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High-Throughput CD36 Phenotyping on Human Platelets Based on Sandwich ELISA and Mutant Gene Analysis

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Keywords

CD36 deficiency · Phenotyping · Sandwich ELISA · pCD36-negative phenotype database

Abstract

Background: CD36 deficiency is closely associated with fetal/neonatal alloimmune thrombocytopenia, platelet transfusion refractoriness, and other hemorrhage disorders, particularly in Asian and African populations. There is a clinical need for rapid and high-throughput methods of platelet CD36 (pCD36) phenotyping to improve the availability of CD36 typing of donors and assist clinical blood transfusions for patients with anti-CD36 antibodies. Such methods can also support the establishment of databases of pCD36-negative phenotypes. ***Study Design and Methods:*** A sandwich enzyme-linked immunosorbent assay (ELISA) for CD36 phenotyping of human platelets was developed using anti-CD36 monoclonal antibodies. The reliability of the assay was evaluated by calculating the intra-assay and inter-assay coefficients of variation (CV). A total of 1,691 anticoagulant whole blood samples from healthy blood donors were randomly selected. PCD36 expression was measured using a sandwich ELISA. PCD36 deficiency was confirmed by flow cytometry (FC). Mutations underlying pCD36 deficiency were identified using polymerase chain reaction sequence-based typing (PCR-SBT). ***Results:*** The sandwich ELISA for pCD36 phenotyping had high reliability (intra-assay CV, 2.1–4.8%; inter-assay CV, 2.3–5.2%). The sandwich ELISA was used to screen for CD36 expression on platelets isolated from 1,691 healthy blood donors. Of these, 36 samples were pCD36-negative. FC demonstrated absence of CD36 expression on monocytes in three of the 36 cases. In the present study population, the frequency of CD36 deficiency was 2.13% (36/1,691), of which 0.18% (3/1,691) was type I deficiency and 1.95% (33/1,691) was type II deficiency. In addition, we used PCR-SBT to characterize the gene mutations in exons 3–14 of the CD36 gene in 27 cases of CD36 deficiency and discovered 10 types of mutations in 13 pCD36-negative samples. ***Conclusion:*** The present study describes the development and characterization of a highly reliable sandwich ELISA for high-throughput screening for pCD36 expression. This novel method is feasible for clinical applications and provides a useful tool for the establishment of databases of pCD36-negative phenotype donors.

Introduction

CD36, a highly glycosylated 88-kDa protein also known as platelet glycoprotein IV (GPIV) or GPIIIb, is one of the most important glycoproteins expressed by platelets. CD36 is also expressed by monocytes,

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erythroblasts, adipocytes, endothelial cells, skeletal myocytes, and cardiac myocytes. CD36 belongs to the class B scavenger receptor family [1], which plays an important role in platelet aggregation, atherosclerosis, cardiopathy, diabetes, and malaria [2–6].

The frequency of CD36 deficiency varies widely between ethnic groups, with a prevalence of 3–11% in Asians, 8% in sub-Saharan Africans, and less than 0.4% in Caucasians [7–10]. CD36 deficiency can be categorized into two types: type I is characterized by a lack of CD36 expression on both platelets and monocytes, and type II is characterized by the lack of CD36 surface expression on platelets only. The frequency of type II CD36 deficiency is significantly higher than type I CD36 deficiency. Patients with type I CD36 deficiency are more likely to produce anti-CD36 antibodies [11, 12]. Individuals with CD36 deficiency are prone to the development of anti-CD36 isoimmunization during blood transfusion, pregnancy, or organ transplantation, which can lead to serious clinical conditions such as platelet transfusion refractoriness, post-transfusion purpura, fetal/neonatal alloimmune thrombocytopenia (FNAIT), and transfusion-related acute lung injury (TRALI) [13–18]. Accordingly, research on CD36 antigens and antibodies is of great clinical significance.

Flow cytometry (FC) is currently the most widely used and direct method of detecting pCD36 expression in laboratory settings [12], but it is rarely used in clinical transfusion departments. Accordingly, there is a clinical need for the development of novel high-throughput methods for pCD36 phenotyping to meet the routine needs of clinical laboratories and blood centers and improve the effectiveness of platelet transfusion.

