Original Article

Frequencies of human neutrophil antigen-4 and human neutrophil antigen-5 among Thai blood donors

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Abstract:

Context: Antibodies against human neutrophil antigens (HNAs) are implicated in immune-mediated neutropenia, transfusion-related acute lung injury and febrile transfusion reactions. **Aims:** This study aimed to determine HNA gene frequencies of the HNA-4 and HNA-5 systems among Thai populations and compare these frequencies with those previously reported for other populations. **Materials and Methods:** 800 DNA samples obtained from 500 unrelated healthy blood donors from Bangkok and 300 samples from Chiang Mai, Thailand were included. Samples were typed for each *HNA* allele including *HNA-4a*, *HNA-4b*, *HNA-5a,* and *HNA-5b* using an in-house polymerase chain reaction with sequence-specific primer technique. **Results:** The frequencies of *HNA-4a* and *HNA-4b* alleles in central Thais were 0.975 and 0.025, respectively and for Northern Thais, their frequencies were 0.965 and 0.035, respectively. For *HNA-5a* and *HNA-5b* alleles, their frequencies were 0.771 and 0.229; 0.748, and 0.252 in central and Northern Thais, respectively. The frequencies of HNA-4 and HNA-5 systems in central Thais are closely related to those in Northern Thais ($P > 0.05$). However, their frequencies were different from other populations ($P < 0.001$), except *HNA-5a* and *HNA-5b* gene frequencies in Thais were similar to Caucasians ($P > 0.05$). **Conclusion:** This study could contribute to predict the risk of alloimmunization to HNA-4 and HNA-5 systems, especially in feto-maternal incompatibility in Thais.

Key words:

Gene frequencies, human neutrophil antigen-4, human neutrophil antigen-5, Thais

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Introduction

Human neutrophil antigens (HNAs) are glycoproteins located in the neutrophil membranes,[1] and HNA antibodies are implicated in alloimmune neutropenia (ANN), autoimmune neutropenia (AIN) in childhood, febrile nonhemolytic transfusion reactions, and transfusion-related acute lung injury.[2,3] Nowadays, the International Society of Blood Transfusion has established the HNA nomenclature for well-defined neutrophil alloantigens based on glycoprotein location of the antigens to five systems; HNA-1 to HNA-5.[2-4]

Antigens of HNA-4 and HNA-5 arise from single nucleotide polymorphisms in the integrin alpha M and integrin alpha L genes, respectively.[5-8] Cases of ANN and AIN caused by anti-HNA-4a were previously reported;[3,9,10] whereas, the occurrence of ANN because of maternal HNA-5a antibodies was found.[11] In addition, a multitransfused patient with aplastic anemia who produced only anti-HNA-5a had prolonged survival of a skin graft from human leukocyte antigen (HLA) unidentical donor, because the leukocyte interactions in the graft had been blocked.[12]

Gene frequency of *HNA-4a* and *HNA-5a* are high among different populations.[13-18] This study aimed to report the gene frequencies of the HNA-4 and HNA-5 systems determined by polymerase chain reaction with sequence-specific primer (PCR-SSP) and to estimate the potential risk for alloimmunization in Thai populations.

Materials and Methods

Subjects and controls

Peripheral venous blood was collected in ethylenediaminetetraacetic acid (EDTA)-anticoagulated vacutainer from 800 unrelated healthy blood donors. 500 samples were from the National Blood Centre, Thai Red Cross Society, Bangkok and 300 samples were from the Blood Bank, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. 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, Pathum Thani, Thailand. For Genomic DNA extraction, EDTA blood samples were extracted using the Genomic DNA extraction kit (REAL Genomics, RBC Bioscience, Taipei, Taiwan) and the salting out method, as previously described,[19] then stored at –20°C until use for genotyping. Known HNA-4a,-4b,-5a, and-5b DNA samples were provided by Dr. Núria Nogués, Laboratori d'Immunohematologia,

