Identification of cryptic splice site, exon skipping, and novel point mutations in type I CD36 deficiency

H Hanawa, K Watanabe, T Nakamura, Y Ogawa, K Toba, I Fuse, M Kodama, K Kato, K Fuse, Y Aizawa

CD36 is an 88 kDa glycoprotein IV expressed in platelets, monocytes, erythroblasts, capillary endothelial cells, and mammary epithelial cells. CD6 was reported to be a receptor for collagen, thrombospondin, P. falciparum infected red blood cells, apoptotic neutrophils, oxidised low density lipoproteins, and as a transporter for long chain fatty acids. CD36 serves many functions in coagulation, host defence, lipid metabolism and scavenging. CD36 deficiency was first identified in a patient who showed refractoriness to HLA matched platelet transfusion. CD36 deficiency can be divided into two subgroups: type I neither platelets nor monocytes/macrophages express CD36, and in type II monocytes/macrophages express CD36 but platelets do not. CD36 deficiency is more often observed in Japanese, Thais, and Africans than in Americans of European descent. The incidence of type II CD36 deficiency is approximately 4.0% in Japanese, whereas type I CD36 deficiency is 0.56%. However, approximately 17% of Japanese patients with coronary heart disease have CD36 deficiency and 40% of Japanese patients with HCM with asymmetrical hypertrophy (ASH) have CD36 deficiency. It has been reported that CD36 deficiency might result in ischaemic heart disease and hypertrophic cardiomyopathy (HCM). Recently, CD36 has been reported to play an important role in atherogenicity. It was reported that CD36 deficiency could promote defective insulin action and disorders fatty acid metabolism in spontaneous hypertension and antidiabetic thiazolidinedione drug and peroxisome proliferator activated γ (PPAR-γ) regulated CD36 expression. To understand the role of CD36, it is important to clarify the condition of CD36 deficient subjects.

Kashiwagi et al. and Yanai et al. reported three mutations responsible for CD36 deficiency, a substitution of T for C at nt 478 of CD36 cDNA, a dinucleotide deletion at nt 539, and a single nucleotide insertion at nt 1159. The substitution of T for C at 478 leads directly to CD36 deficiency via defects in post-translational modification. The dinucleotide deletion at nt 539 or the single nucleotide insertion at nt 1159 caused a frameshift leading to the appearance of a translation stop codon and a marked reduction in the level of CD36 mRNA. Furthermore, the dinucleotide deletion at nt 539 may affect the splicing of exon 4.

In the present study, we performed a molecular analysis of 11 type I CD36 deficiency subjects (including one family). The full lengths of translated CD36 cDNA were sequenced and the CD36 gene was analysed at the exon that was thought to show the mutation. In addition, quantification of monocyte CD36 mRNA was examined. Some types of genomic CD36 mutation often affect the splicing abnormality and their amounts of CD36 mRNA were similar to normal subjects.

**MATERIALS AND METHODS**

**Patients**

We studied 11 type I CD36 deficiency subjects (including one family) who were identified by flow cytometry. Their platelets and monocytes did not express CD36. Cardiovascular disease and other patient characteristics are shown in table 1.

**Amplification of CD36 cDNA in monocytes**

Peripheral blood cells from subjects were centrifuged at 400 × g for five minutes to collect leucocytes. Leucocytes were isolated after haemolysis in 0.17 mol/l Tris supplemented with 0.83% NH4Cl. Total RNA was isolated from them using Trizol (Life Technologies, Tokyo, Japan). cDNA was synthesised from 5 μg of total RNA with random primers and murine Moloney leukaemia virus reverse transcriptase in a final volume of 20 pl. CD36 cDNA was amplified with AmpliTaq polymerase.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of 11 patients with type I CD36 deficiency</th>
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<tbody>
<tr>
<td>Patients</td>
<td>Age</td>
</tr>
<tr>
<td>1</td>
<td>SI 2</td>
</tr>
<tr>
<td>2</td>
<td>SII 2</td>
</tr>
<tr>
<td>3</td>
<td>SII 2</td>
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</tr>
<tr>
<td>10</td>
<td>KS</td>
</tr>
<tr>
<td>11</td>
<td>MS</td>
</tr>
</tbody>
</table>

**Abbreviations:** HCM, hypertrophic cardiomyopathy; BMIPF, β-methyl-p-iodophenyl pentadecanoic acid; RT, reverse transcription; ASH, asymmetrical hypertrophy; PBGID, paraplatinogen deaminase; LDL, low density lipoprotein

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(TOYOBO, Osaka, Japan), each primer (table 2A, fig 1A) from 1 µl of leucocyte cDNA according to the following amplification profile: 35 cycles at 94°C for 60 seconds, 58°C for 90 seconds, and 73°C for 120 seconds. Amplified products were separated on 3% agarose gels and stained with ethidium bromide.