We therefore developed a sandwich enzyme-linked immunosorbent assay (ELISA) for high-throughput pCD36 phenotyping. This novel method was also evaluated by specifically screening for pCD36-deficient individuals in Suzhou city. To validate the results, we also determined the CD36 phenotype and genetic background of the study subjects. This method may have utility in establishing regional pCD36-negative phenotype databases to ensure the safety and effectiveness of clinical platelet transfusions.

Materials and Methods

Blood Donors

A total of 1,691 whole blood samples were randomly collected from healthy blood donors during routine blood donation at the Suzhou Blood Center from October 2020 to May 2021. All blood donors were ethnic Han and met the national standards for blood donation following clinical examination. All donors recruited to the present study provided written informed consent.

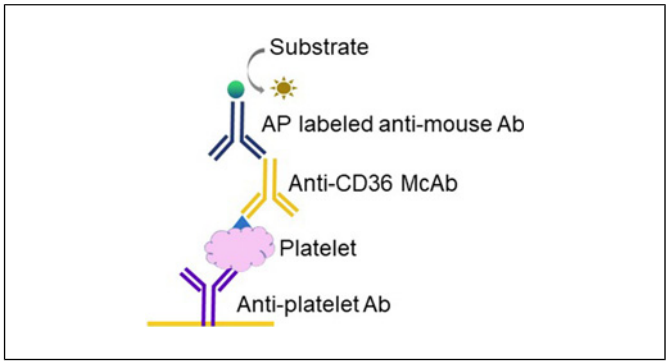


Fig. 1. Experimental schematic of the proposed method of sandwich ELISA for CD36 phenotyping.

Platelet Preparation

Anticoagulant whole blood samples from donors were collected in EDTA buffer and centrifuged at 400 *g* for 10 min. Platelet-rich plasma (PRP) was obtained, and the top three quarters of PRP samples were transferred into clean tubes. Platelet concentration was adjusted to approximately 100×10^9/L in an isotonic saline solution containing 0.1% NaN_3 and stored at 4°C for up to 3 days.

Antibodies Used for ELISA and FC

Rabbit anti-human integrin beta 3 antibody (ab197662, Abcam; Cambridge, UK) was used as a coating antibody to immobilize platelets onto the surface of microplates. Murine anti-human monoclonal antibody specifically targeting glycoprotein CD36 (clone: TR9, IgG1, SAB4700165, Sigma-Aldrich; St. Louis, Missouri, USA) was used to detect CD36. Alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific, A1418, Sigma-Aldrich) was used as the enzyme-linked antibody in ELISA. Phycoerythrin (PE)-conjugated anti-human CD36 (clone 5–271, Biolegend; San Diego, CA, USA) and FITC anti-human CD14 antibody (clone HCD14, Biolegend; San Diego, CA, USA) were used for FC detection.

Sandwich ELISA for CD36 Phenotyping

The wells of flat-bottom 96-well microplates were coated with rabbit anti-human integrin beta 3 antibodies (10 μg/mL) in 0.05-M sodium carbonate buffer and incubated overnight at 4°C. Microplates were then blocked for 2 h with 1% bovine serum albumin (BSA) to prevent non-specific binding, and then washed five times with Tris-buffered saline containing 0.05% Tween-20 (TBST). Then, 50 μL of platelet suspension was added to each well, and plates were centrifuged at 50 *g* for 5 min. Excess unbound platelets were removed by washing. Immediately after washing, 100 μL of murine anti-human CD36 monoclonal antibody (5 μg/mL) was added to each well, and plates were incubated at 37°C for 30 min. After washing five times, the antigen and antibody complexes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific, 1:6,000 dilution in 100 μL) at 37°C for 30 min. After washing, p-nitrophenyl phosphate (PNPP) was used as a chromogenic substrate for the alkaline phosphatase to allow chromogenic detection. Absorbance was measured at 405 nm using an ELISA reader after the addition of a 3 M NaOH solution (Fig. 1).

This method was validated using platelets from eight pCD36-positive and two pCD36-negative samples (each independently confirmed by FC). The intra-assay and inter-assay coefficients of variation (CVs) were calculated to evaluate the repeatability of the assay.