Banc de Sang i Teixits, Passeig Taulat, Barcelona, Spain. Primers for HNA-4 and-5 genotyping in this study are shown in Table 1. The specific primers were identical to those previously described.[1,20,21]

Human neutrophil antigen-4 genotyping by polymerase chain reaction with sequence-specific primer

Human neutrophil antigen-4 genotyping was performed by PCR-SSP technique, as previously described[1,10,21] with some modifications. Briefly, 1 μL of genomic DNA (50 ng/μL) was amplified in a total volume of 10 μL using 1 μL of HNA-4ab (forward, F) and 1 μL of HNA-4a (reverse, R) primers for *HNA-4a* genotyping. For *HNA-4b* genotyping, 1 μL of HNA-4ab (F) and 1 μL of HNA-4b (R) primers were used. Co-amplification of the human growth hormone (*HGH*) gene using 1 μL of HGH (F) primer and 1 μL of HGH (R) primer was run as an internal control. The PCR was performed with 5 μL of DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc., Glen Burnie, MD, USA) consisting of 2X DreamTaq green buffer, 0.4 mM of each dNTP, and 4 mM $MgCl_2$ in a G-STORM GS1 thermal cycler (Gene Technologies Ltd., Somerset, UK). PCR was performed under the following conditions; 95°C for 5 min (initial denaturation), 30 cycles of 95°C for 30 s (denaturation), 56°C for 40 s (annealing), 72°C for 30 s (extension), and 72°C for 5 min (final extension) and the sample was kept at 4°C. After amplification, the PCR products were electrophoresed at 100 V with 1.5% agarose gel using 1X Tris borate ethylenediaminetetraacetate (TBE) buffer containing 0.5 μg/mL ethidium bromide and were visualized under ultraviolet (UV) illumination. The PCR product size of both HNA-4a and-4b alleles was 124 bp, whereas that of the internal control the *HGH* gene was 434 bp, as shown in Figure 1.

Human neutrophil antigen-5 genotyping by polymerase chain reaction with sequence-specific primer

Human neutrophil antigen-5 genotyping was performed by PCR-SSP technique, as previously described[1,20] with some modifications. Briefly, 1 μL of genomic DNA (50 ng/μL) was amplified in a total volume of 10 μL using 1 μL of HNA-5ab (R) and 1 μL of HNA-5a (F) primers for *HNA-5a* genotyping. For *HNA-5b* genotyping, 1 μL of HNA-5ab (R) and 1 μL of HNA-5b (F) primers were used. Co-amplification of the *HGH* gene using 1 μL of HGH (F) primer and 1 μL of HGH (R) primer was run as an internal control. The PCR was performed with 5 μL of DreamTaq™ DNA polymerase (Thermo Fisher Scientific Inc., Glen Burnie, MD, USA) consisting of 2X DreamTaq green buffer, 0.4 mM of each dNTP, and 4 mM $MgCl_2$ in a G-STORM GS1 thermal cycler (Gene Technologies Ltd., Somerset, UK). The cycle parameters of the PCR program began with the first step of 1 cycle of 30 s at 95°C and 60 s at 69°C, followed by 9 cycles of 10 s at 95°C and 60 s at 69°C, then 20 cycles of 10 s at 95°C and 50 s at 60°C and 30 s at 72°C. The last step was final extension 5 min at 72°C and the sample was kept at 4°C. After amplification, the PCR products were electrophoresed at 100 V with 1.5% agarose gel using 1X TBE buffer containing 0.5 μg/mL ethidium bromide and were visualized under UV illumination. The PCR product size of both *HNA-5a* and-*5b* alleles was 283 bp, whereas that of the internal control the *HGH* gene was 434 bp, as shown in Figure 2.

