Defect in human myocardial long-chain fatty acid uptake is caused by *FAT/CD36* mutations

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Abstract **Because of the importance of long-chain fatty acids (LCFAs) as a myocardial energy substrate, myocardial LCFA metabolism has been of particular interest for the understanding of cardiac pathophysiology. Recently, by using radiolabeled LCFA analogues, myocardial LCFA metabolism has been clinically evaluated, which revealed a total defect of myocardial LCFA accumulation in a small number of subjects. The mechanism for the cellular LCFA uptake process is still disputable, but recent results suggest that fatty acid translocase (FAT)/CD36 is a transporter in the heart. In the present study, we analyzed mutations and protein expression of the *FAT/CD36* gene in 47 patients who showed total lack of the accumulation of a radiolabeled LCFA analogue in the heart. All the patients carried two mutations in the *FAT/CD36* gene, and expression of the FAT/CD36 protein was not detected on either platelet or monocyte membranes. Our results showed the link between mutations of the *FAT/CD36* gene and a defect in the accumulation of LCFAs in the human heart.**—Tanaka, T., T. Nakata, T. Oka, T. Ogawa, F. Okamoto, Y. Kusaka, K. Sohmiya, K. Shimamoto, and K. Itakura. **Defect in human myocardial long-chain fatty acid uptake is caused by *FAT/CD36* mutations. *J. Lipid Res.* 2001.** 42: **751–759.**

Supplementary key words fatty acid translocase • human heart • cardiac pathophysiology

A family of long-chain fatty acids (LCFAs) is an important energy source and plays many roles in diverse cellular processes. The cellular uptake is the first step in the utilization of LCFAs by organs. However, controversy exists concerning the mechanisms responsible for LCFA transport across plasma membranes. Although diffusion of LCFAs is an accepted fact (1), there is some doubt that diffusion is sufficient to provide the rates of LCFA uptake observed in vivo and can allow adaptive changes in the process. Thus, in addition to diffusion, a facilitated protein-mediated LCFA transport across membranes has been proposed (2–5). The myocardium rapidly takes up LCFAs and derives 60–90% of their energy through β-oxidation (6). Accordingly, it is reasonable to assume that a specific protein-mediated LCFA transport system exists on the myocardial plasma membrane.

CD36 is known as a receptor for a variety of molecules, including collagen, thrombospondin, malaria-infected erythrocytes, a 37-kDa agglutination protein (PAP p37) (7), and oxidized low density lipoprotein (LDL) (8). Additionally, Abumrad et al. (4) proposed another function for CD36 a transporter of LCFAs. They cloned an 88-kDa mouse adipocyte membrane protein of which covalent modification with sulfo-*N*-succinimidyl oleate irreversibly inhibited fatty acid transport, and designated this protein as fatty acid translocase (FAT). The amino acid sequence of FAT is 85% homologous with that of human CD36. By using a similar LCFA analogue, sulfo-*N*-succinimidyl palmitate, we also demonstrated the inhibition of myocardial LCFA uptake into the isolated perfused rat heart. To obtain the amino acid sequence of the cardiac membrane protein that is specifically modified with sulfo-*N*-succinimidyl palmitate, we purified the protein from bovine hearts. Its partial amino acid sequence showed a high homology to human CD36 (9). These results strongly suggest that CD36 (also referred to as FAT/CD36) is a transporter protein of myocardial LCFAs. We therefore hypothesized that the defect in the uptake of human myocardial LCFAs links to mutations in the *FAT/CD36* gene, and we have already reported the association between the defect in the myocardial LCFA uptake and mutations of the *FAT/CD36* gene (10–13). However, our previous work had two limitations: 1) a small number of patients was examined, and 2) only the base substitution mutation C to T at nucleotide position

Abbreviations: HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; AP, angina pectoris; MI, myocardial infarction; HT, hypertension; AS, aortic stenosis; MS, mitral stenosis.

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478 (C478T), the two-base deletion A and C at nucleotide positions 539 and 540 (Del539AC), and the single-base insertion of A at nucleotide position 1159 (Ins1159A) were analyzed by polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) (10–13).

