Studies on CD36 deficiency in South China: Two cases demonstrating the clinical impact of anti-CD36 antibodies

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Introduction

CD36, also known as platelet glycoprotein IV (GPIV), is a highly glycosylated 88-kDa protein and is expressed widely in different cells such as platelets, monocytes, macrophages, endothelial and epithelial cells. CD36 belongs to the class B scavenger receptor family, which binds many ligands including, collagen, thrombospondin, erythrocytes parasitised with Plasmodium falciparum, and oxidised low-density protein (1, 2).

CD36 deficiency was first reported in a patient who developed platelet-transfusion refractoriness caused by anti-CD36 isoantibody (termed anti-Nakα antibody) after multiple platelet transfusions (3, 4). The frequency of CD36 deficiency on platelets has been reported in 4–8% of Africans, about 3–4% of Japanese, 2.4% of African Americans and 0.3% of Caucasians (3, 5–8). Two types of CD36 deficiency are known: type I lacking CD36 on platelets only (6), it has been suggested that individuals with type I deficiency may be at risk of developing anti-Nakα isoantibodies.
after receiving transfusions or during pregnancy (6). Meanwhile, more than 20 mutations underlying type I CD36 deficiency have been described. In contrast, the molecular basis of type II CD36 deficiency is still unclear (9).

The impact of anti-Nak\(^a\) antibodies has been reported in several clinical conditions of immune-mediated thrombocytopenia including, fetal/neonatal alloimmune thrombocytopenia (FNAIT), post-transfusion purpura (PTP) and platelet-transfusion refractoriness (PTR) (8, 10-13). Recently, Nakajima et al. (14) reported that anti-Nak\(^a\) antibodies are also responsible for the development of transfusion-related acute lung injury (TRALI).

Currently, the relevance of CD36 in immune-mediated platelet disorders among Chinese is not known. Here, we studied the frequency of GPIV deficiency in healthy blood donors in South China and describe two cases of CD36 immunisation associated with FNAIT and PTR.

**Materials and methods**

**Study population**

**Blood donors**

Blood samples from healthy blood donors (n = 998) were collected in Guangzhou Blood Center, China, consisting of 78.86% men and 21.14% women with an average age of 32.5 ± 9.5 years (range, 18-60 years). According to geographical dividing line of China (Qinling Mountain-Huaihe River line), our cohort comprises 88.48% southern and 11.52% northern Chinese populations.

**Case 1**

A 30-year-old mother (Mo) with no history of blood transfusion or transplantation experienced four abortions. The last pregnancy ceased with foetal death at 27 weeks of gestation. Autopsy of the foetus showed oedema of foetal skin with bleeding spots in the lower extremities, severe intracranial haemorrhage and hydrops foetalis. Screening of antibodies against erythrocytes by Coombs test was negative.

**Case 2**

A 22-year-old male patient (Li) with T lymphoblastic cell tumour received induction chemotherapy. After several platelet transfusions, he failed to respond to apheresis random donor platelet transfusion (24 hours post-transfusion CCI < 2,600).

**Isolation of platelets**

Platelets were isolated from 10 ml EDTA anti-coagulated blood. After centrifugation at 120 g for 30 minutes (min), the top 3/4 of platelet-rich plasma (PRP) was isolated, and washed three times with PBS/EDTA buffer (Dulbecco’s Phosphate Buffered Saline containing 10 mM EDTA, pH 7.0-7.2; Gibco BRL). Finally, washed platelets were suspended in PBS/EDTA buffer and adjusted to a concentration to 1.0×10\(^5\) platelets/µl.

**Flow cytometry analysis of CD36 expression on platelets and monocytes**

Aliquots of 50 µl platelets suspension were incubated with 10 µl fluorescein isothiocyanate (FITC)-labelled anti-human CD36 monoclonal antibody (mab FA6-152; Immunotech, Marseille, France) for 30 min at room temperature. After washings, labelled platelets were suspended in 1 ml PBS/EDTA buffer and analysed by FACS Canto II system (BD Biosciences, San Jose, CA, USA). For the analysis of CD36 expression on monocytes, 100 µl of EDTA anti-coagulated blood were incubated with 10 µl FITC-labeled mab FA6-152 and phycoerythrin (PE)-labelled anti-CD14 (mab RMO52, Immunotech) at room temperature for 20 min. Subsequently, 500 µl lysis solution (Optilyse C; Immunotech) was added for 10 min at room temperature to remove erythrocytes from cell suspension. Remaining cells were then washed twice with 4 ml PBS/EDTA buffer, resuspended in 500 µl PBS/EDTA buffer and analysed as described above.

