Variants of *CD36* gene and their association with CD36 protein expression in platelets

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Background. The relationship between CD36 expression level in platelets and polymorphism of the *CD36* gene still needs to be explored. Here, we investigated polymorphisms of the *CD36* gene and CD36 expression level in platelets in the Chinese Han population.

Materials and methods. A total of 477 samples were sequenced for exons 2 to 14 of the *CD36* gene using a polymerase chain reaction sequence-based typing method. In 192 of these individuals the expression levels of CD36 antigen were analysed by flow cytometry. The genotype-phenotype relationship in platelets was analysed.

Results. A total of 22 variants of the *CD36* gene were identified, of which five variants (111 A>T, 681 C>A, 1172-1183 del12b, 1236 delT and 1395 A>C) were novel variations, and nine were also found in single nucleotide polymorphism database (dbSNP) but had not been confirmed in individuals with CD36 deficiency. Two variants (329-332 delAC and 1228-1239 del12bp) in the coding region are the most frequent mutations in the Chinese population. Type II CD36 deficiency was identified in seven of 192 individuals, giving a frequency of 3.6%. Individuals with CD36 variations or wild-type genotypes both showed CD36 antigen negative, low-level and high-level expression patterns in platelets. The frequency of the nt-132 A>C polymorphism in the 5'-UTR is relatively high in the Chinese population (0.3516): the expression of CD36 was lower in individuals with nt-132 A>C than in those with the wild-type genotype.

Discussion. The distribution of *CD36* gene variants in the Chinese population is different from that previously reported. The levels of expression of CD36 antigen in platelets are not determined directly by the genotypes of the *CD36* coding region. This suggests that the molecular basis of type II CD36 deficiency may be derived from combined effects of coding region and potential cis-regulatory elements in the 5'-UTR of the *CD36* gene.

Keywords: platelet glycoprotein IV, type II CD36 deficiency, variant.

Introduction

CD36, also known as platelet glycoprotein IV (GPIV), plays a role in various cellular processes such as coagulation, immune regulation, lipid transport and atherosclerosis. It has been proposed as one of the thrombospondin receptors on platelets[1,2]. It has also been shown that CD36 is one of receptors for oxidized low density lipoproteins and fatty acids, suggesting that CD36 may play a part in atherogenesis and lipid metabolism[3,4].

It has been shown that the Nak^a antigen is located on GPIV or CD36 and that individuals who do not express CD36 on their platelets can probably be immunized with CD36 antigen, producing anti-Nak^a, by transfusion or other pathways[5,6]. The first described individual with deficiency of CD36 was a Japanese woman who was refractory to HLA-matched platelet transfusions[7]. Currently, CD36 deficiency (or Nak^a-negative blood group) is divided into two subgroups according to the phenotype. In type I deficiency, CD36 is not expressed in either platelets or monocytes, whereas in type II deficiency, CD36 is expressed in monocytes but not in platelets[8]. According to previous reports, approximately 90% of Nak^a-negative individuals lack CD36 expression only in platelets; the remaining 10% are presumably CD36 null (type I deficiency) and do not express CD36 in any cells or tissues[9].

The *CD36* gene is located on chromosome 7q11.2 and has 15 exons. Exons 1, 2, and 15 are non-coding. Exons 3 and 14 encode the N-terminal and C-terminal domains of the CD36 protein, respectively[10]. Interestingly, the 5′-untranslated region (UTR) of *CD36* mRNA is encoded by three exons. Exon 3 contains the last 89 nucleotides of the 5′-UTR and encodes the N-terminal

cytoplasmic and transmembrane domain. The 3'-UTR is contained in exon 14 only or in exons 14 and 15 in various cells or tissues[11]. The *CD36* gene is highly polymorphic and more than 20 sites of polymorphism have been described in the coding sequence leading to type I deficiency. Some of them have been reported to be associated with type I deficiency in Asians[12-17]. However, there is little information on polymorphisms of the *CD36* gene and the frequency of individuals with CD36 deficiency in the Chinese population[18].

In this study we established a polymerase chain reaction sequence-based typing (PCR-SBT) assay combined with high-throughput DNA sequencing to detect polymorphisms of the *CD36* gene in the Chinese Han population. We also analysed the levels of CD36 expression in platelets by flow cytometer in some individuals.