Table 1. Specific primers for CD36 genotyping

Exon	Sequence of primer (5′-3′)	PCR product length (bp)
Exon 3	F: 5′-AATAAAACATCTGTTACCATAC-3′ R: 5′-ATAAAGCAGGTAAATACAAAGC-3	753
Exon 4	F: 5′-TTTTATTCTGGCTGACTC-3′ R: 5′-ATGACATTTGCCAAGTAG-3′	335
Exon 5	F: 5′-AGATCTAATGTTCACATATG-3′ R: 5′-GATTAATTACATGAGTTCTAG-3′	267
Exon 6	F: 5′-TTGTATTAAGCTCAATATTAGC-3′ R: 5′-ATAAAATTATGCCTTGCC-3′	350
Exon 7	F: 5′-AAGTAACATTTTCCCATAC-3′ R: 5′-ATGAATACTATTCCTGCT-3′	187
Exon 8	F: 5′-TGCAATAAGATAAAAGGTTC-3′ R: 5′-AATTTTGTTGTGGGGATA-3′	356
Exon 9	F: 5′-ATGGACTACACTGGAGGAG-3′ R: 5′-CTGGACTTGATCGTTAATAGAC-3′	400
Exon 10	F: 5′-AGTTCAGGTTCCTGGAATGC-3′ R: 5′-ATGGACTGTGCTACTGAGGT-3′	262
Exon 11	F: 5′-TAGACATATTACTGCCTGAA-3′ R: 5′-AGGAAGAAATCGACCTAA-3′	485
Exon 12	F: 5′-CCTTAAGTTACTACCTTCTC-3′ R: 5′-AAGTGTGGATTTTTCCTTTCA-3′	201
Exon 13	F: 5′-TATTTCAGTTCCCCGAGA-3′ R: 5′-TTTGTTCAATTGGATCAT-3′	463
Exon 14	F: 5′-CTTGCCTTATAGATACTG-3′ R: 5′-TACTTTAGTGATCTGCGT-3′	470

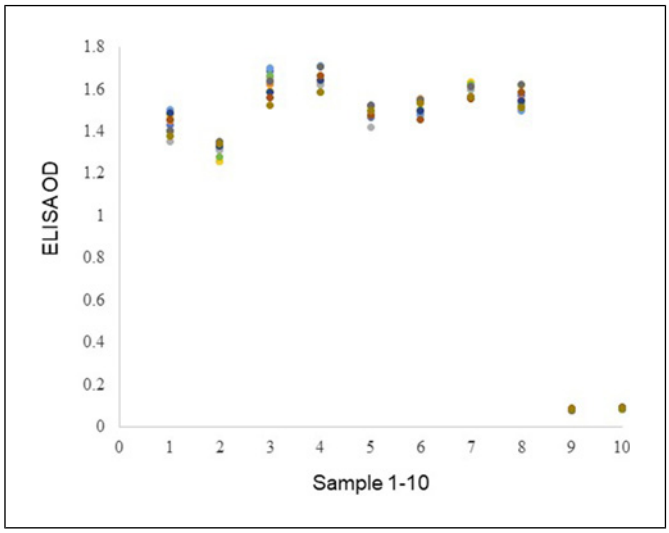


Fig. 2. Intra-assay variability of sandwich ELISA for CD36 phenotyping.

Evaluation and Application of the Sandwich ELISA

The proposed sandwich ELISA method for detecting pCD36 expression was used to screen 1,691 whole blood samples. To evaluate the feasibility of the sandwich ELISA, the ELISA results were subsequently validated using FC. Platelet suspensions of pCD36-negative specimens were first incubated with PE-conjugated anti-human CD36 antibody for 30 min at room temperature and then washed with 1% BSA in PBS. Platelet pellets were then resuspended in 300 μL of PBS. Mean CD36 fluorescence intensity was analyzed using FC (Beckman, DxFLEX, Beckman Coulter, Inc.; CA, USA) to confirm pCD36 expression.

CD36 Phenotyping

Peripheral blood mononuclear cells were isolated from confirmed pCD36-negative specimens by standard density gradient centrifugation using Lymphoprep™ (Stemcell Technologies; Vancouver, Canada). Cells were resuspended and incubated with PE-conjugated anti-human CD36 and FITC-conjugated anti-human CD14 antibody at 4°C for 30 min. Cells were then washed with 1% BSA in PBS and centrifuged. Finally, cells were resuspended in 300 μL of PBS for FC analysis to identify the phenotype of CD36-deficient samples. A PE-labeled isotype antibody was used as a control.