To substantiate our hypothesis, in this study we selected patients with a total defect in myocardial LCFA uptake, which was defined by a total lack of myocardial iodine-123 15-(*p*-iodiphenyl) 3-R,S-methyl-pentadecanoic acid (^{123}I-BMIPP) accumulation, from a larger number of patients with heart diseases, and investigated mutations of the *FAT/CD36* gene and its protein expression. Mutations of the *FAT/CD36* gene were analyzed thoroughly with PCR-preferential homoduplex formation assay (PCR-PHFA) (14) and DNA sequencing in addition to PCR-RFLP, and expression of the mutated FAT/CD36 proteins was examined by flow cytometry on both platelets and monocytes. Our results revealed that all the patients lacking myocardial ^{123}I-BMIPP accumulation carried two mutations in the *FAT/CD36* gene and their proteins were not detected on either platelet or monocyte membranes. On the contrary, no mutations of the gene were found in any of the control subjects showing normal uptake of ^{123}I-BMIPP in the heart. These results strongly support the hypothesis that FAT/CD36 functions as a transporter of human myocardial LCFAs.

SUBJECTS AND METHODS

Study population

Six thousand, nine hundred seventy patients at 12 medical centers in Hokkaido, Japan, who underwent myocardial ^{123}I-BMIPP scintigraphy between March 1993 and December 1997 for evaluation of suspected or known cardiac diseases, were retrospectively surveyed. Thirty three patients (0.47%) were identified as having a total defect in myocardial LCFA uptake, and 28 of them whose blood samples were available were enrolled in this study. Apart from this study, 19 patients who showed the same phenomenon were found at several other medical centers and included in this study. Thus, a total of 47 blood samples from patients who showed a total defect in LCFA uptake in the heart were obtained. for control subjects showing normal myocardial ^{123}I-BMIPP accumulation, we examined the same number of subjects randomly selected from the pool and 8 healthy volunteers without any apparent cardiac disorders. After informed consent was obtained in every case, blood samples were kindly donated and were sent to our laboratory. The study protocol was approved by the Research Council of our institution and the investigation conformed with the principles outlined in the Declaration of Helsinki.

Evaluation of myocardial LCFA uptake with ^{123}I-BMIPP and myocardial perfusion

The total defect in myocardial LCFA accumulation was defined as described (12). Myocardial ^{123}I-BMIPP single-photon emission computed tomography (SPECT) was done 20 min after an intravenous injection of 111 MBq of ^{123}I-BMIPP (Nihon Medi-Physics, Nishinomiya, Japan) at resting and overnight fasting conditions. A negative accumulation of radioactivity in the heart was considered a total defect in myocardial LCFA uptake. To exclude the possible influence of myocardial blood supply on the incorporation of myocardial ^{123}I-BMIPP, myocardial perfusion single-photon emission computed tomography (SPECT) with thallium-201 (74–111 MBq), technetium-99m sestamibi (600–740 MBq), or technetium-99m tetrofosmin (600–740 MBq) was also performed on all the subjects, using a standard rotating gamma-camera and dedicated minicomputer system.

FAT/CD36 Molecule flow cytometry

FAT/CD36 expression on platelets and monocytes was analyzed by flow cytometry using an anti-human CD36 monoclonal antibody (OKM5; Ortho Diagnostic Systems, Raritan, NJ) as described previously (10).

Preparation of genomic DNA and total RNA

Genomic DNA was isolated from peripheral blood samples using a QIAamp Blood Kit (QIAGEN, Hilden, Germany), and total RNA was isolated from peripheral blood samples by the acid guanidium thiocyanate-phenol-chloroform extraction method (15).

PCR-RFLP of genomic DNA

PCR-RFLP analysis was carried out as described previously (13). Briefly, to detect C478T (16), Del539AC (17), and Ins1159A (18), 1.0 μg of genomic DNA was amplified using the primer sets, NAK386(+)-ID(−), ID(+)-SSPI(−), and XMNI(+)-XMNI(−)RE (0.4 μmol/l), and ExTaq polymerase (TaKaRa, Kyoto, Japan) (0.025 U/μl) or AmpliTaq Gold (Perkin-Elmer) (0.02 U/μl) in a DNA thermal cycler (MiniCycler; MJ Research, Inc., MA) or GeneAmp PCR System 9600 (Perkin-Elmer), and all PCR products were digested with the restriction endonucleases, *Cfr* 13I (TaKaRa), *Ssp* I (TaKaRa), and *Xmn* I (New England BioLabs, Beverly, MA), respectively.