Materials and methods

Subjects

Peripheral blood samples (n=477) were collected from unrelated volunteer blood donors in the Blood Centre of Zhejiang Province, China. All these donors were of Han ethnicity and all gave informed consent to participation in this study. All 477 samples were analysed for *CD36* gene polymorphisms, while platelet CD36 protein expression was analysed in a subgroup of 192 patients. Genomic DNA was extracted from peripheral blood using QuickGene DNA whole blood kits (QuickGene, Kurabo, Japan) according to the manufacturer's instructions and preserved at −20 °C until genotyping.

Primer design and amplification

The specific primers for PCR amplification and sequencing were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). The nucleotide sequences, annealing temperature, cycle number, Mg^{2}+ concentration and the size range of amplicons are listed in Table I. All oligonucleotide primers were synthesised by Shenery Biocolour Co. Ltd (Shanghai, China).

All PCR reaction parameters were optimised and are listed in the table I. The PCR reactions were performed in a 10 μL volume, containing about 50-100 ng genomic DNA in 10×LA PCR buffer II (TaKaRa, Dalian, China), 0.5 μmol/L of each primer, 200 μmol/L of each dNTP, 1.5 to 2.5 mmol/L of $MgCl_2$ and 0.5 unit of LA-Taq DNA polymerase (TaKaRa). The amplification was performed in a PTC-240 thermocycler (MJ Research, Waltham, MA, USA).

DNA sequencing for exons 2 to 14 of the *CD36* gene

To degrade excess primers and nucleotides from the PCR amplicons for the subsequent sequencing reaction, 5 U of exonuclease I (TaKaRa) and 1 U of shrimp alkaline phosphatase (Promega, Madison, WI, USA) were applied to each 10 μL of PCR products, then the mixture was incubated at 37 °C for 30 minutes, followed by 15 minutes at 80 °C in the PCR thermocycler. The purified PCR amplicons were sequenced using a BigDye® 3.0 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The thermocycling conditions were as follows: 95 °C for 5 minutes, followed by 30 cycles at

Table I - The nucleotide sequence of primers and amplification parameters for *CD36* gene.

CD36 gene	Nucleotide sequence(5'-3')	PCR parameters (annealing temperature × cycles)	$Mg2^{+}$ (mM)	Amplicon size (bp)
Exon 2, exon 3	E23F: ATGGTGATATTAGAGAGTGT	63 °C×35 cycles	2.5	1,070
	E23R: TTTAAGACAGCAATGGAGTC			
Exon 4	E4F: GTAAAAGGCTAAAAAGACTG	58 °C×35 cycles	2.5	672
	E4R :ACTTCATAAACATAGGGAAG			
Exon 5	E5F: CCCCTTCTCGTTAGTTTGCT	66 °C×35 cycles	2.5	707
	E5R: TTTCTTACAGGCTGCGTTTG			
Exon 6, exon 7	E67F: AGAGAGCCTCGTGCCGTTAG	64 °C×35 cycles	1.5	2,539
	E67R: TGAAAGCAGTTGCCTGGATTC			
Exon 8	E8F: TCTGCCTGCCTTGTACTTAGC	65 °C×35 cycles	1.5	921
	E8R: AATGGATGGTTCTGTCTTGCA			
Exon 9	E9F: GTATCCGCCTCCTGGGTGC	64 °C×35 cycles	1.5	906
	E9R: GCTTGGGCTCAAGGGTAGTG			
Exon 10, exon 11	E10-11F: AGACACTTGGTAAACGATGG	61 °C×35 cycles	1.5	2,140
	E10-11R: ACAGCAGAAGAGAAGGTAGT			
Exon 12, exon 13, exon 14	E12-14F: AGGTCGATTTCTTCCTATGG	59 °C×35 cycles	2.5	2,243
	E12-14R: TTGTTCAGGACTTTTCTGGA			

95 °C for 10 seconds, 50 °C for 10 seconds, 60 °C for 4 minutes, and cooling at 4 °C in the PCR thermocycler. The ethanol/acetate sodium method was applied to purify the sequencing reaction products as previously reported[19], and the products were then processed on an ABI 3730 DNA analyser and the sequence data were analysed using SeqScape 2.5 software (Applied Biosystems, Carlsbad, CA, USA). All nucleotides sequences obtained were compared with *CD36* reference sequences from the GenBank database (ID number NG_008192.1). Every variation of the *CD36* gene was analysed and recorded. The novel variations were further verified by cloning using a TOPO cloning sequencing kit (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions.