Molecular Background Analysis of CD36-Deficient Samples

Twenty-seven CD36 deficiency samples were used for polymerase chain reaction (PCR) sequence-based typing. Genomic DNA was extracted from EDTA-anticoagulated blood using blood

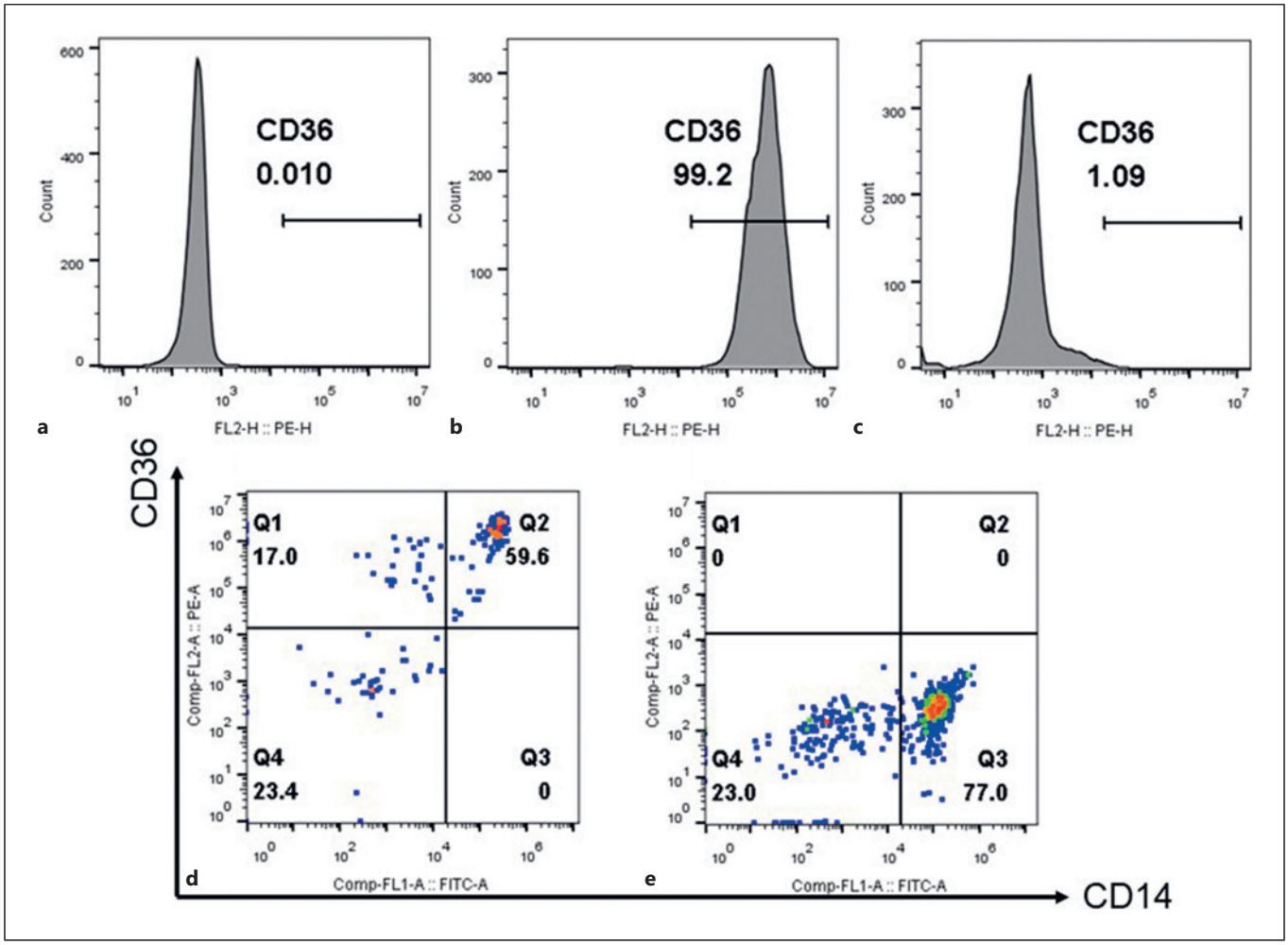


Fig. 3. Flow cytometric confirmation of CD36 expression on platelets and monocytes. **a** pCD36 expression in the isotype control group. **b** pCD36 expression in the positive control group. **c** Samples with confirmed pCD36 deficiency. **d** Positive expression of CD36 antigen on the surface of monocytes. **e** Negative expression of CD36 antigen on the surface of monocytes.