PCR-PHFA of genomic DNA

PCR-PHFA, developed by Oka et al. (14), is a powerful method for searching mutations in 200–300 bp DNA based on competition between double-labeled PCR amplicon and unlabeled amplicon during hybridization. The brief protocol is as follows: Oligonucleotide primers flanking every exon, from exon III to XIV of the *FAT/CD36* gene (19), were synthesized as shown in **Table 1.** To detect unknown mutations in a PCR amplicon, double-labeled PCR amplicons from all of the DNA samples and a large amount of unlabeled PCR amplicons from a control DNA with the wild-type sequence were mixed to prepare the annealing mixture. For detection of known mutations, double-labeled PCR amplicons from a control DNA, wild-type sequence and known mutant, and a large amount of unlabeled PCR amplicons from every sample DNA were mixed to prepare the annealing mixture. Equal volumes of annealing mixtures from the double-labeled PCR amplicon and the unlabeled amplicon were denatured at 98°C for 10 min, then slowly cooled to 85°C in 90 min with a thermal cycler. Then the hybridization mixtures were transferred to the streptavidin-coated microtiter wells containing alkaline phosphatase conjugated anti-DNP antibody (Wakunaga Pharmaceutical, Hiroshima, Japan) solution and incubated for 30 min at room temperature. After washing the microtiter wells, the chromogenic substrate solution containing *p*-nitrophenyl phosphate (*p*NPP) and diethanolamine buffer were added. The coloring reaction proceeded at room temperature for 10 min, and the absorbance at 405 nm was measured with a microtiter plate reader (Titertek Multiskan® MCC/340; Labsystems Oy, Helsinki, Finland).

Cloning of PCR amplified products and sequencing

PCR amplification of every mutated exon DNA was carried out using unlabeled sets of the same primers as were used for

TABLE 1. Nucleotide sequences of primers used in this study

Primers Used in PCR-RFLP

Mutation	Upstream Primer		Downstream Primer	
C478T	NAK386(+):	5′-CAGAAGTTTACAGACAGTTTTGGACCTTTG-3′	NAK583(−):	5′-AAGGTTCGAAGATGGCACCATTGG-3′
Del539AC	ID(+):	5′-AGATCTAATGTTCACATATG-3′	SSPI(−):	5′-CCATTGGGCTGCAGGAAAGAGAATAT-3′
Ins1159A	XMNI(+):	5′-CGTTAATCTGAAAGGATTCC-3′	XMNI(−)RE:	5′-TGTACAATTTTTTGAGAGAA-3′

Primers Used in PCR-PHFA and Sequencing of Genomic DNA

Exon		Upstream Primer		Downstream Primer	
III	*CD36-3U*	(Bio or Am.):	5′-TTCTGTTTTATGATCTCTTTCTAAT-3′	CD36-3L	(DNP or Am.): 5′- AATGAGAGGATATTCTTTGACTAC-3′
IV	*CD36-4U*	(Bio or Am.):	5′-CATAACCCAAACTTATTTTCTTTTCC-3′	CD36-4L	(DNP or Am.): 5′-AGTGTCTCATATTTGTGGGCACTCA-3′
V	*CD36-5U*	(Bio or Am.):	5′-TTTGAATTTTGTTTACTGCTGTTTC-3′	CD36-5L	(DNP or Am.): 5′-AATAGATAACTTTGTTGTTTGTCAC-3′
VI	*CD36-6U*	(Bio or Am.):	5′-TTGTCTTAAACAGTGACTTTGTTTT-3′	CD36-6L	(DNP or Am.): 5′-ATAATATTGCCATTCATATTTGGTA-3′
VII	*CD36-7U*	(Bio or Am.):	5′-AAGTAACATTTTCCCATACATATAT-3′	CD36-7L	(DNP or Am.): 5′-CATACATGCACATTTTACCAGAATA-3′
VIII	*CD36-8U*	(Bio or Am.):	5′-TGTTTATTCATTGTCTTTTTCTATT-3′	CD36-8L	(DNP or Am.): 5′-CTGTGATGACCACAAAACAAATATT-3′
IX	*CD36-9U*	(Bio or Am.):	5′-CTAATCATTTGCCACTCGATTTTTA-3′	CD36-9L	(DNP or Am.): 5′-AGCATACTTATACTTCAGTATCTGT-3′
X	*CD36-10U*	(Bio or Am.):	5′-TGGAATGCAGCTCTTTTTTCTCTGT-3′	CD36-10L	(DNP or Am.): 5′-ATGGACTGTGCTACTGAGGTTATTT-3′
XI	*CD36-11U*	(Bio or Am.):	5′-TTCCAATTGACTCTTAAAACTTGTC-3′	CD36-11L	(DNP or Am.): 5′-CCAAATCAGATCAATAAGGTGTTTT-3′
XII	*CD36-12U2*	(Bio or Am.):	5′-TTGGTAATTATTTAGTTGTTCTCTTTTTAG-3′	CD36-12L2	(DNP or Am.): 5′-TATCAAAATAACCATTTTCAAGAGACTCAC-3′
	CD36-12Uint[a]	(Bio or Am.):	5′-TTACTACCTTCTCTTCTGCTGTAAG-3′		
XIII	*CD36-13U*	(Bio or Am.):	5′-GTTCATAATTATTTTCAACGTATTA-3′	CD36-13L2	(DNP or Am.): 5′-TTTTAATGACTAACAGCTGCAAATACAAAC-3′
	CD36-13U3[b]	(Bio or Am.):	5′-AGTTTATATGTTCATAATTATTTTCAACGT-3′		
XIV	*CD36-14U*	(Bio or Am.):	5′-AAATAATGTTGATTATTAACTTGAT-3′	CD36-14L	(DNP or Am.): 5′-TGAAGCAATATTTTTTGGTACATAC-3′