Expression of CD36 determined by flow cytometry

The expression of CD36 was determined by flow cytometry using monoclonal antibodies. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD36 (clone: SMΦ), R-phycoerythrin (R-PE)-conjugated anti-human CD41 (clone: VIPL3), R-PE-conjugated anti-human CD14 (clone: TüK4) and FITC-mouse anti-human IgG (isotype control) were purchased from Invitrogen (Invitrogen Co, Carlsbad, CA, USA).

The level of expression of CD36 protein in platelets was determined within 4 hours of collecting the blood samples. Fresh whole blood was collected and centrifuged (200 g for 10 minutes) to prepare platelet-rich plasma, then washed with phosphate-buffered saline containing 0.2% K2-EDTA solution and the platelet concentration adjusted to 1×10^7/mL. The 100 µL platelet-rich plasma suspensions were incubated with 10 µL FITC-anti-CD36 and 10 µL PE-anti-CD41 (a specific marker for platelets) for 20 minutes at room temperature and protected from light. The samples were analysed on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) with at least 10,000 platelets assessed in each sample. The platelet population was gated on the basis of forward scatter, side scatter and CD41 characteristics. The mean fluorescence intensity (MFI) of platelet CD36 was measured using CellQuest1.1 software (Becton Dickinson).

For those individuals with CD36-negative platelets, CD36 antigen expression on monocytes was analysed by flow cytometry. Samples of whole blood (100 µL) were stained doubly with 10 µL FITC-anti-human CD36 and 10 µL PE-anti-human CD14 (a specific marker for monocytes) for 20 minutes at room temperature and protected from light. The cells were then washed three times with phosphate-buffered saline-EDTA solution. After washing, FACS lysing solution (Becton Dickinson) was added and mixed together. The mixture was left at room temperature for 30 minutes to lyse erythrocytes and then analysed. The MFI of CD36 on CD14-positive monocytes was measured using CellQuest1.1 software.

Statistical analysis

The allelic frequencies were calculated by the maximum likelihood gene-counting method. Each polymorphism was tested for Hardy-Weinberg equilibrium using the classical chi-square test as described previously[20]. Flow cytometric parameters were statistically analysed by the Kruskal-Wallis test and Mann-Whitney test using GraphPad software version 5.01 (GraphPad Software Inc., San Diego, CA, USA). All data are presented as mean ± standard deviation (SD), and p values <0.05 for differences between the experimental groups are considered to be statistically significant.

Results

The distribution of polymorphisms in the *CD36* gene coding region

Twenty variations were identified in the coding region of the *CD36* gene in the 477 DNA samples, including 13 single nucleotide polymorphisms, four deletions, two insertions and one 43-bp duplication. Table II show the characteristics of these variations, whose positions were calculated from the start codon ATG, and minor allele frequencies. The minor allele frequencies for these variations ranged from 0.0010 to 0.0136 in the Chinese Han population. All variation alleles were heterozygous with wild-type alleles and no homozygous or compound heterozygous variants were found in the coding region of any of the 477 samples. The genotype distributions of the variations sites were in Hardy-Weinberg equilibrium ($p>0.05$).

Of the twenty variations, six (268C>T; 329-332 delAC; 410 T>C; 944-949 insA; 1204-1246 43bp dupl and 1228-1239 del12bp) had been reported previously to be responsible for CD36 deficiency[12-16]. Another nine variations were also confirmed in the single nucleotide polymorphism database (dbSNP) and we further provided the frequencies of these variations in the Chinese Han population (Table II). Moreover, five novel variations have been identified and partial DNA sequencing chromatograms of these five novel variations, which were verified by cloning sequencing, are presented in Figure 1. Three of them are synonymous mutations with 111 A>T in exon 3, 681 C>A in exon 7 and 1395 A>C in exon 14 (Figure 1). Two of them are deletions: a 12 basepair (TATTGGTCAAGC) deletion from position 1172 to 1183 in exon 12 (Figure D1) which lead to the deletion of Leu-leu-Val-Lys at amino acid position 391-394, and one T deletion at nucleotide position 1236 in exon 13 (Figure E1) which causes a frameshift at amino acid position 412 and appearance of a new premature stop codon at nt 1,301-1,303.