**Analysis of anti-CD36 antibodies against platelets**

**Flow cytometry**

Platelets were isolated from group O blood donors as described above. Fifty µl of platelets suspension were incubated with 50 µl test serum for 30 min at 37°C. After washings with 4 ml PBS/EDTA buffer, platelets were incubated with 50 µl of FITC-labelled rabbit anti-human IgG (dilution 1:50; DAKO, Hamburg, Germany) for 30 min at room temperature. After washings, labelled platelets were suspended in 500 µl PBS/EDTA buffer and analysed by flow cytometer as described above.

**ELISA**

Anti-Nak\(^a\) antibodies were identified by the use of a commercial ELISA Kit (PAKPLUS, GTI Diagnostics, Waukesha, WI, USA) as recommended by the manufacturer. Fifty µl appropriate diluted sera (1:3) were added to microtitre wells coated with different platelet glycoproteins (HLA class I, GPIIb/IIIa, GPIa/IIa, GPIb/IX and GPIV). Bound antibodies were detected with alkaline phosphatase labelled anti-human IgG (dilution 1:100). In some cases, antibodies specificities were determined by the use of antigen capture ELISA (MACE; GTI Diagnostics).

**Immunoprecipitation**

Washed platelets (10\(^6\) cells) were incubated with 1 ml sulfo-NHS-LC biotin (1 mg/ml; Thermo Scientific, Rockford, IL, USA) for 30 min at room temperature. Labeled cells were then resuspended in 2 ml lysis buffer. After centrifugation at 10,000 x g for 10 min, cell lysates were precleared for 30 min with 50 µl of 20% protein G-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) in the...
presence of 30 µl normal human serum for 30 min. Aliquots of 50 µl precleared cell lysates were incubated with 50 µl AB serum (as negative control), anti-HPA-1a (as positive control) and patient’s serum overnight at 4°C. Immunocomplexes were then washed five times with 10 mM Tris HCl at pH 7.4. Bound proteins were released by boiling in SDS buffer for 5 min at 95°C. After centrifugation at 10,000 x g for 2 min, samples were analysed on 7.5% SDS-PAGE under nonreducing conditions. Proteins were then transferred to PVDF membrane by blotting procedure. Membrane were incubated with 8 µl streptavidin horseradish peroxidase (1:8,000 dilution; Amersham Life Sciences) for 30 min at room temperature and visualized by using an enhanced ECL chemiluminescence kit (Calbiochem, Darmstadt, Germany).

Preclearing experiment
Aliquots of 50 µl biotin-labelled platelet lysates were incubated with 10 µl mab against CD36 for 45 min at 4°C, and precipitated with protein G as described above. After centrifugation, cell supernatant was incubated again with anti-CD36 mab and precipitation was repeated. After three times precipitation, cell lysates were incubated with 30 µl patient’s serum for overnight at 4°C. Immunocomplexes were then washed five times with washing buffer (10 mM Tris HCl, pH 7.4). Bound proteins were released by boiling in SDS buffer for 5 min at 95°C. After centrifugation at 10,000 x g for 2 min, samples were analysed by SDS-PAGE and immunoblot, as described above.

Nucleotide sequencing analysis
Genomic DNA was extracted from EDTA anti-coagulated blood using a QIA Mini kit (Qiagen, Hilden, Germany) and subjected to sequencing from exon 3 to exon 15. Each exon (primers were shown in Table 1) including a part of flanking introns was amplified with CD36 specific primers (reference sequence: NG_008192; see Table 1) under following conditions: denaturation (30 seconds [sec], 95°C), annealing (30 sec, 57°C), extension (1 min, 72°C) for 35 cycles, followed by a final extension (5 min, 72°C). PCR products of CD36 gene were purified using MultiScreen Filter plate (Millipore, Carrigtwohill Co., Cork, Ireland). Purified PCR products and CD36 constructs were sequenced with an ABI BigDye Terminator cycle sequencing kit and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Amplification of CD36 transcript
Total RNA was isolated from platelets using peqGOLD RNAPure FILM (PEQLAB Biotechnologie, GmbH, Erlangen, Germany). cDNA was synthesised using Ready-To-GoTM You-Prime Frist–Strand Beads (GE Healthcare, Buckinghamshire, UK) with pd(N)6 primer in a final volume of 33 µl and was amplified with PCR GPIV-F sense primer (5’-GTTGCTTAACTGATTTATATTGCCCTCC-3’) and GPIV-R antisense primer (5’-TTTATTTGTTTCGATCTGCGATC-3’). PCR products were purified by gel extraction (Qiagen), and cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen, Karlsruhe, Germany). Subsequently, positive clones were screened by PCR, and amplified plasmid was purified using a QIAprep Miniprep kit (Qiagen).

