Genotyping of Human Platelet Antigens in Ethnic Northeastern Thais by the Polymerase Chain Reaction-Sequence Specific Primer Technique†

AMORNRAT V ROMPHRUK, M.Sc.*, JONGKOL AKAHAT, M.Sc.*,
PRAMOTE SRIVANICHRAK, M.Sc.*, CHINTANA PUAPAIROJ, M.D.*,
ARUNRAT ROMPHRUK, M.Sc.**, CHANVIT LEELAYUWAT, Ph.D.**

Abstract

Human platelet antigens (HPA) are important in neonatal alloimmune thrombocytopenia (NAITP), post-transfusion purpura (PTP), refractoriness to platelet transfusion therapy and population genetics. The distribution of HPA in a Northeast Thai population was studied. 300 healthy, unrelated, and ethnic Northeastern Thais were randomly selected. Using the polymerase chain reaction-sequence specific primer technique (PCR-SSP), the frequency of HPA-1, -2, -3, -4, -5 and -6 were determined. The phenotype frequencies were 100 per cent for HPA-1a, 4a, 5a, and 6a. For HPA-1b, 2a, 2b, 3a, 3b, 5b and 6b, the frequencies were 5.7, 99.7, 12.3, 78.0, 71.3, 7.3 and 3.0 per cent, respectively. The HPA-4b was not found. The HPA frequencies in our subjects were quite similar to other Asian populations but were different from Caucasians. The distribution of HPA genotypes encountered in our study indicate that HPA-1a, -4a, -4b, -5a and -6a will not be involved in NAITP, PTP and refractoriness to platelet transfusion therapy in Northeastern Thais. Moreover, HPA-1b, -2a, -2b, -3a, -3b, -5b and -6b may induce alloantibodies in these patients.

Key word : Platelet Antigens, PCR-SSP

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* Blood Transfusion Centre, Faculty of Medicine, Khon Kaen University,
** Department of Clinical Immunology and CMII-KKU Institutional Co-operative Centre, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand.
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Human platelet antigens include eight recognized biallelic systems and Nak^a antigen. The polymorphism in HPA-1 to 8 is derived from a single base pair substitution which leads to a single amino acid difference in the glycoprotein expressed on the platelet[1-7]. Knowing the distribution of HPA is important when identifying and treating neonatal alloimmune thrombocytopenia (NAITP), post-transfusion purpura (PTP), refractoriness to platelet transfusion therapy and population genetics[8]. Therefore, HPA typing and detection of platelet antibodies are essential in the diagnosis and further treatment of patients suffering from these conditions.

Serological typing for HPA is well-developed but the practice is limited by a shortage of well-characterized typing sera, and only a few platelets can be collected from patients with thrombocytopenia. Based on nucleotide sequence polymorphism, there are several DNA-based typing methods such as polymerase chain reaction (PCR) allele-specific oligonucleotide probe (ASO)[9] and PCR-restriction fragment length polymorphism (PCR-RFLP)[10-12]. Subsequently PCR-single strand conformation polymorphism (PCR-SSCP)[13] and PCR-sequence specific primer (PCR-SSP)[14-19] were introduced. Using serology, the phenotype frequencies of HPA have been reported in many populations including in Thais[20,21]. Previous studies could not determine some antigens due to limited typing sera. The purpose of this study, therefore, was to investigate the distribution of HPA-1 to 6 in healthy ethnic Northeastern Thais using the PCR-SSP technique.

MATERIAL AND METHOD

Samples

The samples were collected from 300 unrelated healthy subjects. They were all ethnic Northeastern Thais of at least two generations and currently living in Northeast Thailand. Genomic DNA was extracted from the buffy coat by the proteinase K digestion and salting out technique[22].

DNA standards

The known HPA-1 to 6 DNA samples were used as standards in the PCR-SSP method. These DNA standards were provided by Dr. Sentot Santoso of the Institute for Clinical Immunology and Transfusion Medicine, Justus-Liebig University Giessen, Germany.

HPA-genotyping

The PCR-SSP technique was used to characterize HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, -6a and -6b. The primers used in this study are listed in Table 1. The PCR reactions were carried out in 13 µl aliquots containing 150 ng of genomic DNA and PCR buffer (67 mM Tris HCl pH 8.8, 17 mM ammonium sulfate, 0.1 per cent Tween 20, 200 µM each of dNTP, 2 mM $MgCl_2$). Each PCR reaction contained 0.1 µM of the control primers, 0.5 to 3.5 mM of the allele specific primers and 0.35 units of Taq DNA polymerase (Promega, Medison, WI, USA.). PCR amplifications were carried out in a 480 or 9600 DNA thermal cycler (Perkin-Elmer Instrument, Cetus Corp., Norwalk, CT, USA.).