Table II - Characteristics of the polymorphisms in coding regions of the *CD36* gene in 477 Chinese individuals.

Location	mRNA§	Amino acid position	Nucleotide change	Amino acid change	N.	MAF	dbSNP number(ref)
Exon 3	−89 to +120	1-40	14 G>T	Arg5Leu	1	0.0010	rs13306227
			*111 A>T	Thr37Thr	1	0.0010	-
Exon 4	121-281	41-94	268 C>T	Pro90Ser	1	0.0010	rs75326924[12]
Exon 5	282-429	94-143	287 G>C	Arg96Pro	2	0.0021	rs70961715
			329-332 delAC	frameshift at AA 110	13	0.0136	-[13]
			410 T>C	Val137Ala	2	0.0021	rs2272350[14]
Exon 6	430-609	144-203	538 T>C	Trp180Arg	3	0.0031	rs201759307
Exon 7	610-701	204-234	*681 C>A	Ile227Ile	1	0.0010	-
Exon 10	819-1006	273-336	944-949 insA	frameshift at AA 317	1	0.0010	rs70961716[15]
Exon 12	1126-1199	376-400	1156 C>T	Arg386Trp	2	0.0021	rs148910227
			1157 G>A	Arg386Gln	1	0.0010	rs187500047
			1163 A>T	Gln388Leu	2	0.0021	rs201355711
			*1172-1,183 delTATTGGTCAAGC	Deletion of Leu-leu-Val-Lys at AA 391-394	1	0.0010	-
Exon 13	1200-1254	400-418	1204-1246 43bp dupl	frameshift at AA 416	1	0.0010	-[14]
			*1236 delT	frameshift at AA 412	3	0.0031	-
			1228-1239 delATTGTGCCTATT	Deletion of Ile-Val-Pro–Ile at AA 410-413	5	0.0052	-[16]
Exon 14	1255-1419	419-472	1343-1344 insTCTT	frameshift at AA 446	1	0.0010	rs201958707
			*1395 A>C	Ala465Ala	1	0.0010	-
			1404 G>A	Ser468Ser	1	0.0010	rs141626483
			1409 C>T	Thr470Ile	2	0.0021	rs200771788

§: The sequence from the GenBank database (ID number NM_001001547.2) was used for the reference sequence and the first mRNA nucleotide encoding CD36 protein is +1; MAF: minor allele frequency; -: no dbSNP number; *: novel variation.

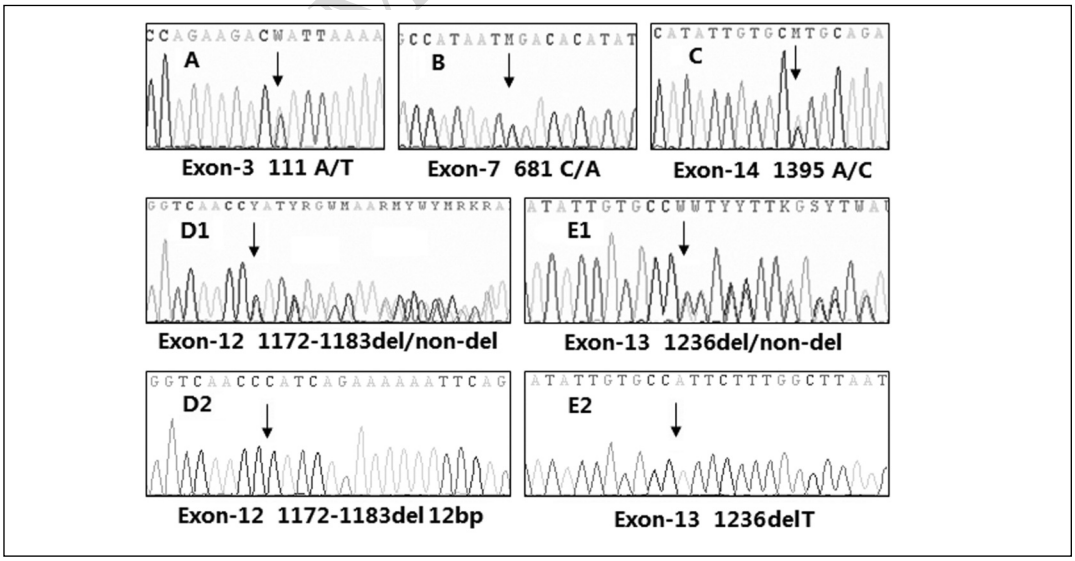

Figure 1 - Partial DNA sequencing chromatograms for five novel variations, including 3 SNPs and 2 deletions in *CD36* gene coding region.