**Amplification of genomic CD36**

DNA was isolated from leucocytes by SepaGene (Sanko, Tokyo, Japan). The CD36 gene was amplified with each primer (table 2B, fig 1B) according to the following amplification profile: 35 cycles at 94°C for 60 seconds, 58°C for 90 seconds, and 73°C for 120 seconds.

**Subcloning of short RT-PCR products**

For subcloning of short RT-PCR products, they were purified from agarose gel and directly inserted into the pGEM-T vector (Promega, Madison, WI). The recombinant plasmids were then used to transform *Escherichia coli* JM109 competent cells (Takara, Kyoto, Japan). Individual ampicillin resistant colonies were isolated.

**Sequencing analysis**

PCR products of CD36 cDNA and the CD36 gene were purified with microcon-100 (Millipore, Bedford, MA). Purified PCR products and plasmid inserted short RT-PCR products were sequenced with a *Taq* Dye Deoxy terminator cycle sequencing kit (PE Applied Biosystems, Tokyo, Japan) and a DNA sequencer (model 310, PE Applied Biosystems).

**Quantitative RT-PCR**

For the purposes of making templates of CD36 and porphobilinogen deaminase (PBGD) as a housekeeping gene to calculate the standard concentration curve, their cDNA was amplified with each primer (CD36NAK5-5′, CD36NAK6-3′, or CD36 4-5′, CD36 6-3′ for CD36; PBGD-5′, PBGD-3′ for PBGD)

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**Table 2** Primer list

<table>
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<tr>
<th>Primer name</th>
<th>Sequence</th>
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<td>a CD36NAK5-5′</td>
<td>ccttcttagccattttaaagatagc</td>
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</tr>
<tr>
<td>b CD36NAK6-3′</td>
<td>aggccaggacagcaccaatgac</td>
<td>18</td>
</tr>
<tr>
<td>c CD36 2-5′</td>
<td>aaggtaggtagagacagacagac</td>
<td>18</td>
</tr>
<tr>
<td>d CD36 3-5′</td>
<td>aggccaggacagcaccaatgac</td>
<td>18</td>
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<tr>
<td>e CD36 4-5′</td>
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</tr>
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</tr>
<tr>
<td>g CD36 6-3′</td>
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<td>l CD36 14-3′</td>
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<tr>
<td>m CD36 13-5′</td>
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<tr>
<td>n CD36 14-3′</td>
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</tr>
<tr>
<td>o PBGD-5′</td>
<td>agccaggacagcaccaatgac</td>
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</tr>
<tr>
<td>p PBGD-3′</td>
<td>agccaggacagcaccaatgac</td>
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</tbody>
</table>

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**Figure 1** (A) Schematic presentation of the CD36 cDNA is shown. Arrows indicate the positions and directions of the primers. (B) Schematic presentation of the CD36 gene structure around the amplified regions is shown. Arrows indicate the positions and directions of the primers. Letters indicate primers in table 2.
RESULTS

Amplification of CD36 cDNA

St.2 and her two children (SII.1 and SII.2) had short RT-PCR products using the CD36 2-5′- and CD36 5-3′- primers or the CD36 8-5′- and CD36 11-3′- primers, respectively (fig 2B) and IK had short RT-PCR products using the CD36 8-5′- and CD36 14-3′-1 primers (fig 2C).