Validity testing for human neutrophil antigen-4 and human neutrophil antigen-5 genotyping by polymerase chain reaction with sequence-specific primer

Validity of HNA genotyping in Thai populations, the PCR-SSP was performed using known HNA-4a,-4b,-5a, and-5b genotype panels. Moreover, to confirm the results of HNA-4 genotyping, four HNA-4 genotyped samples (2, *HNA-4a/4a* and 2, *HNA-4a/4b*) were sequenced. In addition, four HNA-5 genotyped samples (2, *HNA-5a/5a*, 1, *HNA-5a/5b* and 1, *HNA-5b/5b*) were sequenced.

Statistical analysis

Gene frequencies were calculated by the gene counting method. The Chi-square (χ^2) test was used to test for Hardy–Weinberg equilibrium for the HNA-4 and HNA-5 systems and to compare the

Table 1: Sequences of the primers for HNA-4a, HNA-4b, HNA-5a, HNA-5b, and HGH

Primer	Sequence (5'→3')	Product size (bp)	Final concentration (μM)
HNA-4ab (F)[21]	CTCCCCACAGGGTGGTG		0.1
HNA-4a (R)[21]	AGTGACTCACCCTGCATGC	124	0.1
HNA-4b (R)[21]	AGTGACTCACCCTGCATGT		0.1
HNA-5ab (R)[20]	AGGTTGAGGCAGGAGAATGG		0.1
HNA-5a (F)[20]	CAGTTAGACGCAGGGCTC	283	0.1
HNA-5b (F)[20]	CAGTTAGACGCAGGGCTG		0.1
HGH (F)[1]	TGCCTTCCCAACCATTCCCTTA	434	0.1
HGH (R)[1]	CCACTCACGGATTTCTGTTGTGTTTC		0.1

Abbreviations: HNA: Human neutrophil antigens; HGH: Human growth hormone; F: Forward; R: Reverse

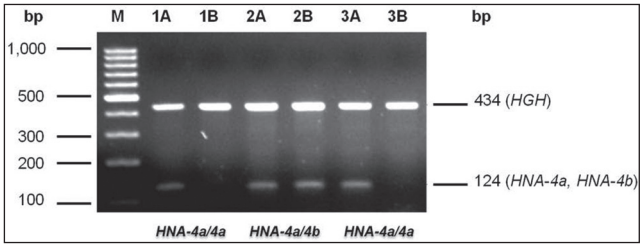

Figure 1: Results of HNA-4 genotyping by PCR-SSP. Lane M: 100 bp DNA molecular weight marker (Fermentas, Carlsbad, CA). The 434 bp amplification product of the HGH control primer is presented in all lanes, which indicate successful PCR amplification. The HNA-4 genotype was deduced from the presence of amplification product specific for *HNA-4a* and *HNA-4b* (124 bp). Lanes 1A&1B = *HNA-4a/4a*, Lanes 2A&2B = *HNA-4a/4b* and Lanes 3A&3B = *HNA-4a/4a*, respectively

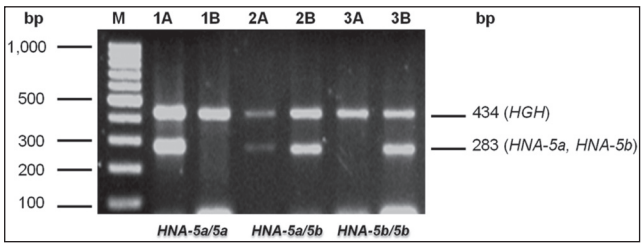

Figure 2: Results of HNA-5 genotyping by PCR-SSP. Lane M: 100 bp DNA molecular weight marker (Fermentas, Carlsbad, CA). The 434 bp amplification product of the HGH control primer is presented in all lanes. The HNA-5 genotype was deduced from the presence of amplification product specific for *HNA-5a* and *HNA-5b* (283 bp). Lanes 1A&1B = *HNA-5a/5a*, Lanes 2A&2B = *HNA-5a/5b* and Lanes 3A&3B = *HNA-5b/5b*, respectively

published HNA genotype frequencies among different populations. The analysis was performed using SPSS (Version 15.0, SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant, as previously described.[13]

Results

In this study, the determined HNA-4 and HNA-5 genotypes were consistent with the Hardy–Weinberg equilibrium and the χ^2 test results for observed and expected frequencies in central Thai and Northern Thai populations and showed no significant difference for any HNA allele as shown in Table 2.

