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Original Article

Platelet immunology of patients with hemophilia

Shu-Hong Yu*, Zong-Zheng Han*, Min Wang

*Hanchuan People's Hospital, Hanchuan 432300, Hubei Province, China. *Equal contributors.*

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Abstract: Platelets are important in hemostasis and participate widely in metabolism. Glycoproteins (GPs) on the surface of platelets can fold into different spatial structures caused by single-nucleotide polymorphisms (SNPs) and perform as antigens. Human platelet antigen (HPAs) immunology has been reported to have a close relationship with many clinical issues, suggesting the importance of HPA genotyping and platelet antibody detection. In this study, we aimed to detect human platelet antigen (HPA) and HPA antibody characters of hemophilia patients in Hanchuan, China. Totally, 127 hemophilia A (HA) and 33 hemophilia B (HB) patients were included in this study. We examined the HPA genotypes of hemophilia patients by PCR with specific primers (PCR-SP). Then we calculated the frequencies of HPA alleles in hemophilia patients using the gene-counting method and compared them with data from normal people in China. The results showed no significant differences except that the prevalence of HPA-2b was significantly higher in HA patients when compared to healthy people (0.0827 vs. 0.0485, P=0.0212) and the prevalence of HPA-4b was significantly higher in HB patients when compared to both HA patients (0.0758 vs. 0.0079, P=0.0008) and healthy people (0.0758 vs. 0.0045, P<0.0001). Finally, using Luminex assay, we detected the features of HPA antibody in hemophilia patients and found ratios of anti-HPA-3a (7.87% in HA patients, 9.09% in HB patients, and 1.26% in healthy people), anti-HPA-5b (3.15% in HA patients, 3.03% in HB patients, and 0.42% in healthy people), and anti-HPA-2b (3.15% in HA patients, 3.03% in HB patients, and 0.21% in healthy people) were all higher in hemophilia patients. To conclude, our data indicate that the detection and identification of clinically relevant platelet antibodies are important for patients with hemophilia to prevent immune-mediated thrombocytopenia.

Keywords: Human platelet alloantigen, antibody, hemophilia, Chinese

Introduction

Hemophilia is an X-linked recessive, inherited bleeding disease and can be divided into hemophilia A (HA) or hemophilia B (HB). HA patients lack coagulation factor VIII (cFVIII) while HA patients lack coagulation factor IX (cFIX) [1]. The prevalence of HA ranges from 1/5000 to 1/10,000 [2], and the prevalence of HB ranges from 1/25000 to 1/30,000 [3]. Hemophilia patients are divided into mild level (cF arranging from 5% to 30%), moderate (cF ranging from 1% to 5%), or severe (cF less than 1%) [4-6]. China has at least 130,000 hemophilia patients, who need transfusions regularly to avoid blood loss caused by various accidents [7].

HPAs are a family of glycoproteins (GP) expressed on the surface of platelets and play a role in blood coagulation through ligand-receptor interactions. HPAs on the surface of platelets can fold into different spatial structures induced by single-nucleotide polymorphisms (SNPs). To date, 36 human platelet antigens (HPAs) have been identified and are involved in six platelet GPs: GPIa, GPIbα, GPIbβ, GPIIb, GPIIIa, and CD109 [8]. Moreover, platelets have other antigens, including CD36. HPAs are variant in the frequency of genotypes between different populations [9-12]. Of the 36 human platelet antigens, HPA-1a to HPA-16b are most widely identified through PCR with specific primers (PCR-SP) [13-15].

Due to the polymorphic HPA subtypes, different GPs may perform as antigens to stimulate the production of specific antibodies when an individual is exposed to transfusion [16]. HPA antibodies are associated with numerous clinical conditions, including transplantation-associated alloimmune thrombocytopenia, alloimmune

thrombocytopenia, posttransfusion purpura (PTP), and platelet transfusion refractoriness (PTR) [17-19], seriously threatening people's lives. Thus, detection and identification of HPA antibodies are important for patients who tend to be affected by platelet antibodies, for example, peoples who often need transfusions.

Previous studies have reported the HPA features and low frequencies of HPA antibodies of normal people belonging to Ethnic Hans in China, indicating little threat of HPA antibodies to normal people [20-23]. Because of regular transfusions, hemophilia patients may be more easily affected by platelet antibodies. However, the HPA and HPA antibody findings of patients with hemophilia have never been studied. Here we report the HPA and HPA antibody findings of patients with hemophilia in Hanchuan, China, and compare them to normal controls.

