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

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CD36 is an 88 kDa glycoprotein IV expressed in platelets, monocytes, erythroblasts, capillary endothelial cells, and mammary epithelial cells.¹ CD6 was reported to be a receptor for collagen, thrombospondin, *P. falciparum* infected red blood cells,² apoptotic neutrophils,³ oxidised low density lipoproteins,⁴ and as a transporter for long chain fatty acids.⁵ 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.⁶ CD36 deficiency can be divided into two subgroups; in type I neither platelets nor monocytes/macrophages express CD36, and in type II monocytes/macrophages express CD36 but platelets do not.⁷ CD36 deficiency is more often observed in Japanese, Thais, and Africans than in Americans of European descent.^{8–9} The incidence of type II CD36 deficiency is approximately 4.0% in Japanese, whereas type I CD36 deficiency is 0.56%.⁸ However, approximately 17% of Japanese patients with coronary heart disease have CD36 deficiency¹⁰ and 40% of Japanese patients with HCM with asymmetrical hypertrophy (ASH) have CD36 deficiency.¹¹ It has been reported that CD36 deficiency might easily result in ischaemic heart disease¹⁰ and hypertrophic cardiomyopathy (HCM).¹¹

Recently, CD36 has been reported to play an important role in atherogenicity. It was reported that CD36 deficiency could promote defective insulin action and disordered fatty acid metabolism in spontaneous hypertension^{12–13} and antidiabetic thiazolidinedione drug and peroxisome proliferator activated receptor- γ (PPAR- γ) regulated CD36 expression.^{14–15} To understand the role of CD36, it is important to clarify the condition of CD36 deficient subjects.

Kashiwagi *et al*¹⁶ and Yanai *et al*¹⁷ 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,^{17–18} and a single nucleotide insertion at nt 1159.^{17–19} The substitution of T for C at 478 leads directly to CD36 deficiency via defects in post-translational modification.¹⁶ 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.^{18–19} Furthermore, the dinucleotide deletion at nt 539 may affect the splicing of exon 4.¹⁸

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 $\times$ 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 μ g of total RNA with random primers and murine Moloney leukaemia virus reverse transcriptase in a final volume of 20 μ l. CD36 cDNA was amplified with AmpliTaq polymerase

Abbreviations: HCM, hypertrophic cardiomyopathy; BMIPP, β -methyl-p-iodophenyl pentadecanoic acid; RT, reverse transcription; ASH, asymmetrical hypertrophy; PBGD, porphobilinogen deaminase; LDL, low density lipoprotein

Table 1 Characteristics of 11 patients with type I CD36 deficiency

Patients	Age	Gender	Diagnosis	CD36 gene mutation
1 SI.2	60	F	IHD (post CABG)	Homozygous 1159A insertion
2 SII.1	30	F	HT	Heterozygous 1159A insertion + heterozygous 478T
3 SII.2	35	M	HT, LVH	Heterozygous 1159A insertion + heterozygous 478T
4 FK	57	M	Dilated HCM, DM, HT	Homozygous 970C
5 IK	78	F	HT	Heterozygous 1447C + 478T
6 TT	59	M	HCM, HT	Homozygous 478T
7 AT	87	M	AR, CHF	Homozygous 478T
8 SY	74	M	IHD	Homozygous 478T
9 KT	70	M	OMI, CHF	Homozygous 478T
10 KS	62	F	IHD, LVH	Homozygous 478T
11 MS	74	M	CHF, lymphoma	Homozygous 478T

IHD, ischaemic heart disease; CABG, coronary artery bypass graft; HT, hypertension; LVH, left ventricular hypertrophy; AR, aortic regurgitation; DM, diabetes mellitus; CHF, congestive heart failure; OMI, old myocardial infarction.

