∂
Chinese[14, 17] (n=138), (n=119)	0.68∂ NA	0.32∂ NA	0.00 NA	NA 0.62∂	NA 0.38∂	NA NA	NA NA
Japanese[11, 18] (n=400)	0.62∂	0.38∂	0.00	NA	NA	NA	NA
German[11] (n=119)	0.39	0.60	0.025	0.74∂	0.26∂	0.91∂	0.73
Turkish[11] (n=118)	0.42	0.56	0.03	0.74∂	0.26∂	0.88∂	0.75

*Thai blood donors, ∂P <0.001.

German and Turkish populations[11]. However, *HNA-5a* is significantly more frequent in Koreans than in Thais and other populations[11,13]. Hence, additional genotyping for *HNA-5bw*, using a commercially available kit, is needed in the Thai population. The selected DNA sequencing results of some HNA-5a negative donors by PCR-RFLP confirmed a single nucleotide polymorphism at position G2466C, as shown in Figure 1.

In conclusion, the implementation of *HNA* genotyping in blood donors and patients is beneficial not only to provide more effective blood transfusions but also to increase the capability for HNA alloantibody investigations.

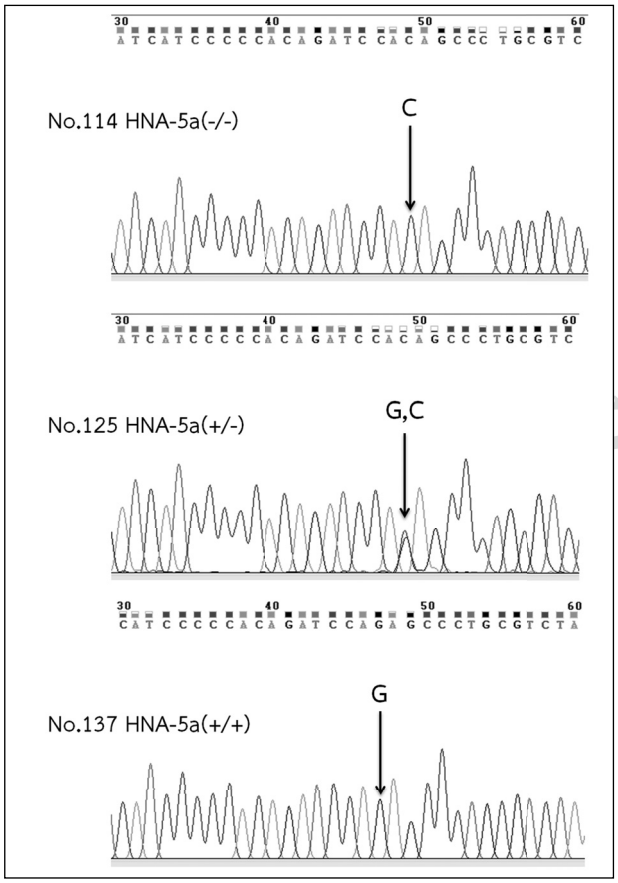

Figure 1 - Chromatogram of HNA-5a sequencing results in samples of 5a(−/−), 5a(+/−) and 5a(+/+).

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The Authors declare no conflicts of interest.

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