Materials and methods

Patients

This study was conducted from 2015 to 2020 in the Hanchuan People's Hospital. Human samples involved in this study were managed using protocols approved by the Ethical Committee of the Hanchuan People's Hospital (E2015003). Informed consent was obtained from all patients. Hemophilia patients that belonged to ethnic Hans and whose factor level was less than 30% were included.

DNA extraction

Blood samples (1 ml) from 127 hemophilia A and 33 hemophilia B patients were collected using EDTA anticoagulation tubes. Collected samples were frozen in liquid nitrogen and stored at -80°C. DNA was extracted with a Pure Link Genomic DNA Kit (Thermo Fisher).

Data from normal Chinese people

The data of healthy persons' HPAs were obtained from a previous study by Feng, et al. [13]. The data of healthy persons' HPA antibodies were obtained from a previous study by Wu Guoguang, et al. [9].

Genotyping

PCR with specific primers (PCR-SP) was used to perform the genotyping as described in previous studies [13, 24, 25]. The primers used are shown in **Table 1**. Sequences were obtained from GenBank or published papers [13]. Briefly, for each HPA subtype, coupled specific primer and common primer were used and the PCR was performed with a Prime STAR GXL DNA Polymerase kit (Takara). All PCRs were run for 35 cycles with an annealing temperature of 60°C. PCR products were separated by agarose electrophoresis and visualized by UV transillumination.

Anti-HPA antibody detection (Luminex assay)

Luminex assay was used for anti-HPA antibody detection as described previously [26]. Briefly, antibodies anti-GPIIb/IIIa (Acris), anti-GPIa/IIa (Beckman Coulter), anti-GPIb/IX (Santa Cruz), and anti-CD109 (MBL) were separately added to Luminex xMAP beads (5.0×10^6) while human IgG (Acris) was used as a positive control. 500 µl buffer was added to the beads and incubated at room temperature for 2 h. Then the beads were washed with PBS-TBN solution 2 times and diluted to a final concentration of 1×10^3 beads/µl. Thereafter, the platelets whose HPA genotypes were already validated were washed and resuspended. 5×10^6 platelets were mixed with 15 µl target serum samples at 37°C for 0.5 h. After washing 2 times, treated platelet samples were reacted with goat-anti-human IgG at 37°C for 0.5 h. Then the samples were lysed and 20 µl lysate was reacted with 5 µl beads at 37°C for 0.5 h. 50 µl phycoerythrin-conjugated goat-anti-human IgG was added to the samples and incubated at 37°C for 0.5 h. Finally, 50 µl PBS-TBN buffer was added and the samples were analyzed on a Luminex 100 instrument.

Statistical analysis

Gene-counting method was used for gene frequency calculation. Data are presented as mean ± SD or numbers. Statistical analysis was performed using SPSS18.0 software. Comparisons of gene frequencies between different groups were performed using the χ^2 test.

Result

HPA types of HA and HB patients

127 HA and 33 HB patients included in this study all belonged to the ethnic Hans. The age

Table 1. Primer sequences for HPA genotyping

HPA allele	Specific primer sequence	Common primer sequence
1a	1aF ACTTACAGGCCCTGCCTCT	1R GTGCAATCCTCTGGGGACT
1b	1bF ACTTACAGGCCCTGCCTCC	
2a	2aF CCCCCAGGGCTCCTGAC	2R GCCAGCGACGAAAATAGAGG
2b	2bF GCCCCCAGGGCTCCTGAT	
3a	3aR GGGGGAGGGGCTGGGGA	3F GAAAGACCTGGGAAGGCGG
3b	3bR GGGGGAGGGGCTGGGGC	
4a	4aF GCTGGCCACCCAGATGCG	4R GCTGTCCTGGCGTCTGGAG
4b	4bF AGCTGGCCACCCAGATGCA	
5a	5aF AGTCTACCTGTTTACTATCAAAG	5R CTCTCATGGAAAATGGCAGTA
5b	5bF AGTCTACCTGTTTACTATCAAAA	
6a	6aF GACGAGTGCAGCCCCCG	6R TAGCGGACACAGGAGAAGTC
6b	6bF GGACGAGTGCAGCCCCCA	
7a	7aF CCAAGGTGCGAGGCTGTC	7R CGGCATACCCCACACTCAA
7b	7bF CCAAGGTGCGAGGCTGTG	
8a	8aR ACTGACTCAATCTCGTCACA	8F TGTGTGTGTGTTTTAATGGAGG
8b	8bR GGCAGCCCCCAGTCCAC	
9a	9aR GGGCAGCCCCCAGTCCAT	9F CTGGATATACAGCCCCAGGG
9b	9bR CTGAGCTACTTCCCCAAGAC	
10a	10aF CCCAGTGAGTGAGGCCCG	10R ACTGACTCAATCTCGTCACG
10b	10bF TCCCAGTGAGTGAGGCCCA	
11a	11aF ACCGAAAATACCTGCAACCG	11R CCAGCTCACATCAAGTGTCC
11b	11bF GACCGAAAATACCTGCAACCA	
12a	12aF GACGCTCGTGGACTGCGG	12R GCAACGCAGGTCGCGGTAG
12b	12bF GACGCTCGTGGACTGCGA	
13a	13aF CAAAAGGTTAACATTTTCAGTAAC	13R TACCGGTAGGGAGAATGATGC
13b	13bF CAAAAGGTTAACATTTTCAGTAAT	
14a	14aR CAGACTCCACACTCACTTCTT	14F GACTCCGACTGGACCGGC
14b	14bR CAGACTCCACACTCACTTAAA	
15a	15aF TTCAAATTCTTGGTAAATCCTGG	15R ATGACCTTATGATGACCTATTC
15b	15bF TTCAAATTCTTGGTAAATCCTGT	
16a	16aR GTGAGCTTTCGCATCTGGG	16F GGGAGAAGAAGATAAAAACTAAC
16b	16bR GGTGAGCTTTCGCATCTGGA	