Table 2 Primer list

Primer name	Sequence	Reference
<i>(A) Primers for cDNA analysis</i>		
a CD36NAK5-5'	ccttcttagcattttaagatagc	18
b CD36NAK6-3'	agccaggacagcaccaatgac	18
c CD36 2-5'	aaaatcatgtctgtctgt (exon 2)	
d CD36 3-5'	ctggctgtgttggagggtattct (exon 3)	
e CD36 5-3'	agcgtcctgggttacatttcc (exon 5)	
f CD36 4-5'	agtgtatgtgaacagcagcaaca (exon 4)	
g CD36 6-3'	agttcttcaaagttctgacttgg (exon 6)	
h CD36 6-5'	gatactcaattcacttattaacaa (exon 6)	
i CD36 9-3'	tacctggcttttcaacaa (exon 9)	
j CD36 8-5'	tattgggaagtcactgcgac (exon 8)	
k CD36 11-3'	acttgatcagaaaatgaggaag (exon 11)	
l CD36 14-3'-1	aggagggttattttccagttac (exon 14)	
m CD36 13-5'	actatattgtcctattcttgg (exon 13)	
n CD36 14-3'-2	gcctaattgtactctcttggat (exon 14)	
PBGD-5'	agattggagagaaagcctgtta	
PBGD-3'	cgaatactcctgaactccagatg	
<i>(B) Primers for genomic DNA analysis</i>		
o CD36 IC(+)	ggctctttatctggctgactcaaggctgc	18
p CD36 ID(-)	taagtacatttcaatacaatgac	18
q CD36 IH(+)	ctaactcattgacactcgatt	
r CD36 II(-)	agcatactatacttcagtatctgttta	
s CD36 II(+)	tctctgtattagggtcaatctatgctg	
t CD36 IJ(-)	tggactgtgctactgaggttattt	
u CD36 IL(+)	gttcataatttttcaacg	
v CD36 IM(-)	tttaatgactaacagctgc	

(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)

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.

Figure 2 (A) RT-PCR products for cDNA of CD36 in normal subjects, SI.2, SII.1, and SII.2 were fractionated on a 2% agarose gel and stained with ethidium bromide. (a-d) PCR products with CD36 2-5' and CD36 5-3'. (e-h) PCR products with CD36 8-5' and CD36 14-3'-1. (a and e) Normal subjects. (b and f) SI.2. (c and g) SII.1. (d and h) SII.2. (1) and (4) are wild types. (2), (3), (5), and (6) are shorter PCR products than the wild types. (B) RT-PCR products for cDNA of CD36 in a normal subject and FK were fractionated on a 2% agarose gel and stained with ethidium bromide. (a and b) PCR products with CD36 3-5' and CD36 5-3'. (c and d) PCR products with CD36 8-5' and CD36 11-3'. (a and c) Normal subjects. (b and d) IK. (7) and (9) are wild types. (8) and (10) are shorter PCR products than the wild types. (C) RT-PCR products with CD36 8-5' and CD36 14-3'-1 for cDNA of CD36 in normal subjects and IK were fractionated on a 2% agarose gel and stained with ethidium bromide. (a) Normal subjects. (b) IK. (11) is wild type and (12) is a shorter PCR product than the wild type.

(table 2A) and directly inserted into the pGEM-T vector and the recombinant plasmids were isolated after transforming *Escherichia coli* JM109 competent cells. cDNA from leucocytes of each subject was diluted 100-fold with DNase free water and 5 μ l were then used for CD36 and PBGD real time PCR, respectively. cDNA and diluted plasmid were amplified with the same primer used for making plasmid and LightCycler-FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN). After an initial denaturation step of 10 minutes at 95°C, a three step cycle procedure was used (denaturation 95°C for 0 second, annealing 62°C for 10 seconds, and extension 72°C for 13 seconds) for 40 cycles. The LightCycler Software calculated a standard curve using five CD36 or PBGD plasmid standards. The standard curve was created by plotting the cycle numbers at which the fluorescent signals enter the log-linear phase versus the concentrations of the standards. The relative amounts of PBGD and CD36 transcripts of all samples were calculated by the LightCycler software using this standard curve. The final results were the normalised CD36 value, expressed as the ratio CD36/PBGD.