Gene frequencies of *HNA-4a* and *HNA-4b* in 500 central Thai blood donors and 300 Northern Thai blood donors were determined. From the genotyping results, *HNA-4a* gene frequencies were 0.975 (975/1000) and 0.965 (579/600), whereas *HNA-4b* frequencies were 0.025 (25/1000) and 0.035 (21/600) in Central and Northern Thai blood donors, respectively. Interestingly, *HNA-4b/4b* genotype was not found in both populations. Moreover, *HNA-4a* and *HNA-4b* frequencies in central Thais were compared with Northern Thais and no significantly difference was found ($P > 0.05$).

In addition, *HNA-5a* and *HNA-5b* gene frequencies in 500 Central Thai blood donors and 300 Northern Thai blood donors were also determined. From the genotyping results, *HNA-5a* gene frequencies were 0.771 (771/1000) and 0.748 (449/600), whereas *HNA-5b* frequencies were 0.229 (229/1000) and 0.252 (151/600) in central and Northern Thai blood donors, respectively. A comparison of *HNA-5a* and *HNA-5b* gene frequencies between Central Thais and Northern Thais showed no significantly difference ($P > 0.05$). In addition, the DNA controls of HNA-4a, HNA-4b, HNA-5a, and HNA-5b were simultaneously tested by in-house PCR-SSP and the genotyping results were in agreement. Confirmation testing by DNA sequencing of the HNA-4 and HNA-5 genotyped samples showed 100% concordance.

Human neutrophil antigen-*4a, HNA-4b*, *HNA-5a*, and *HNA-5b* gene frequencies in Thai populations were compared with those reported from previous studies among Asian and other ethnical populations as shown in Table 3.[13-18] Even though *HNA-4a* has a

Table 2: Hardy-Weinberg equilibrium in observed vs. expected HNA genotypes in Thai populations

	Genotypes	Observed number	Observed frequency (%)	Expected number	Remarks
Central Thais (*N*=500)					
HNA-4	*a/a*	475	95.0	475.0	χ^2=0.0000
	a/b	25	5.0	25.0	*P*=1.0000
	b/b	0	0.0	0.0	
HNA-5	*a/a*	321	64.2	297.2	χ^2=0.9042
	a/b	129	25.8	176.6	*P*=0.3417
	b/b	50	10.0	26.2	
Northern Thais (*N*=300)					
HNA-4	*a/a*	279	93.0	279.4	χ^2=1.0012
	a/b	21	7.0	20.3	*P*=0.3170
	b/b	0	0.0	0.3	
HNA-5	*a/a*	168	56.0	167.9	χ^2=0.0001
	a/b	113	37.7	113.1	*P*=0.9920
	b/b	19	6.3	19.0	

Abbreviations: HNA: Human neutrophil antigens

Table 3: HNA-4a, HNA-4b, HNA-5a, and HNA-5b gene frequencies in different populations

Populations	HNA gene frequencies				References
	HNA-4a	HNA-4b	HNA-5a	HNA-5b	
Central Thais (*N*=500)	0.975	0.025	0.771	0.229	Present study
Northern Thais (*N*=300)	0.965	0.035	0.748	0.252	Present study
English[18]					
N=140	0.882*	0.118*	0.736	0.264	Cardoso, *et al*., 2013
Danish[14]					
N=210	0.881*	0.119*	0.724	0.276	Nielsen, *et al*., 2012
Zambian[14]					
N=181	0.892*	0.108*	NA	NA	Nielsen, *et al*., 2012
N=189	NA	NA	0.500*	0.500*	Nielsen, *et al*., 2012
German[13]					
N=119	0.908*	0.092*	0.731	0.269	Hauck, *et al*., 2011
Turkish[13]					
N=118	0.881*	0.119*	0.754	0.246	Hauck, *et al*., 2011
Japanese[17]					
N=570	1.000*	0.000*	NA	NA	Matsuhashi, *et al*., 2012
N=508	NA	NA	0.840*	0.160*	Matsuhashi, *et al*., 2012
Chinese[15,16]					
N=493	0.996*	0.004*	0.854*	0.146*	Xia, *et al*., 2011
N=400	1.000*	0.000*	0.896*	0.104*	He, *et al*., 2014