The arrows indicated the variant nucleotides position. A: Synonymous mutation 111 A>T; B: Synonymous mutation 681 C>A; C: Synonymous mutation 1395 A>C; D. Deletion of 12bp (TATTGGTCAAGC) at nt1172-1183; E. Deletion T at nt1236. D1 and E1 are the chromatograms by directly sequencing, and D2 and E2 are the chromatograms of the deletion allele by cloning sequencing.

Variations in exons 2 and 3 upstream of the start codon

Two variations were detected upstream of the start codon of the *CD36* gene. One is at nt-132 A>C (rs1049654) in exon 2 and the other is nt-18 insA (rs75112981) in exon 3. The allele frequencies of nt-132C and nt-18 insA are 0.3516 and 0.0105, respectively.

Flow cytometric analysis of CD36 protein expression

The levels of expression of CD36 in platelets were highly variable among the 192 individuals assessed. According to MFI values of CD36 antigen expression in platelets, the individuals were divided into three groups: no CD36 expression, i.e., CD36-negative (n=7), low-level expression (n=59) and high-level expression (n=126). The average MFI of the three groups was 14.7±6.4, 48.0±11.9 and 132.7±54.3, respectively, and CD36 expression was significantly different among these three groups ($p<0.05$). The reaction patterns for CD36 expression in platelets containing different groups by flow cytometry are shown in Figure 2.

For individuals with CD36-negative platelets, CD36 antigen expression was further analysed on monocytes. All these individuals expressed CD36 antigen on monocytes and were, therefore, defined as having type II CD36 deficiency. The frequency of type II CD36 deficiency in the Chinese Han populations studied was, therefore, 3.6%. In the seven individuals with type II CD36 deficiency, CD36 expression in monocytes from three subjects with *CD36* gene variants (MFI=271.0±49.4) was lower than that in monocytes from four subjects without gene variants (MFI=555.0±87.7), but the difference was not statistically significant ($p>0.05$).

The relationship between *CD36* gene variants and *CD36* expression levels in platelets

Both genotype and levels of expression of CD36 antigen were analysed in 192 individuals, 176 of whom had a wild-type (WT) genotype and 16 with a heterozygous variant according to the sequence of the coding region (Table III). These 16 individuals were heterozygous with a single mutation and comprised three individuals with CD36 negative expression in