The PCR-cycling conditions were as follows: 1) 1 cycle denaturation at 94°C for 2 minutes. 2) 5 cycles of : denaturation at 94°C for 30 seconds, annealing at 65°C for 60 seconds and extension at 72°C for 40 seconds. 3) 21 cycles of: denaturation at 94°C for 30 seconds, annealing at 60°C for 60 seconds and extension at 72°C for 40 seconds. 4) 4 cycles of: denaturation at 94°C for 30 seconds, annealing at 55°C for 75 seconds and extension at 72°C for 120 seconds. 5) a final extension cycle at 72°C for 10 minutes.

PCR products were electrophoresed through 1.0 per cent agarose gel containing 0.5 µg/ml ethidium bromide. The gels were run for 30 minutes at 15 V/cm in 0.5X TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA pH 8.0) and visualized under UV transilluminator.

Reproducibility of SSP typing

Ten samples were randomly repeated for HPA-1 to 6 SSP-typing to test reproducibility.

Statistical analysis

Genotype frequencies were calculated using the formula:

% genotype frequencies = $\sum$ particular allele ÷ N x 100 (where N = total number of individual tested).

Gene frequencies (GF) were calculated by direct counting. The distributions of HPA phenotypes between the two groups were tested for significance by χ^2 or Fisher's exact test.

RESULTS

Reproducibility

In order to test reproducibility of the assay, 10 DNA samples were randomly selected and re-

Table 1. Sequence and length of the primers for HPA-1-6 and internal control.

HPA	specificity	Primer sequences 5'——————————>3'	Length	Concentration of primer (μM)	Reference
HPA-1	1a	ACTTACAggCCCTgCCTCT	19-mer	0.5	17
	1b	ACTTACAggCCCTgCCTCC	19-mer	0.5	
	common	gTgCAATCCTCTggggACT	19-mer		
HPA-2	2a	gCCCCCAgggCTCCTgAC	18-mer	0.5	19
	2b	gCCCCCAgggCTCCTgAT	18-mer	0.5	
	common	TCAgCATTgTCCTgCAgCCA	20-mer		
HPA-3	3a	ggACTgggggCTgCCCAT	18-mer	0.75	19
	3b	ggACTgggggCTgCCCAg	18-mer	0.75	
	common	TCCATgTTCACTTgAAgTgCT	21-mer		
HPA-4	4a	gCTggCCACCCAgATgCg	18-mer	0.5	19
	4b	gCTggCCACCCAgATgCA	18-mer	0.5	
	common	CAggggTTTTCgAgggCCT	19-mer		
HPA-5	5a	AgTCTACCTgTTTACTATCAAAg	23-mer	3.5	19
	5b	AgTCTACCTgTTTACTATCAAAA	23-mer	3.5	
	common	CTCTCATggAAAATggCAgTA	21-mer		
HPA-6	6a	gACgAgTgCAgCCCCCg	17-mer	0.75	18
	6b	ggACgAgTgCAgCCCCCA	18-mer	1	
	common	CTATgTTTCCCAgTggTTgCA	21-mer		
internal control	HGH I	CAgTgCCTTCCCAACCATTCCCTTA	25-mer	0.1	23
	HGH II	ATCCACTCACggATTTCTgTTgTgTTTC	28-mer	0.1	

Table 2. Distributions of HPA-1-6 in 300 healthy, unrelated, ethnic Northeastern Thais.

Genotype	no.	Genotype frequencies (%)	Gene	Gene frequencies (%)
HPA-1a/1a	283	94.33	HPA-1a	97.16
HPA-1a/1b	17	5.67	HPA-1b	2.83
HPA-1b/1b	0	0.00		
HPA-2a/2a	264	88.00	HPA-2a	93.83
HPA-2a/2b	35	11.67	HPA-2b	5.83
HPA-2b/2b	1	0.33		
HPA-3a/3a	86	28.67	HPA-3a	53.33
HPA-3a/3b	148	49.33	HPA-3b	46.67
HPA-3b/3b	66	22.00		
HPA-4a/4a	300	100.00	HPA-4a	100.00
HPA-4a/4b	0	0.00	HPA-4b	0.00
HPA-4b/4b	0	0.00		
HPA-5a/5a	278	92.67	HPA-5a	96.33
HPA-5a/5b	22	7.33	HPA-5b	3.67
HPA-5b/5b	0	0.00		
HPA-6a/6a	291	97.00	HPA-6a	98.50
HPA-6a/6b	9	3.00	HPA-6b	1.50
HPA-6b/6b	0	0.00		

tested for HPA-1 to 6 typing. The results of the repeated assays were comparable with the first round of testing.