Table 2. CD36 deficiency types and frequency

Type of CD36 deficiency	Number	Frequency, %
I	3	0.18
II	33	1.95
Normal	1,655	
Total	1,691	2.13

DNA kits. Exon 3 to exon 14 of the CD36 gene, including the relevant flanking introns, were amplified using exon-specific primers (Table 1). PCR was performed using the following cycling steps and parameters: 35 cycles of denaturation (30 s, 94°C), annealing (30 s, 60°C), and extension (30 s, 72°C), followed by a final extension step (5 min, 72°C). PCR products of the CD36 gene were purified and sequenced.

Statistical Analyses

The intra-assay and inter-assay mean CV was calculated for the sandwich ELISA based on the variation in OD values (CV = SD/mean). Coefficient of variability was expressed as percentages. The intra-assay CV was assessed using 10 repeated measurements of 10 selected samples in the same assay by the same technician under the same conditions. Inter-assay variation was calculated by measuring the same 10 samples on the same day by three different technicians. All statistical analyses were performed using GraphPad Prism 8.0. p values <0.05 were considered statistically significant. Flow cytometry data were analyzed using FlowJo V10. PCR sequence-based typing data were analyzed using Chromas software.

Results

Evaluation of the Sandwich ELISA

The results of the proposed sandwich ELISA for samples with known CD36 status are shown in Figure 2. Among the eight pCD36-positive samples, the mean OD was 1.512 (range, 1.332–1.607), which allowed clear discrimination from the two pCD36-negative samples