of the patients ranged from 12 to 50 years (20.3±13.6 years), All patients were male, of which 41.87% (67 persons) had moderate disease with factor level ranging from 1% to 5%, and 36.87% (59 persons) had severe disease with factor level less than 1%. Detailed information about the patients is shown in **Table 2**.

Detailed information on the patients' HPA genotypes is listed in **Table 3**. For HPA-7 to HPA-14 and HPA-16, all the patients were of aa genotype. For HPA-1, HPA-2, HPA-4, HPA-5, and HPA-6, the vast patients were of aa genotype (ranging from 85.83% to 98.43%) while the rest were almost heterozygous of ab genotype. Homozygotes for gene b were very rare only HPA-2bb and HPA-5bb were observed with ratios of 2.36% and 0.79%, respectively, in HA patients. The frequencies of gene a and b were relatively balanced in HPA-3 and HPA-15, with similar ratios of aa and bb.

Comparison of HPA types between HA and HB patients, and healthy people

Based on the information of HPA genotypes, we calculated frequencies of the 32 HPA alleles in the HA and HB patients collected by us, and then we compared the data with healthy persons' HPA characters which were reported

Table 2. Basal information of the patients

Variable	Statistics
Age (Years)	20.3±13.6
Sex	
Male	160
Female	0
Height (cm)	153.1±13.7
Weight (kg)	50.6±7.2
Hemophilia subtype	
Hemophilia-A	127
Progression	
Mild	23
Moderate	55
Severe	49
Hemophilia-B	33
Progression	
Mild	11
Moderate	12
Severe	10

by Feng, et al. (**Table 4**) [13]. The majority of the HPA alleles had no significant differences between different groups (**Table 3**). We observed that the prevalence of HPA-2b was slightly increased in HA patients with a significant difference (χ^2=5.306, P=0.0212) when compared to healthy people, and it is worth noting that only HPA-2bb was detected in HA patients (**Tables 3** and **4**). Nevertheless, we found increased HPA-4b in HB patients which was significant compared to both HA patients (χ^2=11.28, P=0.0008) and healthy people (χ^2=48.2, P<0.0001).

Detection of platelet antibodies in HA and HB patients

With the data of the HPA types in HA and HB patients, we needed to detect the HPA antibodies in HA and HB patients because HPA antibodies are crucial for the safety of transfusion [18]. Our data revealed that there is no significant difference between hemophilia patients and healthy people in the prevalence of anti-CD36 (**Table 5**). The data of healthy people were obtained from a previous study by Wu Guoguang, et al. [9]. We could not observe a positive signal of anti-HPA-15 (data not shown), consistent with the conclusion of a previous report [26]. Surprisingly, we found that hemophilia patients had a significantly higher prevalence of anti-HPA-3a, anti-HPA-5b, and anti-HPA-2b when compared to healthy people (**Table 5**). In detail, the prevalence of anti-HPA-3a was 7.87%, 9.09%, and 1.26% in HA patients, HB patients, and healthy people, respectively; the prevalence of anti-HPA-5b was 3.15%, 3.03%, and 0.42% in HA patients, HB patients, and healthy people, respectively; the prevalence of anti-HPA-2b was 3.15%, 3.03%, and 0.21% in HA patients, HB patients, and healthy people, respectively. Even though there are no data about anti-HPA-3b in healthy people, we do believe that the prevalence of anti-HPA-3b was also higher in hemophilia patients. Moreover, all the three HPA-2bb patients or the single HPA-5bb patient in the HA group had been detected positive for anti-HPA-2a or anti-HPA-5a, respectively (**Table 5**), shedding light on that the low frequencies of anti-HPA-2a, and anti-HPA-5a may be limited by the low frequencies of HPA-2bb and HPA-5bb in the whole population.