Sequencing analysis of short RT-PCR products

Plasmid inserted short RT-PCR products were sequenced. Each short RT-PCR product of SI.2, SII.1, and SII.2 was a cryptic splice of exon 4 (the 52 bp deletion from 331 to 382, abnormal splicing leads to a frameshift with the stop codon TGA) and skipping of exons 4 (the 161 bp deletion from 331 to 491, stop codon TGA), 9 (the 70 bp deletion from 959 to 1028, stop codon TGA), and both 9 and 11 (the 70 bp deletion from 959 to 1028 and the 119 bp deletion from 1217 to 1335, stop codon TGA) (fig 3A). FK showed a cryptic splice site of exon 4 and skipping of exon 9 (fig 3B) and IK showed skipping of exon 13 (the 55 bp deletion from 1410 to 1464, stop codon TTA) (fig 3C). However, eleven normal subjects showed no splicing abnormality of exons 4, 9, 11, and 13 (data not shown).

Analysis of genomic CD36

SI.2 was homozygous for an insertion of A at 1159 (a stop codon at nt 1263) as previously shown (fig 3D, b). SII.1 and SII.2 were heterozygous for T478 (a Pro90→Ser mutation) and heterozygous for an insertion of A at 1159 (fig 3D, c and d). The husband (SI.1) of SI.2, who showed normal CD36 expression, was heterozygous for T478 and did not have an insertion of A at 1159 (fig 3D, a). FK showed a novel homozygous mutation for C970 (a Phe253→Leu mutation) (fig 3E) and IK showed a novel homozygous mutation for C1447 (a Ile413→Leu mutation) and was heterozygous for T478 (fig 3F). The other six subjects of type I CD36 deficiency showed the T478 homozygous mutation as previously reported (data not shown). The findings of the genomic CD36 mutation are summarised in table 1.

Sequencing analysis of CD36 cDNA in SII.1 and SII.2

To examine the quantity of mRNA in the insertion of A at 1159 is similar to wild type, we tested using real time RT-PCR with two kinds of primers sets in SII.1, SII.1 and SII.2. Ten-fold serial dilutions of plasmids (10 copies, 10 copies, 10 copies, 10 copies, and 10 copies/test) were used to determine the standard curve. The quantities of CD36 and PBGD mRNA from the normal subjects SII.1, SII.1, and SII.2 were calculated from these standard curves. The melting curve showed specific products and its specificity was confirmed by electrophoresis (data not shown). The ratios of CD36/PBGD from SII.1, SII.1, and SII.2 were similar to those from normal subjects (fig 4).

DISCUSSION

We identified many splicing abnormalities of CD36 in CD36 deficient patients. SI.2 and her two children (SII.1 and SII.2) showed the same abnormal splicing of CD36, but 11 normal subjects showed no abnormal splicing; therefore, this suggested that the genomic CD36 mutation (maybe an insertion of A at 1159) might induce abnormal splicing. CD36 has been shown to produce alternatively spliced mRNA molecules with skipping exons in normal CD36 positive subjects, but we could not detect any cryptic splice sites or skipping of exons 4, 9, and 11 in normal CD36 positive subjects. Mutations of...
Figure 3   (A) Sequences of subclones from shorter PCR products for CD36 cDNA in Sl.2, Sl.1, and Sl.2 are shown, (2), (3), (5), and (6) are the sequences of subclones from the same numbered bands in fig 2A. (B) Sequences of subclones from shorter PCR products for cDNA of CD36 in FK are shown, (8) and (10) are the sequences of subclones from the same numbered bands in fig 2B. (C) The sequence of the subclone from the shorter PCR product for CD36 cDNA in FK is shown, (12) is the sequence of the subclone from the same numbered band in fig 2C. (D) Sequences of amplified genomic CD36 with CD36 IC(+) and CD36 ID(+) or CD36 II(+) and CD36 II(+) in Sl.1 (a), Sl.2 (b), Sl.1 (c), and Sl.2 (d) are shown. (E) The sequence of amplified genomic CD36 with CD36 IH(+) and CD36 II(+) in FK is shown. (F) The sequence of amplified genomic CD36 with CD36 IC(+) and CD36 ID(+) or CD36 ID(+) and CD36 IM(+) in IK is shown. (G) Sequence of PCR products with CD36 8-5' and CD36 14-3' for CD36 cDNA in Sl.1 is shown. CD36 11-3' was used for sequencing. (H) Sequence of PCR products with CD36 8-5' and CD36 14-3' for CD36 cDNA in Sl.2 is shown. CD36 11-3' was used for sequencing.
genomic DNA sometimes induce cryptic splice sites or exon skipping.\textsuperscript{1, 2} Kashiwagi et al\textsuperscript{5} reported that dinucleotide deletion at nt 539 might affect exon 4 skipping. The new CD36 mutations of C970 in KF and the C1447 mutation in KI might also have induced abnormal splicing.