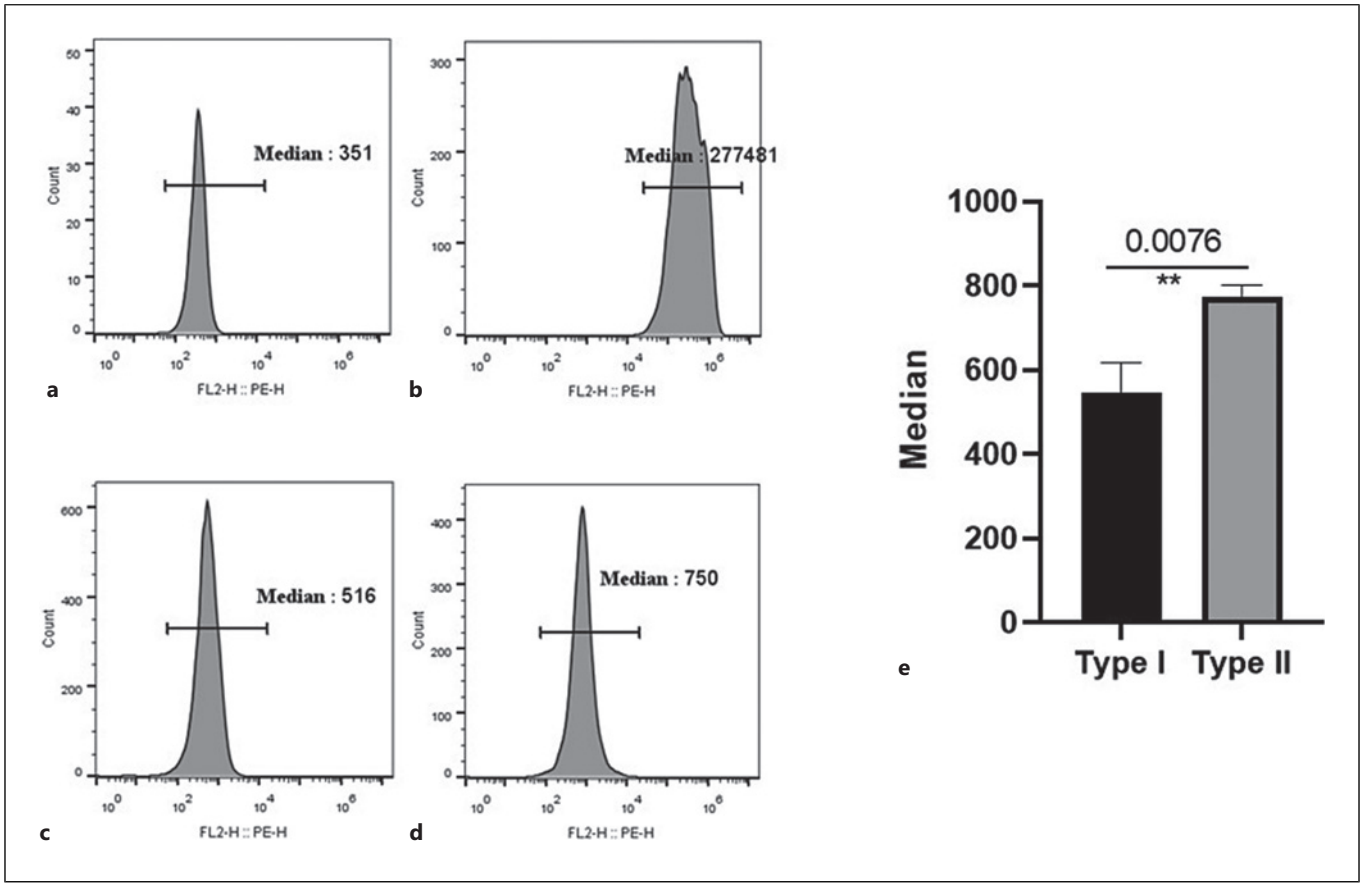


Fig. 4. Anti-CD36 reactivity of platelets with types I and II CD36 deficiency. **a** Isotype control group. **b** Positive control group. **c** Anti-CD36 reactivity of platelets with type I CD36 deficiency. **d** Anti-CD36 reactivity of platelets with type II CD36 deficiency. **e** GraphPad was used to compare the anti-CD36 reactivity of platelets with types I and II CD36 deficiency.

(OD values, 0.081 and 0.093). Based on these results, the cut-off value of the assay was set at 0.104 (mean + 2 SD). Intra-assay variation, assessed as the CV of 10 repeated measurements of these 10 samples, was low (CV, 2.1–4.8%). The inter-assay variation, based on three independent measurements of the same 10 samples, was also comparable (CV range, 2.3–5.2%). In addition, this method could be used to test more than 600 samples in 2 h using a fully automated detection system, and it be could flexibly adjusted to allow testing of clinical samples.

Detection of CD36 Expression in Donor Samples

Platelet samples obtained from 1,691 blood donors were screened using the sandwich ELISA. Of these, 36 cases (2.13%) with pCD36 antigen deficiency were detected (ELISA OD <0.104). These pCD36-negative samples were subjected to an independent validation using FC, which revealed that three had absence of CD36 expression on monocytes (Fig. 3). According to these results, the estimated frequencies of CD36 deficiency in the Suzhou population were 0.18% for type I and 1.95% for type II CD36 deficiency (Table 2). In addition, differences in the reactivity of platelets to the anti-CD36 antibody were observed between samples with type I and type II CD36 deficiency ($p < 0.01$; Fig. 4). Platelets with type II CD36 deficiency had higher anti-CD36 reactivity compared to platelets with type I CD36 deficiency. This finding indicates that glycoprotein expression on platelets differs between type I and type II CD36 deficiency, thereby explaining the observed difference in reactivity to anti-CD36.

Molecular Background Analysis of pCD36-Negative Samples

Exons 3–14 of the CD36 gene were amplified and sequenced from randomly selected 27 of the 36 samples from pCD36-negative donors. The sequence analysis revealed mutations in these exons in 13 donors (13/27, 48.1%). Of these, 10 samples with type II CD36 deficiency had single nucleotide mutations or gene deletions, and three samples with type I CD36 deficiency had homozygous or compound heterozygous mutations. A total of 10 types of CD36 gene mutations were detected in the present study. The amino acid changes caused by each mutation are summarized in Table 3.