HPA-1 to 6 genotyping in Northeastern Thais

A total of 300 samples were determined for HPA-1 to 6 alleles (Table 2). HPA-1a, -4a, -5a and -6a alleles were present in all samples. HPA-1b, -2b, -5b, and -6b were rare and HPA-4b was not found. HPA-3a and -3b showed frequencies of 53.3 and 46.7 per cent, respectively.

Comparisons of HPA phenotype frequencies from 8 Asian studies[12,14,20,21,24-30] and 3 Caucasian studies[10,31,32] are presented in Table 3. The ethnic Northeastern Thais' HPA frequencies are similar to those of other Asians rather than those of Caucasians.

DISCUSSION

The phenotype and genotype frequencies of HPA are important in both clinical and population genetics, but, perhaps due to technical difficulties, the frequency data for HPA have been defined for only a few ethnic populations. The conventional serological technique requires a number of patients' platelets and well-defined antisera, however, various DNA-typing methods have been developed for HPA typing[9-11] that overcome these logistical obstacles.

The use of the PCR-SSP method for human leukocyte antigen (HLA) genotyping is well established at our center[33,34]. We therefore extended the PCR-SSP assay to type HPA. In the previously published PCR-SSP protocol, the assay required the utilization of a wax hot start[17] or AmpliTaq Gold in the reaction[35] to ensure specificity. Others have used AmpliTaq DNA polymerase but the amplification conditions were different in some primer pairs[18]. In our study, the amplification condition was optimized for all HPA-1 to 6 genotyping. In addition to the flexibility and simplicity of the modified test, the PCR-buffer from the HLA-protocol was adopted, making the test simple, reliable and rapid.

This is the first report of the distribution of HPA genotypes by DNA-typing in the Thai population. The 300 healthy subjects were from the 19 provinces of Northeast Thailand. We compared these Northeastern Thais with other Asian and Caucasian populations (Table 3). The distributions of HPA-1a, -2a, -4a, -5a and -6a from the other

Table 3. Comparison of phenotype frequencies of HPA-1-6 in 300 healthy NET and various populations.

populations	n	HPA-1a	HPA-1b	HPA-2a	HPA-2b	HPA-3a	HPA-3b	HPA-4a	HPA-4b	HPA-5a	HPA-5b	HPA-6a	HPA-6b	Reference
NET	300	100.0	5.7	99.7	12.3	78.0	71.3	100.0	0.0	100.0	7.3	100.0	3.0	this study
Northeastern Thais	483	100.0	ND	ND	15.9	60.2*	ND	98.8	1.9	ND	5.4	ND	ND	20
Thais	132	100.0	ND	ND	12.5	66.6*	ND	100.0	0.0	ND	ND	ND	ND	21
Indonesian	168	100.0	1.8	ND	ND	72.9	80.7*	100.0	0.6	100.0	9.3	ND	ND	24
Taiwan	100	100.0	ND	ND	9.0	77.0	ND	100.0	0.5	ND	ND	ND	ND	25
Hong kong	100	100.0	1.0*	100.0	5.0	75.0	70.0	100.0	0.0	100.0	7.0	ND	ND	26
Korean	126	100.0	11.5	ND	ND	87.3*	ND	100.0	1.6	ND	ND	ND	ND	27
Japanese	254	ND	ND	99.2	19.7*	85.1	66.2	100.0	2.0	ND	ND	ND	ND	14
Japanese	331	100.0	0.3*	ND	ND	ND	ND	100.0	2.1	ND	ND	99.7	5.1	28
Japanese	100	100.0	0.0*	98.0	22.0*	83.0	57.0*	100.0	0.0	99.0	1.0*	100.0	2.0	29
S.American Indian	112	100.0	ND	ND	ND	89.3*	ND	100.0	0.9	ND	4.9	ND	ND	30
Ameridian	132	100.0	0.0*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	31
Dutch	200	97.9	28.8*	100.0	13.2	81.0	69.8	100.0	0.0	100.0	19.7*	ND	ND	10
Finns	200	99.0	26.5*	99.0	16.5	83.5	66.5	ND	ND	99.5	10*	ND	2.4	32

ND = not done
* = phenotype frequencies showing a significant difference between NET and other groups at $P<0.05$.

groups were not statistically significant. However, HPA-1b and -5b are more frequent in Caucasians, whereas, HPA-2b is more prevalent in the Japanese. The frequency of HPA-3a previously measured in Thais[20,21] was significantly lower than in our study. The difference might be due to the typing method used. HPA-3a was more prevalent than -3b in all populations except Indonesians.