Discussion

In China, numerous hemophilia patients are threatened by HPA antibodies for their crucial need for transfusions. It is urgently necessary to make certain of the HPA and anti-HPA characters of hemophilia patients. Our study revealed the HPA and anti-HPA characters among hemophilia patients in China, filling the gap.

In this study, we focused on patients who belonged to ethnic Hans. Firstly, ethnic Han constitute the majority of people in China, and more than 99% of people belong to ethnic Hans at Hanchuan. The study on Ethnic Hans could cover most populations. Secondly, previous studies have reported that different nationalities have different HPA types [9], so integrated analysis of different nationalities may lead to a confusing result. However, research about minorities' HPA and HPA antibody types is worth study in future work.

Our data revealed a slightly significant increase in the prevalence of HPA-2b in HA patients, indicating there may exist a relationship between HPA-2b with the occurrence of HA. This has never been reported. A previous study has indicated a relationship between ABO groups with VWF in hemophilia patients [27]. The phenomenon obtained by us may have a similar mechanism. However, the result still

Table 3. HPA types of HA and HB patients

Subtype	Hemophilia-A Patients (n=127)			Hemophilia-B Patients (n=33)			Hemophilia-A Patients			Hemophilia-B Patients		
	aa	ab	bb	aa	ab	bb	aa (%)	ab (%)	bb (%)	aa (%)	ab (%)	bb (%)
HPA-1	125	2	0	33	0	0	98.43	1.57	0.00	100.00	0.00	0.00
HPA-2	109	15	3	31	2	0	85.83	11.81	2.36	93.94	6.06	0.00
HPA-3	47	57	23	13	13	7	37.01	44.88	18.11	39.39	39.39	21.21
HPA-4	125	2	0	28	5	0	98.43	1.57	0.00	84.85	15.15	0.00
HPA-5	124	2	1	31	2	0	97.64	1.57	0.79	93.94	6.06	0.00
HPA-6	122	5	0	33	0	0	96.06	3.94	0.00	100.00	0.00	0.00
HPA-7	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00
HPA-8	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00
HPA-9	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00
HPA-10	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00
HPA-11	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00
HPA-12	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00
HPA-13	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00
HPA-14	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00
HPA-15	34	66	27	10	15	8	26.77	51.97	21.26	30.30	45.45	24.24
HPA-16	127	0	0	33	0	0	100.00	0.00	0.00	100.00	0.00	0.00

needs careful further confirmation. Firstly, the sample size of this study may be too small. Secondly, a previous study has reported that the frequency of gene HPA-2b may be basally high in some minorities [9]. To preclude these influencing factors, a larger sample size and a multi-center study may be needed. Despite HPA-2b, we also found HPA-4b was significantly higher in HB patients compared to HA patients or healthy people. It should be noted that the sample size of the HB group was even smaller than the HA group, so a bigger sample size is also needed. Even though those two findings are interesting, further study is needed.

Sudan, et al. have reported that anti-HPA-15 could not be detected in vitro based on existing technology [26]. Our experiments confirm their study. Nowadays, restricted to the detection technology, only anti-HPA-1, anti-HPA-2, anti-HPA-3, anti-HPA-5, and anti-CD36 are available and convincing [26], arresting the application of anti-HPA antibody detection in the clinic, and calling for technical optimization. Moreover, there is no HPA-1bb reported in ethnic Han in any research, so there may not exist any anti-HPA-1. Therefore, we used the mono-antibody (anti-GPIIb/IIIa, clone: P2), which could detect HPA-1a and HPA-3a by the same time, to detect anti-HPA-3a.

We note that the prevalence of anti-HPA-3a or anti-HPA-3b is predominantly higher in healthy people or hemophilia patients. This may be explained by their almost half-and-half frequencies, which directly induces a higher risk for the HPA-3aa or HPA-3bb individuals to receive the blood from the other type of homozygotes. The same logic is also appropriate for HPA-15, whose genotype's constitution is similar to HPA-3. Moreover, all the three HPA-2bb patients or the single HPA-5bb patient were positive for anti-HPA-2a or anti-HPA-5a, respectively. These findings show the importance of anti-HPA antibody detection for homozygous individuals, since they may suffer a much higher risk of accidents caused by anti-HPA antibodies.