Primers Used for Detecting Skip exon XII (Case 47) and Skip exon XIII (Case 43)

Upstream Primer		Downstream Primer	
NAK5(+):	5′-CCTTCTTAGCCATTTTAAAGATAGC-3′	NAK4(−):	5′-CAGTGGCTAGACATGTCTAG-3′
CMP-1:	5′-TCATATCCAGGAGTTTGCAAG-3′	CMP-2:	5′-TTGGCTAGAAAACGAACTCTG-3′
CMP-3:	5′-TTGATGTGCAAAATCCACAGG-3′	CMP-4:	5′-ATGGATCCCTATAGCCCC-3′
CMP-5:	5′-TTGTTCAAATGATCCTCAATTCAC-3′	CMP-6:	5′-GACCTGCAAATATCAGAAGAAAAG-3′
CMP-7:	5′-TGGTACAGATGCAGCCTC-3′	CMP-8:	5′-TTCTGAAACATCAGGACTTGC-3′
CMP-9:	5′-GTACATCATATGGTGTGCTAG-3′	CMP-10:	5′-GCCTTCTCATCACCAATGG-3′
CMP-11:	5′-CCTATTGGTCAAGCCATCAG-3′	CMP-12:	5′-GGCCTAATATGTAACTTCTCTTTG-3′
IN12F:	5′-GAAAGGAAAAATCCACACTTGTG-3′	IN13R:	5′-AAGACAAATGTTACAACAAGAAAATGTG-3′

Biotin and DNP labeled primers were used to prepare labeled amplicons. Aminoalkylated primers were used to prepare unlabeled amplicons. Primers in *italic* were newly designed, and those in plain text were obtained from previous publications (15–18). Am., aminoalkylated; Bio, biotin labeled; DNP, DNP labeled; PCR-PHFA, PCR-preferential homoduplex formation assay; PCR-RFLP, polymerase chain reaction-restriction fragment polymorphism.

[a] Re-synthesized to detect Skip exon XII.

[b] Re-synthesized to detect Skip exon XIII.

PCR-PHFA. Cloning of PCR products was performed using the pCR-Script AmpSK(+) Cloning Kit (STRATAGEN, CA) or pT7Blue(R) (Novagen, WI) respectively, following the manufacturer's instructions. DNA sequencing was performed using unlabeled sets of the same primers that were used for PCR-PHFA, with the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster, CA).

Identifications of mutations in cases 43 and 47

cDNA was synthesized from 0.5 μg of blood total RNA using oligo-dT primer (25 μg/ml) and SuperScript™ II RNase H- Reverse Transcriptase (GIBCO BRL, Rockville, MD) (10 U/μl). First-round PCR was performed using NAK5(+) and NAK4(−) primers, which amplified from nt.8 to 1745 of the *FAT/CD36* gene. The second round PCR was carried out using the five sets of primer as follows: CMP1–CMP4 for nt.149-765, CMP3–CMP6 for nt.413-1031, CMP5–CMP8 for nt.668-1275, CMP7–CMP10 for nt.951-1490, and CMP9–CMP12 for nt.1175-1715. All the PCR products were directly sequenced using the same primers as were used for the second-round PCR.