RESULTS

Amplification of CD36 cDNA

SI.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 14-3'-1 primers, respectively (fig 2A), although RT-PCR products of other kinds of primers were only

wild type bands. In addition, FK had short RT-PCR products using CD36 3-5' 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 ⁴³⁷tga) and skipping of exons 4 (the 161 bp deletion from 331 to 491, stop codon ⁵³¹tga), 9 (the 70 bp deletion from 959 to 1028, stop codon ¹⁰⁶⁴tga), and both 9 and 11 (the 70 bp deletion from 959 to 1028 and the 119 bp deletion from 1217 to 1335, stop codon ¹⁰⁶⁴tga) (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 ¹⁵¹¹taa) (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 heterozygous 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, we analysed sequences of PCR products amplified with CD36 8-5' and CD36 14-3'-1 using primer CD36 11-3'. We found an insertion of A at 1159 but no insertion in SII.1 and SII.2 (fig 3G, H) which suggested that the amount of mRNA in the insertion of A at 1159 was similar to the mRNA without an insertion.

Quantities of CD36 mRNA

To confirm that the amount 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 SI.1, SII.1, and SII.2. Ten-fold serial dilutions of plasmids (10⁷ copies, 10⁶ copies, 10⁵ copies, 10⁴ copies, and 10³ copies/test) were used to determine the standard curve. The quantities of CD36 and PBGD mRNA from the normal subjects SI.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 SI.2, 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 skipped exons in normal CD36 positive subjects,^{20 21} 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 4 The ratio of CD36/PBGD from five normal subjects, SI.2, SII.1, and SII.2. To calculate the ratio, quantification of CD36 mRNA was examined with CD36NAK5-5' and CD36NAK6-3' (A) or with CD36 4-5' and CD36 6-3' (B).

genomic DNA sometimes induce cryptic splice sites or exon skipping.^{22, 23} Kashiwagi *et al*¹⁸ 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*¹⁹ 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.²⁻⁵ CD36 has binding sites for thrombospondin, collagen, long fatty acid apoptotic neutrophils, or oxidised LDL. Pearce *et al*²⁴ 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.¹² However, Yanai *et al*²⁵ reported that type I CD36 deficiency in humans was not associated with insulin resistance. Watanabe *et al*¹⁰ 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*²⁷ 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*¹¹ reported that CD36 deficiency might be one aetiology of hereditary HCM, but Nakamura *et al*²⁸ 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,

- CD36 serves many functions in coagulation, lipid metabolism, and scavenging. It was reported that CD36 deficiency might underlie refractoriness to HLA matched platelet transfusions, ischaemic heart disease, or insulin resistance.
- We conducted a molecular analysis of 11 subjects of type I CD36 deficiency which showed that five of 11 had abnormal spliced CD36 cDNA. The genomic DNA from one subject (SI.2) was homozygous for an insertion of A at 1159. Two children of SI.2 (SII.1 and SII.2) were also type I CD36 deficient and heterozygous for T478 and an insertion of A at 1159. One subject (FK) showed a novel homozygous mutation for C970 and one subject (IK) showed a novel heterozygous mutation for C1447 and was heterozygous for T478. SI.2, SII.1, and SII.2 showed cryptic splice sites or skipping of exons 4, 9, and 11. Furthermore, their CD36 mRNA amounts were similar to normal subjects. IK showed skipping of exon 13 and FK showed a cryptic splice site of exon 4 and skipping of exon 9.
- These findings suggested that some types of genomic CD36 mutation often affect the splicing abnormality.

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.

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ECHO

Limited role for HLA DR15 in MS

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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), with clinically definite (89%), laboratory supported definite (6%), or clinically probable (5%) MS. Hensiek *et al* grouped patients according to whether they had primary progressive MS (no remissions) (69 probands) or bout onset MS (relapsing-remitting and secondary progressive) (660) and opticospinal (61) or diffuse (668) disease.

In all, 59% of the patients had one or more HLA DR15 alleles. MS was diagnosed at an earlier age in patients with HLA DR15 (30.3 v 32.3 years, $p=0.001$), and more women than men had this phenotype (66% v 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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