*$P < 0.001$; Abbreviations: HNA: Human neutrophil antigens; NA: Not assessed

high incidence among populations, its frequencies among Thais was significantly different than those in Asian and other populations ($P < 0.001$). The *HNA-5a* shows higher frequency than *HNA-5b* in Thai populations; however, their frequencies were significantly different than those in Chinese, Japanese, and Zambian populations ($P < 0.001$).[14-17] On the contrary, *HNA-5a* and *HNA-5b* frequencies in Thai populations were similar to those in English, Danish, German, and Turkish populations ($P > 0.05$).[13,14,18]

Discussion

At present, HNA genotyping is an effective alternative technique to genotype using different PCR techniques, because HNA phenotyping using serological techniques have some disadvantages such as it being time-consuming to isolate granulocytes, which may affect test results due to low viability and requiring access to reactive sera. In general, *HNA-4* and *HNA-5* genotyping can be performed by PCR-SSP and PCR-RFLP; however, HNA-2a is only defined by serological techniques.[3,7,22] A previous study reported that the PCR-RFLP for *HNA-5a* genotyping showed a good correlation with the DNA sequencing results, but was expensive and time-consuming.[5] Moreover, the homozygous of *HNA-5b/-5b* genotype could not be determined.[4]

In this study, we determined the alleles of the HNA-4 and HNA-5 systems in Thai populations using our in-house PCR-SSP technique. This technique is widely used in routine laboratory examination for HNA genotyping because of its simplicity and less time consumption. Moreover, DNA sequencing confirmed the results of HNA-4 and HNA-5 genotyped samples. Thus, all samples tested by PCR-SSP were verified as correct results. The gene frequency of *HNA-4a* and *HNA-4b* in the Central Thai population is similar to the Northern Thai population; while, their frequencies were significantly different from Asian and other populations.[13-18] Although ANN caused by antibodies against HNA-4a seems to be a rare event across populations,[3,7,23,24] in this study the frequency of *HNA-4a/-4b* in Thai populations was lower than those in German and Turkish populations,[13] but higher than those in other Asian populations.[15-17] The occurrence of *HNA-4b/-4b*, a rare genotype, may be found in Thai populations if the sample size will be increased; therefore, further study is suggested.

In addition, the *HNA-5a* and *HNA-5b* gene frequency in the central Thai population is similar to the Northern Thai population and Caucasian populations.[13,14,18] In contrast, their frequencies were significantly higher than in Asian populations.[15-17] This finding confirms that the possibility of alloimmunization and HNA clinical disorders, especially in unexplained neutropenia among Thai populations caused by antibodies against HNA-5a may occur more frequently than other Asian populations.

Even though, the granulocyte transfusions are not commonly used, but they are beneficial in neutropenic patients with severe uncontrolled infections.[25] In practical, granulocytes from nonmatched ABO compatible donors can be provided to nonalloimmunized patients; whereas, alloimmunized patients should receive granulocytes from either HLA-matched or HNA-matched donors. Hence, HNA genotyping using PCR-SSP in patients and donors can be used as the method of choice when phenotyping by serological techniques is not feasible.

Conclusion

We have determined the gene frequencies of HNA-4 and HNA-5 systems in Thai populations. The gene frequencies in the Central Thai population are closely related to those in the Northern Thai population. However, their frequencies were different from other populations, except *HNA-5a* and *HNA-5b* frequencies in Thais were similar to Caucasians. In addition, this data would be beneficial to provide more effective blood transfusions and to predict for HNA alloimunization.

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