Results
Frequency of CD36 deficiency in China
When we screened the expression of CD36 on platelets by flow cytometry, 18 out of 998 individuals were negative for CD36 expression indicating the frequency of CD36-deficient individuals is around 1.8%. In order to identify the frequency of type I CD36-deficient individuals, monocytes derived from CD36-negative platelets donors were analysed. Among 12 tested individuals, five showed no CD36 expression on monocytes as well (Table 2A). This result suggested that the frequencies of type I CD36-deficient (negative on platelets and monocytes) and type II CD36-deficient (negative on platelets only) in our cohort are approximately 0.5 and 1.3%, respectively.
Molecular analysis of CD36 deficiency

To characterise the molecular base underlying CD36 deficiency, the entire coding region of CD36 gene was amplified by PCR (Table 1), and was analysed by a direct sequencing approach. In nine individuals no mutation was identified (Table 2A). Among seven type II CD36 deficient individuals, no mutation was found in six subjects. In only one (Donor 18), deletion of 12 nucleotides (1228-1239delATTGTGCTATT in heterozygous form) located in exon 13 leading to four amino acids deletion (Ile-Val-Pro-Ile; AA 410-413) was found. This deletion was also found in other four donors (Donors 2, 4, 10, and 16). Three of them are found as type I CD36 deficiency. Other

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Age/Sex</th>
<th>CD 36 Plt/Mo</th>
<th>Type</th>
<th>Mutations of CD36 gene</th>
<th>Change in amino acid</th>
<th>Reference</th>
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<td>24/F</td>
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<td>n.d</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
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<td>neg/neg</td>
<td>I</td>
<td>1228–1239delATTGTGCTATT; 121–126delCAAGTT§</td>
<td>deletion of Ile-Val-Pro-Ile;</td>
<td>15,19</td>
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<td>frameshift at AA 110;</td>
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<td>This study</td>
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<tr>
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<td>deletion of Ile-Val-Pro-Ile;</td>
<td>15,19</td>
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GenebankNM: 000072; the first mRNA nucleotide encoding CD36 protein is +1; n.t: not tested; n.d: not defined; - not found; inv: inversion; del: deletion; ins: insertion; AA: amino acid; § new mutations.

Table 2B: Type I CD36 deficiency associated with anti-Nak isoantibodies.

<table>
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<tr>
<th>Subjects</th>
<th>Age/Sex</th>
<th>CD 36 Plt/Mo</th>
<th>Type</th>
<th>Antibody Clinic</th>
<th>Mutations of CD36 gene</th>
<th>Change in amino acid</th>
<th>Reference</th>
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</thead>
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<td>neg/neg</td>
<td>I</td>
<td>anti-Nak*none</td>
<td>329–330delAC; C220T§</td>
<td>frameshift at AA 110;</td>
<td>17</td>
</tr>
<tr>
<td>Mo</td>
<td>30/F</td>
<td>neg/neg</td>
<td>I</td>
<td>anti-Nak*FNAIT</td>
<td>C380T§</td>
<td>Ser127Leu;</td>
<td>22</td>
</tr>
<tr>
<td>Li</td>
<td>22/M</td>
<td>neg/neg</td>
<td>I</td>
<td>anti-Nak*PTR</td>
<td>Homozygous 329–330delAC</td>
<td>frameshift at AA 110;</td>
<td>17</td>
</tr>
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</table>

GenebankNM: 000072; the first mRNA nucleotide encoding CD36 protein is +1; del: deletion; ins: insertion; AA: amino acid; § new mutations.
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Antibodies against CD36 in blood donors and patients

All sera derived from blood donors with type I and type II CD36 deficiency (n = 18) were tested for the presence of anti-Nak\(^*\) antibodies by solid phase ELISA (PAKPLUS, GTI Diagnostics). Only one serum (Donor 6) showed specific positive reaction with CD36 (▶Figure 2). This serum originated from a type I CD36-deficient female donor (Donor 6) (see above), who was most probably immunised during her pregnancy.