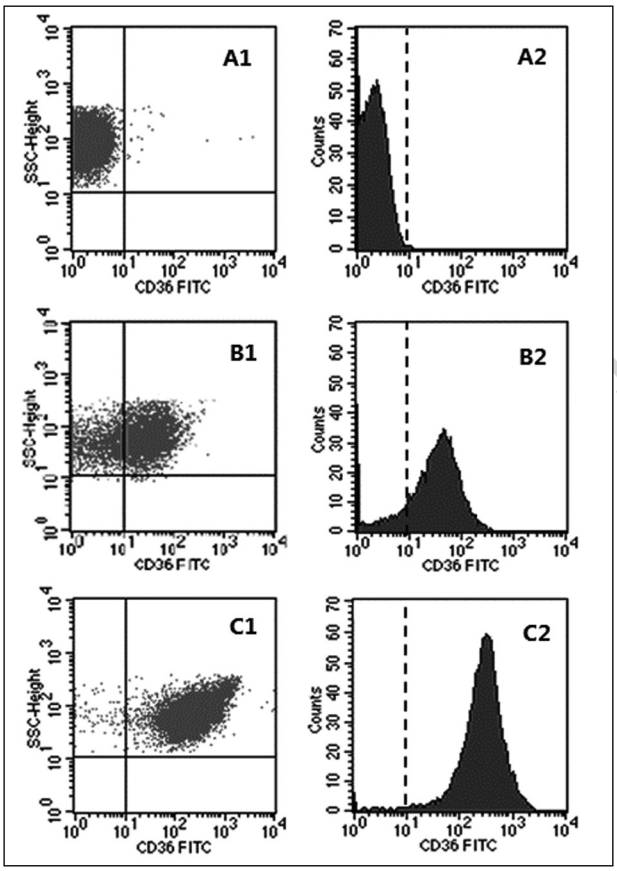

Figure 2 - The CD36 expression level in platelets by flow cytometric analysis.
Left panel, the density dot plots about CD36 expression in platelets. Side-scattered light (SSC) is displayed on Y-axis and CD36-FITC logarithmic scale represented on X-axis. Right panel, histograms are plotted against CD36-FITC fluorescent intensity in platelets. Number of cells on Y-axis and CD36-FITC on X-axis. At least 10,000 platelets were analyzed for each assay. A1 and A2, from an individual expressing CD36 negative; B1 and B2, from an individual with CD36 low expression; C1 and C2, from an individual with CD36 high expression.

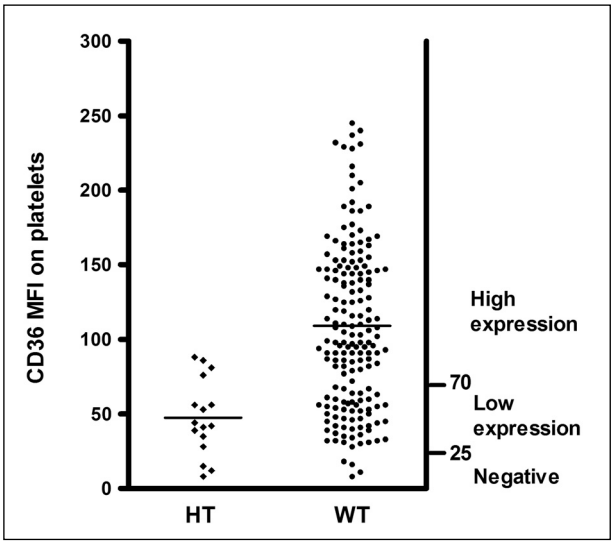

Figure 3 - The MFI distribution of CD36 expression level in platelets of HT (heterozygous-type, n=16) and WT (wild-type, n=176) in coding region.
The right Y axis showed the MFI cut-off values used to divide subjects into three groups according to CD36 expression. In 16 individuals with different mutation, the average MFI is 26.5±20.5 (1,236 delT, n=2), 34.3±23.5 (538 T>C, n=3), 44.0 (1163 A>T, n=1), 49.6±28.4 (329-332 delAC, n=7), 56.0 (410 T>C, n=1), 76.0 (1,228-1,239 del12bp, n=1) and 81.0 (944-949 insA, n=1), respectively.

Table III - The distribution of CD36 expression level in platelets and genotypes in 192 individuals.

CD36 expression	Coding region (exon 3 to exon 14)		Exon 2 and 3 upstream of start codon					Total
			−18 insA		−132 A>C			
	WT	HT*	WT	HT	WT (AA)	HT (AC)	MT (CC)	
Negative	4	3	7	0	3	2	2	7
Weak expression	50	9	57	2	15	33	11	59
High expression	122	4	124	2	63	52	11	126
Total	176	16	188	4	81	87	24	192
MFI (Mean±SD)	109.2±60.2	47.4±25.6	102.7±61.8	86.5±47.1	119.4±66.8	96.0±57.3	69.6±39.7	
p	p<0.0001		p=0.6958		p=0.0010			

WT: wild-type; HT: heterozygous-type; MT: mutation-type; *: Here HT includes all seven variation sites of the coding region.

platelets (329-332 delAC, 538 T>C and 1236 delT), nine individuals with CD36 low expression (4 with 329-332 delAC, 2 with 538 T>C, 1 with 410 T>C, 1 with 1163 A>T and 1 with 1236 delT), and four individuals with CD36 high expression (2 with 329-332 delAC, 1 with 944-949 insA and 1 with 1228-1239 del12bp). Among the 176 WT individuals, four had type II CD36 deficiency and 122 had high CD36 expression on platelets. The MFI distribution of CD36 in platelets from the 192 Chinese Han individuals studied is illustrated in Figure 3, which shows that the MFI of the 16 individuals with heterozygous variants was lower than that of the 176 WT individuals ($p<0.05$).