Table 3. Gene mutation of CD36 deficiency

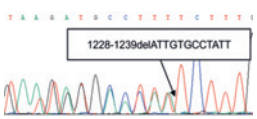
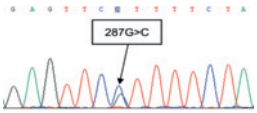
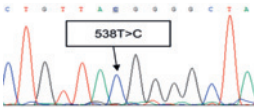
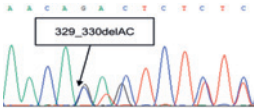
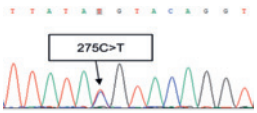
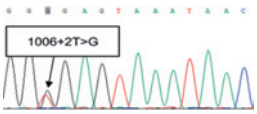
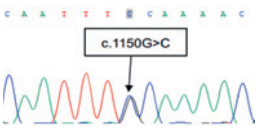
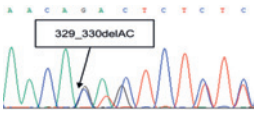
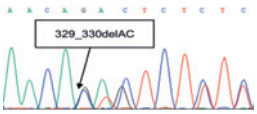
Samples, *n*	Type of CD36 deficiency	Mutant exon	Gene mutation	Gene sequencing map	Amino acid mutation
2	II	Exon 13	1228_1239delATTGTGCCTATT		Amino acid deletion (410_413): Ile-Val-Pro-Ile
2	II	Exon 5	287G>C		Arg96Pro
2	II	Exon 6	538T>C		Trp180Arg (Homozygous mutation)
1	II	Exon 5	329_330delAC		Frameshift at amino acid 110
1	II	Exon 4	275C>T		Thr92Met
1	II	Exon 10	1006+2T>G		Frameshift at amino acid 274 (The mutation point is located in the intron)
1	II	Exon 12	1150G>C		Ala384Pro
1	I	Exon 5	329_330delAC		Frameshift at amino acid 110 (Homozygous mutation)
1	I	Exon 5	329_330delAC		Frameshift at amino acid 110

Table 3 (continued)

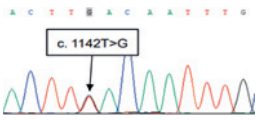
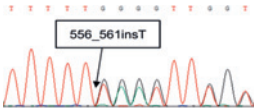
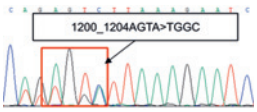
Samples, *n*	Type of CD36 deficiency	Mutant exon	Gene mutation	Gene sequencing map	Amino acid mutation
		Exon 12	1142T>G		Stop codon UGA production
1	I	Exon 6	556_561insT		Frameshift at amino acid 186
		Exon 13	1200_1204AGTA>TGGC		The mutation point is located in the intron

Table 4. The frequency of CD36 deficiency in certain cities in China

Regions	Donors, *n*	Frequency of CD36 deficiency (%)	Type I CD36 deficiency (%)	Type II CD36 deficiency (%)	Reference
Beijing	612	2.12	0	2.12	Ma et al. [12]
Shanghai	1,022	2.2	0.2	2.0	Li et al. [9]
Shenzhen	327	3.06	0.31	2.75	Wu et al. [14]
Guangdong	1,158	1.8	0.6	1.2	Liu et al. [23]
Guangxi	4,621	4.13	1.04	1.49	Wu et al. [14]
The study	1,691	2.13	0.18	1.95	

Table 5. The frequency of CD36 deficiency in northern and southern China

Regions	Donors, *n*	Frequency of CD36 deficiency (%)	Type I CD36 deficiency (%)	Type II CD36 deficiency (%)
North	2,089	1.48	0.048	1.44
South	11,689	2.85	0.63	1.58
Total	13,778	2.64	0.54	1.56

North: Jilin, Shanxi-1, Qinghai, Shanxi-2, Beijing [12, 23]. South: Yunnan, Guizhou, Guangdong, Guangxi, Zhejiang, Shanghai, Shenzhen, Suzhou [9, 14, 23, 24].

Discussion

The frequency of CD36 deficiency is significantly higher in Asian populations than in Caucasian populations. The anti-CD36 antibody, which is the most common platelet antibody after HLA antibodies and certain HPA antibodies of unknown specificity [14], is strongly associated with clinical FNAIT disease in Asian populations. Unlike other types of antibody-mediated FNAIT, cases with anti-CD36 antibodies are often associated with anemia and fetal edema. The reaction between anti-CD36 antibodies and erythroid progenitor cells can lead to the apoptosis of $CD34^+$ erythroid/myeloid precursor cells, resulting in neonatal/fetal anemia and hydrocephalus [16, 19, 20]. In recent years, some provinces in China have performed studies on the frequency of CD36 deficiency. The Guangzhou Blood Center has reported many cases of platelet transfusion refractoriness and neonatal thrombocytopenia caused by anti-CD36 antibodies [19, 21, 22], indicating that the production of anti-CD36 antibodies

is an important risk factor for immune-mediated thrombocytopenia in Chinese populations. Accordingly, the need for databases of donors with CD36 deficiency has recently been highlighted.