In addition, in two patients (Mo and Li) anti-Nak\(^*\) antibodies against CD36 (▶Figure 2) were identified. In case 1 (Mo), FNAIT was suspected. Nucleotide sequencing analysis of maternal genomic DNA showed a point mutation (C380T) located in exon 5 of CD36 gene, which leads to missense amino acid substitution Ser127Leu, and insertion of one nucleotide (429+4insg) at fourth position of intron 5 leading to exon 6 skipping. It was suspected these mutations are directly responsible for the absence of CD36 expression both on maternal monocytes and platelets (▶Table 2B). Indeed, analysis of maternal platelets and monocytes by flow cytometry showed no CD36 expression on the cell surface (▶Figure 3A). In contrast, normal reactions were detected with paternal platelets and monocytes. Cross match analysis between maternal sera with paternal platelets showed strong reaction in MACE (optical density [OD] 1.348) when compared to negative control (OD 0.149; cut-off 0.300). These results indicated that anti-Nak\(^*\) antibodies developed by a type I CD36-deficient mother during the pregnancy reacted with CD36 expressed on foetal platelets leading to FNAIT. These results could be further confirmed by immunoprecipitation analysis. As shown in ▶Figure 3B, maternal serum (Mo) precipitated CD36 from normal donor with an apparent molecular weight of ~95 kDa. This reaction was abolished when CD36 were precleared with mab against CD36 prior to immunoprecipitation with Mo serum.

Figure 1: CD36 mutations found in Chinese blood donors. Mutations located in the region between exon IV – VI (upper panel) and exons XII – XIII (bottom panel) are shown. Grey boxes represent known mutations, white boxes new mutations. Nucleotides substitution, deletion (del.), insertion (ins.) and inversion (inv.) are indicated.
In case 2 (Li), PTR was suspected. Analysis of Li serum showed strong reaction with CD36 (Figure 2). When Li platelets and monocytes were analysed by flow cytometry, no CD36 expression was found (data not shown). This type I CD36 defect could be ascribed to homozygous 329-330 del AC which leads to frame shift at amino acid 110 as described above.

Discussion

In this study, we aimed to determine the frequency of type I and type II CD36 deficiencies among healthy blood donors in South China.

Four major CD36 gene mutations, including C268T, 329-330 del AC, 949 ins A, and 1228-1239 del ATTGTGCCTATT, have been currently reported (9). These four mutations cover almost 90% of CD36 genetic defects characterised so far. Studies in Japanese and Korean populations showed high occurrence of C268T (Pro90Ser) mutation. This mutation was claimed to represent the most common mutation (~50%) among Asians (15, 16). Interestingly, our current study showed that Pro90Ser mutation did not represent the most common cause of type I CD36 deficiency in South China. No individual carrying Pro90Ser was detected in this study. However, similar frequency of the type I CD36 deficiency (>0.5%) was found in our cohort and in Japanese population (6). In contrast, we found fewer individuals carrying CD36 negative platelets (1.8% vs 4.0%).

The most common mutations found in our cohort are 329-330 del AC and 1228-1239 del ATTGTGCCTATT (Table 2A). The impact of these mutations on CD36 gene expression has been reported by Kashiwagi et al. (15, 17). The 329-330 del AC dinucleotide deletion (also known as 539-540 del AC) located in exon 5 results in frame shift with a translation stop at position 396 TGA (also known as 606 TGA) (17). The deletion of 12bp (also known as 1438-1449 del ATTGTGCCTATT) located in the middle of exon 13 leads to four amino acid deletion (Ile-Val-Pro-Ile) at positions 410-413 impairs the transportation of CD36 precursors from the endoplasmic reticulum to Golgi apparatus (15).

In this study, we could identify new CD36 mutations, C220T, 429+4 insg and 1200-5inv49bp) associated with type I CD36-deficient individuals. Analysis of CD36 transcript showed termination of CD36 at Gln74, skipping of exon 6 and exon 13, respectively. Several skippings of CD36 exons associated with CD36 deficiency have been described (11, 15, 18-19). Expression analysis of 1438-1449del associated with exon 9 skipping caused maturation and transport defects of CD36 precursor form. Further study is necessary to unravel the mechanism(s) of the exon 6 and exon 13 skipping found in our CD36-deficient individuals.