To analyze the mutations that could cause skipping of exons, the genomic DNA was sequenced using PCR-amplified fragments. In Case 43 (Skip exon XIII), genomic DNA was amplified using CMP11–CMP10 primers. On the basis of the sequence results, we synthesized a pair of primers, IN12F and IN13R, which primed in intron 12 and intron 13, respectively. After cloning of the PCR product of IN12F–IN13R, sequencing was carried out using T7 primer. In Case 47 (Skip exon XII), the PCR product produced by a pair of primers, CD36-11U-CD36-13L2, was cloned and sequenced using CD36-12U and CD36-12L2 primers.

RESULTS

Evaluation of myocardial LCFA uptake by using ^{123}I-BMIPP scintigraphy

We screened 6,970 patients using myocardial ^{123}I-BMIPP SPECT, and found that 33 patients had a total defect in myocardial accumulation of ^{123}I-BMIPP. In these patients, myocardial perfusion imaging with thallium-201, technetium-99m sestamibi, or technetium 99m tetrofosmin demonstrated no definite perfusion abnormality, except for myocardial hypertrophy and infarct-related perfusion defect. None of the patients exhibited a global myocardial perfusion defect. Thus, the occurrence of this

phenomenon in patients with suspected or known cardiac diseases was estimated to be 0.47% (33/6970). Twenty-eight blood samples were available from these 33 patients. Additionally, 19 blood samples from patients showing the same phenomenon were also available from other sources (see Subjects and Methods), and a total of 47 blood samples were analyzed in the study. **Table 2** shows the clinical backgrounds of the patients, who comprised 29 males and 18 females, mean age of 56.1 ± 17.3 years (range, 16–87). The underlying diagnoses were as follows: 10 patients had angina pectoris, 8 had myocardial infarction, 11 had hypertrophic cardiomyopathy (HCM), 10 had dilated cardiomyopathy (DCM), 6 had hypertension, 1 had aortic stenosis, and 1 had mitral valve disease. **Figure 1** shows a representative ^{123}I-BMIPP scintigram. Figure 1A and B demonstrates a whole body image and a magnified image respectively, of a control subject without *FAT/CD36* mutation, which reveals ^{123}I-BMIPP accumulation in the heart and liver but not in any other organs and tissues. In the case of a patient with two mutations of the *FAT/CD36* gene (Case 4 in Table 2), a total defect in ^{123}I-BMIPP activity was observed only in the region of the heart (Fig. 1C).

TABLE 2. Clinical backgrounds and *FAT/CD36* mutations

	Age	Sex	Diag.[a]	
1.	44	M	HCM	C478T (Pro90Ser) (Homo)
2.	62	M	HCM	C478T (Pro90Ser) (Homo)
3.	55	M	AP	C478T (Pro90Ser) (Homo)
4.	43	M	HCM	C478T (Pro90Ser) (Homo)
5.	37	M	HCM	C478T (Pro90Ser) (Homo)
6.	69	F	AP	C478T (Pro90Ser) (Homo)
7.	71	M	HCM	C478T (Pro90Ser) (Homo)
8.	16	F	DCM	C478T (Pro90Ser) (Homo)
9.	39	M	AP	C478T (Pro90Ser) (Homo)
10.	52	M	MI	C478T (Pro90Ser) (Homo)
11.	63	M	MI	C478T (Pro90Ser) (Homo)
12.	70	M	AS	C478T (Pro90Ser) (Homo)
13.	54	F	DCM	C478T (Pro90Ser) (Homo)
14.	47	M	AP	C478T (Pro90Ser) (Homo)
15.	65	F	MI	C478T (Pro90Ser) (Homo)
16.	65	M	MI	C478T (Pro90Ser) (Homo)
17.	72	F	AP	C478T (Pro90Ser) (Homo)
18.	36	M	HT	C478T (Pro90Ser) (Homo)
19.	20	M	DCM	C478T (Pro90Ser) (Homo)
20.	85	M	HCM	C478T (Pro90Ser) (Homo)
21.	67	F	DCM	C478T (Pro90Ser) (Homo)
22.	69	M	HCM	C478T (Pro90Ser) (Homo)
23.	55	M	HT	C478T (Pro90Ser) (Homo)
24.	50	F	HCM	C478T (Pro90Ser) (Homo)
25.	49	M	DCM	C478T (Pro90Ser) & Del539AC
26.	80	F	DCM	C478T (Pro90Ser) & Del539AC
27.	75	F	HT	C478T (Pro90Ser) & Del539AC
28.	64	M	MR	C478T (Pro90Ser) & Del539AC
29.	34	M	MI	C478T (Pro90Ser) & Del539AC
30.	30	M	HCM	C478T (Pro90Ser) & Del539AC
31.	63	F	AP	C478T (Pro90Ser) & Del539AC
32.	31	M	HCM	C478T (Pro90Ser) & Del539AC
33.	54	M	AP	C478T (Pro90Ser) & Ins1159A
34.	22	F	DCM	C478T (Pro90Ser) & Ins1159A
35.	73	M	MI	C478T (Pro90Ser) & Ins1159A
36.	68	F	AP	C478T (Pro90Ser) & Ins1159A
37.	53	M	MI	C478T (Pro90Ser) & Ins1159A
38.	49	M	HCM	C478T (Pro90Ser) & Ins1159A
39.	51	F	HT	Del539AC & Ins1159A
40.	53	F	DCM	C478T (Pro90Ser) & T970 C (Phe254Leu)
41.	87	M	AP	C478T (Pro90Ser) & 12-bp del. (^{410}Ile-Val-Pro-Ile)
42.	66	F	HT	Del539AC & 43-bp dupl.
43.	65	F	DCM	Ins1159A & skip. exon13
44.	52	M	AP	Ins1159A & 12-bp del. (^{410}Ile-Val-Pro-Ile)
45.	67	M	MI	Ins1159A & 12-bp del. (^{410}Ile-Val-Pro-Ile)
46.	71	F	HT	C478T (Pro90Ser) & Ins770T
47.	76	F	DCM	C478 (Pro90Ser) & skip. exon12