Kashiwagi et al\textsuperscript{5} reported that a single nucleotide 1159A insertion led to a marked reduction in the level of CD36 mRNA in macrophages. However, in the present study, we found that CD36 deficient subjects with the 1159A insertion homozygous or heterozygous for T478 and the 1159A insertion had similar quantities of CD36 mRNA to normal subjects. This discrepancy may be explained by splicing abnormalities, that is, if primers or probes in the skipping exon are used for quantification of CD36 mRNA, the amount of mutant CD36 mRNA may be underestimated. The present findings call into question the previous hypothesis that 1159A insertion led to a marked reduction in the level of CD36 mRNA. CD36 is a multifunctional receptor.\textsuperscript{24} CD36 has binding sites for thrombospondin, collagen, long fatty acid apoptotic neutrophils, or oxidised LDL. Pearce et al\textsuperscript{25} reported that CD36 without the transmembrane region was secreted and bound thrombospondin. Therefore, to clarify the condition of CD36 deficient subjects, not only the types of genomic mutation but also the presence of splicing abnormalities should be examined.

It is unclear what characteristics CD36 deficient subjects have. It was reported that CD36 deficiency underlies insulin resistance, defective fatty acid metabolism, and hyperglyceridaemia in spontaneously hypertensive rats.\textsuperscript{23} However, Yanai et al\textsuperscript{26} reported that type I CD36 deficiency in humans was not associated with insulin resistance. Watanabe et al\textsuperscript{27} previously reported that the ratio of CD36 deficiency subjects in ischaemic heart disease patients was higher than in normal subjects and it was reported that CD36 deficiency increases LDL cholesterol, indicating a contribution of CD36 to LDL metabolism. However, Febbraio et al\textsuperscript{28} reported that in apo E null mice, blockade of CD36 could be protective even in more extreme proatherogenic circumstances. The correlation between HCM and CD36 deficiency is also controversial. Tanaka et al\textsuperscript{29} reported that CD36 deficiency might be one aetiology of hereditary HCM, but Nakamura et al\textsuperscript{30} reported that the incidence of CD36 deficiency in HCM patients is not higher than in the general population. Characteristics of CD36 deficiency must be examined in detail to clarify its role in atherogenicity and other diseases. In the present study, we analysed the molecular abnormality of type I CD36 deficiency and showed novel point mutations, new cryptic splice sites, and exon skipping. The presence of splicing abnormalities as well as the types of genomic mutation should be examined to clarify the condition of CD36 deficiency subjects.

**ACKNOWLEDGEMENTS**

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**REFERENCES**

1. Greenwalt DE, Lipsky RH, Cickenhouse CF, Ikeda H, Tandon NN, Jamieson GA. Membrane glycoprotein CD36: a review of its roles in
9. Curtis BR, Aser RH. Incidence of the Nak(a)-negative platelet phenotype in African Americans is similar to that of Asians. Transfusion 1996; 36:331-4.

ECHO

Limited role for HLA DR15 in MS

HLA DR15 indicates susceptibility to multiple sclerosis (MS) but does not affect its course, Hensiek et al have confirmed. Previous studies have mostly been too small to confirm or refute speculation that HLA type might influence clinical features or course of MS.

In their study Hensiek et al typed 729 patients whose MS was clinically well characterised for HLA DR15 homozygous and heterozygous phenotypes. The patients were all index cases from trio families (affected patient and both parents), white, mean age 38 years, EDSS score 4.3 (range 0–9.3), and more women than men had this phenotype (32.3 years, p=0.001), and more women than men had this phenotype (66% vs 55%, p=0.01). HLA DR15 was linked with female sex and age at diagnosis in regression analyses but not with clinical subgroup, history of autoimmune diseases, family history of MS, or disease progression; nor was it linked with disability or laboratory markers for MS.

These results, the authors say, agree with those of one other large study and suggest that HLA DR15 phenotype affects susceptibility to MS, not its course.

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