It has been showed that type II CD36-deficient individual could develop occasionally anti-Nak\(^a\) antibodies (5). In our cohort, however, no anti-Nak\(^a\) antibodies were found in type II CD36-deficient donors. Anti-Nak\(^a\) antibodies just were found in a female blood donor with type I CD36 deficiency. Molecular analysis showed a compound heterozygous mutation (C220T and 329-330 del AC) in...
The mutation C220T leads to premature stop codon TAG (Gln74Term), and the 329-330delAC causes frame shift at amino acid residue 110 (see above).

Curtis et al. reported the presence of anti-Nakα antibodies in two African-American mothers with FNAIT, who were homozygous for the T1264G mutation (10). This mutation introduces premature stop codon in exon 10 leading to the absence of CD36 on maternal platelets. In South-East Asian, single cases of FNAIT have been reported in Thai and Japanese populations (11, 20). Analysis of the maternal CD36 transcript showed a compound heterozygous mutation associated with skipping of exon 4 and 9 that led to type I CD36 deficiency, and consequently anti-Nakα antibody formation during the pregnancy (11). In addition, anti-Nakα antibodies associated with hydrops fetalis in two siblings have been described (21). These antibodies showed suppressive effects on the CFU-E colony formation of CD36+ cells. Analysis of the CD36 gene of the mother revealed a compound heterozygous mutation, a novel C1366T (also known as C1156T; see Table 2A) mutation in exon 12, corresponding to Arg386Trp, and a 12bp deletion (1438-1449 delATTGTGCCTATT; see above).

In this study, we found a case FNAIT with severe intracranial hemorrhage in the fetus of a type I CD36-deficient multi gravida mother, who has developed anti-Nakα antibodies during pregnancies. Analysis of maternal DNA showed a compound heterozygous mutation: one missense amino acid substitution Ser127Leu and one nucleotide (429+4insg) insertion in intron 5. However, the exact molecular mechanism(s) how these mutations cause CD36 defects are unclear. The Ser127Leu mutation was first described as a minor-allele in Thai patients with malaria (22). Interestingly, the 429+4insg leading to exon 6 skipping were found in two normal donors (Table 2A). Recently, Hori et al. showed that mutation in intron (1248+5g>a) of OXCT1 gene can affect donor splice leading to exons skipping in fibroblasts of patients with a typical ketolytic

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Figure 3: Flow cytometry analysis of CD36 expression of case 1 (Mo). A) Platelets and monocytes isolated from father’s (F) and mother’s blood were tested with FITC-labelled mab against CD36. B) Immunoprecipitation analysis of biotinylated normal platelets before (left panel) and after preclearing with mab FA6–152 (right panel). Left panel: AB-serum (lane 1), anti-HPA-1a (lane 2) and serum Mo (lane 3). Right panel: Preclearing of platelet lysates with mab FA6–152 (lanes 1–3); precleared lysates with serum Mo (lane 4).
What is known about this topic?
- CD36 (GPIV)-deficient individuals (type I and type II) exist in African and Asian populations. CD36-deficient individuals could develop antibodies during pregnancies and multiple platelet transfusion (case reports).
- CD36 deficiencies are caused by different mutations on the CD36 gene including nucleotide substitutions, deletions and insertions.
- However, the frequency of GPIV-deficient and its clinical impact in immune mediated thrombocytopenia is not known, especially in Chinese populations.

What does this paper add?
- This paper reports the first information regarding the frequency of CD36-deficient individuals in China, its molecular bases and its role in immune thrombocytopenia in a large cohort.
- This result suggests that antibodies against GPIV play an important role in the mechanism of immune thrombocytopenia among Asian populations.

defect caused by Succinyl-CoA transferase deficiency (SCOT disease) (23).

Several studies have demonstrated the role of anti-Nak\(^{a}\) antibodies in PTR (10). In most of the cases (87.5%) transfusion with CD36-deficient platelets resulted in satisfactory response. In this study, we could identify a case of PTR associated with strong anti-Nak\(^{a}\) antibodies in a patient receiving multiple transfusions of random platelets. Further analysis documented homozygous 329-330delAC underlying the type I deficiency in this patient.

In conclusion, our results show that CD36 deficiency exists in Chinese population, and CD36 immunisation can occur in these individuals. Based on these observations, screening for anti-Nak\(^{a}\) antibodies should be considered in suspected immune-mediated thrombocytopenia. A donor registry of CD36-deficient individuals should be established to supply patients with these disorders.

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Conflicts of interest
None declared.

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