The relationship between the variants upstream of the start codon and CD36 expression levels in platelets is interesting. The frequency of the nt-132 A>C variant in the Chinese population is relatively high. The average MFI of CD36 expression in individuals with nt-132 CC mutation was lower than that of nt-132 AC heterozygotes and nt-132 CC wild-type ($p<0.05$). The average MFI in nt-18 insA heterozygotes was lower than that of nt-18 wild-type individuals, but the difference was not statistically significant ($p>0.05$) (Table III).

Discussion

CD36 is recognized as a major platelet glycoprotein and plays a complex role in platelets and other cells[21,22]. In recent years, researchers have paid more attention to the molecular basis of CD36 deficiency as this deficiency and the Nak^{a-} phenotype have been reported to be present in some clinical disorders. In this study, a total of 22 variants of *CD36* gene were identified in the Chinese Han population, of which two were located in the upstream ATG while the other 20 variations were located in encoding regions of the *CD36* gene (exons 3 to 7, exon 10 and exons 12 to 14 of the *CD36*). No variants were found in exon 8, exon 9 or exon 11. These results suggest that there are highly polymorphic coding regions of the *CD36* gene and some relatively conserved regions.

The three mutations previously reported to be most commonly detected in subjects with CD36 deficiency are 268 C>T, 944-949 insA, 329-332 delAC, with 268 C>T being the most common and responsible for more than 50% of mutated alleles in Asians[13-16]. However, in this study, 329-332 delAC and 1,228-1,239 del12bp were the most frequent mutations in the Chinese population. This suggests that the *CD36* gene mutation distribution in China is different from that in other countries. Moreover, partial variations found in this report (287 G>C, 538 T>C, 1156 C>T, 1157 G>A, 1163 A>T, 1343-1344 insTCTT, 1404 G>A, 1409 C>T) were also confirmed in the dbSNP, but their effect on CD36 expression were not clarified in previous reports. It should be noted that some of these variations can cause the amino acid substitution and may affect the expression levels of the CD36 protein.

The frequency of CD36 deficiency varies widely among ethnic groups, with the frequency being 3% to 11% in the Chinese and Japanese, 8% in African Americans, and less than 0.4% in white Europeans[23-26]. In this study, seven individuals were confirmed as having type II CD36 deficiency, no individual had type I CD36 deficiency, and the prevalence rate of CD36 deficiency (3.6%) was similar to that of other reports in Asians[23,24]. The mechanism of type I deficiency is reportedly homozygous or compound heterozygous mutations of the *CD36* gene, while the mechanism of type II deficiency is more complex. In this study, of the seven individuals with type II deficiency, three individuals had single heterozygous mutations and four individuals had no detectable mutation in the coding region, so the condition could not be explained based on the sequence results. It has been suggested that the molecular mechanisms of type II CD36 deficiency are very complex and that there may be other genetic regulatory mechanisms. Kashiwagi et al. speculated that a "platelet- specific silent allele" near or at the *CD36* gene would affect the expression of CD36 in

platelets[27]. However, Imai et al. hypothesized the existence of platelet-restricted regulatory factors, which result in genomic heterogeneity of type II CD36 deficiency[14]. Our present study showed that individuals with variations upstream of the start codon (especially -132 A>C) have lower levels of CD36 expression than do individuals with wild-type alleles. It suggests that several potential cis-regulatory elements in the 5'-UTR might contribute to transcriptional regulation and result in type II CD36 deficiency in platelets.

Besides type II CD36 deficiency, three reaction patterns for expression levels of CD36 protein in platelets by flow cytometry were found in the individuals with single heterozygous mutations, while similar reaction patterns were also observed in the wild-type individuals, once again indicating that the mechanisms determining expression levels of CD36 protein in platelets are complicated and need further exploration, for example, of epigenetic changes including methylation of CD36 promoter in platelets. In this study, normal levels of CD36 protein were detected in monocytes from individuals not expressing CD36 in platelets and further studies with large numbers of individuals are required to identify those with type I CD36 deficiency in the Chinese Han population.

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Authorship contributions

Xianguo Xu and Ying Liu contributed equally to this manuscript. They performed the research and wrote the paper. Xiaozhen Hong and Shu Chen performed the flow cytometric analysis. Kairong Ma and Xiaofei Lan performed the DNA sequencing. Yanling Ying and Ji He analysed the data. Faming Zhu and Hangjun Lv designed the research study.

The Authors declare no conflict of interest.

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