In the present study, we describe the development of a reliable, quick, simple, and high-throughput method for detecting CD36 deficiency on platelets using a sandwich ELISA based on anti-CD36 specific antibodies. Due to the cascade amplification effect of ELISA, this method is low cost and also meets the requirements for routine screening of large-scale CD36-negative samples in blood transfusion departments and blood centers.

Initial tests demonstrated adequate reliability of our novel method, with intra-assay and inter-assay CVs of 2.1–4.8% and 2.3–5.2%, respectively. The use of an OD cut-off value of 0.104 (mean + 2 SD) led to good discrimination between CD36-positive and CD36-negative samples. The results of subsequent testing in a much larger sample (from 1,691 blood donors) further supported the use of this discrimination threshold.

The expression of CD36 antigen has been shown to vary according to geographic region (Table 4, Table 5). In the present study, the frequency of CD36 deficiency was estimated to be 2.64% in 13,778 cases of individuals in China, significantly lower than reported in other Asian countries [10, 13]. In addition, the frequency of type I CD36 deficiency was significantly lower in northern regions than in southern regions, with no difference in the frequency of type II CD36 deficiency observed between regions. The estimated frequency of CD36 deficiency among residents of Suzhou in the study was 2.13%, which was close to the reported frequency in the entire population of China [9, 12, 14, 23].

The four most common CD36 gene mutation types in Asian populations are 268C>T, 329_330delAC, 944_949insA, and 1228_1239delATTGTGCCTATT, of which 268C>T is the most frequent allelic mutation [24, 25]. The 329_330delAC and 1228_1239delATTGTGCCTATT mutation types are reported with relatively high frequency in other provinces in China. In comparison, our sequence analysis of exons 3–14 of the CD36 gene among residents of Suzhou identified a homozygous mutation (329_330delAC) and compound heterozygous mutations (556_561insT+1200_1204AGTA>TGGC, 329_330delAC+1142T>G) in three cases of type I CD36 deficiency. Furthermore, we identified a variety of heterozygous mutations, predominantly comprising base deletions and substitutions in 24 cases of type II deficiency. Overall, the results of present study are consistent with previous studies of the genetic background of type I and type II CD36 deficiency that have reported that type I CD36 deficiency is caused by homozygous or compound heterozygous mutations of the CD36 gene, whereas type II CD36 deficiency is associated with mutations in the coding regions, non-coding regions, or 5′-UTR of CD36 and complex molecular regulatory mechanisms [1824, 26, 28]. In addition, previous studies have reported that CD36 gene mutations do not necessarily lead to loss of the CD36 antigen, with some cases of CD36 deficiency caused by reduced antigen expression [29]. Accordingly, the mechanisms underlying CD36 deficiency have yet to be fully elucidated.

Conclusion

In comparison with genetic testing, CD36 phenotyping using specific monoclonal anti-CD36 antibody is simple and inexpensive to perform at scale and well-suited to the establishment of regional pCD36-negative databases and national donor registries. The use of sandwich ELISA for CD36 phenotype analysis in blood centers has considerable advantages over other methods such as flow cytometry and has the potential to greatly improve support for patients with hemorrhagic disorders caused by anti-CD36 antibodies and the effectiveness of platelet transfusion therapy with compatible platelet products. Moreover, the design and application of this technology may facilitate the future standardization of molecular testing methods.

Statement of Ethics

This study protocol was reviewed and approved by the Medical Ethics Committee of Suzhou Blood Center, with approval number SZBC202001. Written informed consent was obtained from all donors prior to participation in the present study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Honghong He and Longhai Tang contributed to the project administration, conception, design, data analysis, and drafting of this paper. Yiming Jin selected and collected the blood samples.

Yujue Wang, Hongmei Wang, Shaohua Ding, Yezhou Chen, and Jingjing Tian contributed to the acquisition of study materials and assistance with compilation of the results. Mingyuan Wang and Shengbao Duan provided financial and technical support. All authors have approved the final version of this paper for publication.

Data Availability Statement

All data generated and analyzed during this study were included in this article. Further inquiries can be directed to the corresponding author.

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