In our study, we divided the hemophilia patients into HA and HB groups. It is worth noting that hemophilia patients can be divided into different levels based on their cF concentration. Therefore, a more refined classification may stratify the findings.

In conclusion, we reported the HPA and anti-HPA antibody types of hemophilia patients in Hanchuan, China. Our data indicate a relationship between HPA-2b with HA, and a relationship between HPA-4b with HB. However, both the findings need further studys. Our data

Table 4. Comparison of HPA types between HA and HB patients, and healthy people

Subtype	Gene frequency of Hemophilia-A		Gene frequency of Hemophilia-B		Gene frequency of normal Chinese people		Hemophilia-A vs. Hemophilia-B		Hemophilia-A vs. Normal		Hemophilia-B vs. Normal	
	a	b	a	b	A	b	χ^2	P	χ^2	P	χ^2	P
HPA-1	0.9921	0.0079	1.0000	0.0000	0.9940	0.0060	0.5230	0.4696	0.1282	0.7203	0.3983	0.5280
HPA-2	0.9173	0.0827	0.9697	0.0303	0.9515	0.0485	2.1540	0.1422	5.3060	0.0212	0.4637	0.4959
HPA-3	0.5945	0.4055	0.5909	0.4091	0.5945	0.4055	0.0030	0.9579	0.0000	0.9997	0.0030	0.9534
HPA-4	0.9921	0.0079	0.9242	0.0758	0.9955	0.0045	11.2800	0.0008	0.5283	0.4673	48.2000	<0.0001
HPA-5	0.9843	0.0157	0.9697	0.0303	0.9860	0.0140	0.6032	0.4374	0.0492	0.8244	1.1870	0.2760
HPA-6	0.9803	0.0197	1.0000	0.0000	0.9565	0.0135	1.3200	0.2506	3.2650	0.0708	2.9970	0.0834
HPA-7	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	NA	NA	NA	NA	NA	NA
HPA-8	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	NA	NA	NA	NA	NA	NA
HPA-9	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	NA	NA	NA	NA	NA	NA
HPA-10	1.0000	0.0000	1.0000	0.0000	0.9995	0.0005	NA	NA	0.1271	0.7215	0.0330	0.8558
HPA-11	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	NA	NA	NA	NA	NA	NA
HPA-12	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	NA	NA	NA	NA	NA	NA
HPA-13	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	NA	NA	NA	NA	NA	NA
HPA-14	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	NA	NA	NA	NA	NA	NA
HPA-15	0.5276	0.4724	0.5303	0.4697	0.5320	0.4680	0.0016	0.9683	0.0178	0.8937	0.0007	0.9783
HPA-16	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	NA	NA	NA	NA	NA	NA

Table 5. Detection of Platelet Antibodies in HA and HB patients

Platelet antibodies	Hemophilia-A Patients (%)	Hemophilia-B Patients (%)	Normal People (%)	Hemophilia-A vs. Hemophilia-B		Hemophilia-A vs. Normal		Hemophilia-B vs. Normal	
				χ^2	P	χ^2	P	χ^2	P
Anti-CD36	2/127 (1.57)	1/33 (3.03)	15/478 (3.14)	0.3016	0.5829	0.8979	0.3433	0.0012	0.9726
Anti-HPA-3a	10/127 (7.87)	3/33 (9.09)	6/478 (1.26)	0.0519	0.8197	17.0700	<0.0001	10.9500	0.0009
Anti-HPA-5b	4/127 (3.15)	1/33 (3.03)	2/478 (0.42)	0.0012	0.9720	7.6230	0.0058	3.6080	0.0575
Anti-HPA-5a	1/127 (0.79)	0/33 (0.00)	1/478 (0.21)	0.2615	0.6091	1.0180	0.3130	0.0691	0.7925
Anti-HPA-2b	4/127 (3.15)	1/33 (3.03)	1/478 (0.21)	0.3016	0.5829	10.5800	0.0011	6.3020	0.0121
Anti-HPA-2a	3/127 (2.36)	0/33 (0.00)	-	0.7944	0.3728	NA	NA	NA	NA
Anti-HPA-3b	13/127 (10.24)	4/33 (12.12)	-	0.2423	0.6225	NA	NA	NA	NA

reveal much higher frequencies of anti-HPA antibodies in hemophilia patients compared to healthy people, indicating a higher risk of thrombocytopenia and severe post-transfusion reactions, calling for the necessity of anti-HPA antibody detection in the hemophilia patients to guide their transfusion plan.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Min Wang, Hanchuan People's Hospital, No. 1, Renmin Avenue, Hanchuan, Hubei Province, China. Tel: +86-189-07292598; E-mail: wangmin7411@163.com

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