The incidences of NAITP, PTP and refractoriness to platelet transfusion therapy in Thais have not been reported. Anti-HPA-1a is the most common cause of NAITP and PTP in Caucasians[36]. Anti-HPA-1b, -HPA-2a, -HPA-2b, -HPA-3a, -HPA-3b, -HPA-4a, -HPA-4b -HPA-5b and -HPA-6a were also found in NATP, PTP and refractoriness to platelet transfusion therapy in Caucasians and Japanese[37-42]. The HPA genotypes from this study suggest that HPA-1a, -4a, -4b and -5a will not be involved in NAITP, PTP and refractoriness to platelet transfusion therapy, in ethnic Northeastern Thais. HPA-typing for HPA-1b, -2a, -2b, -3a, -3b, -5b and -6b in both patients and donors is required in order to reduce the risk of developing platelet alloantibodies in these patients.

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การศึกษาชนิดของ Platelet antigens ในชาวไทยภาคตะวันออกเฉียงเหนือ ด้วยเทคนิค PCR-SSP

อมรรัตน์ ร่มพฤกษ์, วท.ม., จงกล อรรคฮาต, วท.ม.*, ปราโมทย์ ศรีวาณิชรักษ์, วท.ม.*, จินตนา พัวไพโรจน์, พ.บ.*, อรุณรัฐ ร่มพฤกษ์, วท.ม.**, ชาญวิทย์ ลีลายุวัฒน์, ปร.ด.***

ศึกษาความถี่แอนติเจนของเกร็ดเลือดจำนวน 6 ระบบ ในกลุ่มประชากรชาวไทยภาคตะวันออกเฉียงเหนือ โดยใช้ตัวอย่างทดสอบจากประชากรที่มีสุขภาพแข็งแรง มีเชื้อชาติไทย มีภูมิลำเนาอยู่ในภาคตะวันออกเฉียงเหนือ และไม่เป็นเครือญาติกัน จำนวน 300 ราย ทำการตรวจหาแอนติเจนของเกร็ดเลือดโดยใช้ เทคนิค polymerase chain reaction-sequence specific primer (PCR-SSP) พบความถี่ของแอนติเจน HPA-1a, -4a, -5a, -6a เท่ากับ 100% และ HPA-1b(5.7%), -2a(99.7%), -2b(12.3%), -3a(78.0%), -3b(71.3%), -5b(7.3%), -6b(3.0%) โดยที่ไม่พบแอนติเจน HPA-4b ความถี่ของแอนติเจนจากการศึกษาครั้งนี้จะคล้ายกับชนชาวเอเชียด้วยกัน แต่แตกต่างจากชนผิวขาว จากการศึกษาครั้งนี้จะเห็นว่า HPA-1a, -4a, -4b, -5a และ -6a จะไม่เป็นสาเหตุเกี่ยวข้องกับภาวะ neonatal alloimmune thrombocytopenia (NAITP), post-transfusion purpura (PTP) และ refractoriness to platelet transfusion therapy ในประชากรไทยภาคตะวันออกเฉียงเหนือ นอกจากนี้แล้ว พบว่าเทคนิค PCR ที่พัฒนาได้เป็นเทคนิคที่สะดวก รวดเร็ว มีความถูกต้องแม่นยำสูง สามารถนำไปใช้ตรวจหาแอนติเจนของเกร็ดเลือดในผู้ป่วย เพื่อวิเคราะห์หาสาเหตุในผู้ป่วยที่มีปัญหาจากการรับเกร็ดเลือดบ่อย ๆ และการเตรียมเกร็ดเลือดจากผู้บริจาคที่เหมาะสมให้แก่ผู้ป่วยต่อไป

คำสำคัญ : แอนติเจนของเกร็ดเลือด, เทคนิค PCR-SSP

อมรรัตน์ ร่มพฤกษ์, จงกล อรรคฮาต, ปราโมทย์ ศรีวาณิชรักษ์, จินตนา พัวไพโรจน์, อรุณรัฐ ร่มพฤกษ์, ชาญวิทย์ ลีลายุวัฒน์
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* คลังเลือดกลาง, คณะแพทยศาสตร์,
** ภาควิชาภูมิคุ้มกันวิทยาคลินิก, คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น, จ.ขอนแก่น 40002