[a] Diagnosis made at the time ^{123}I-BMIPP scintigraphy was performed.

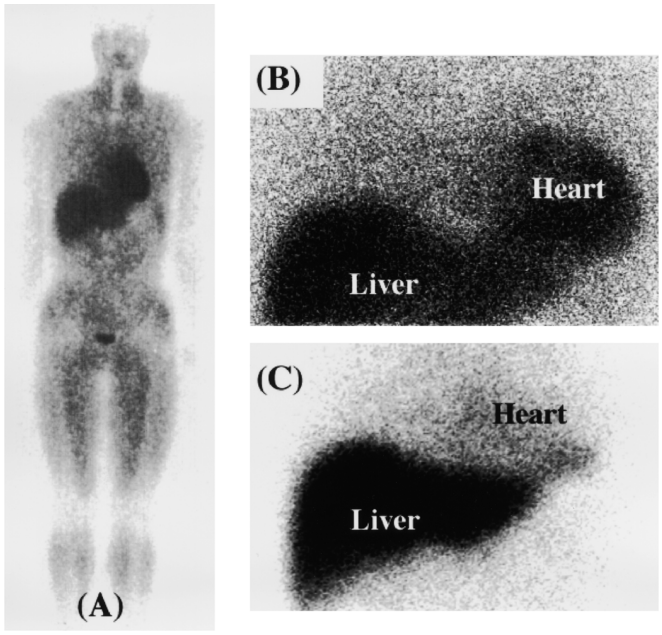

Fig. 1. ^{123}I-BMIPP scintigrams. Myocardial long-chain fatty acid (LCFA) uptake was evaluated using ^{123}I-BMIPP single-photon emission computed tomography (SPECT). A whole body image (A) and a magnified image (B) of a control subject without *FAT/CD36* mutation reveal ^{123}I-BMIPP accumulation in the heart and liver, but not in any other organs or tissues. In the case of a patient with two mutations of the *FAT/CD36* gene (C) (Case 4 in Table 2), total defect in ^{123}I-BMIPP activity was observed only in the region of the heart, even though this picture is exposed more intensively than that in (B). Note that the hepatic accumulation is observed in (C) as well as in (B).

Flow cytometric analysis of the FAT/CD36 protein

We performed flow cytometric analysis of both platelets and monocytes from all 47 cases to evaluate FAT/CD36 expression. **Figure 2** shows the results of representative flow cytometry from a normal (control) subject and 1 of 47 patients (Case 8 in Table 2). Both platelets and monocytes from the normal subject show positive fluorescence intensity (upper panel), but those from Case 8 show no fluorescence intensity (lower panel). In all 47 patients, the FAT/CD36 protein was not detected on either platelet or monocyte membranes by flow cytometric analysis, but it was detected in all the control subjects. Subsequently, we analyzed the *FAT/CD36* gene.

Identification of FAT/CD36 mutation

In all 47 cases, we carried out PCR-RFLP analysis of genomic DNA as published (13) to search for three previously reported mutations, C478T (16), Del539AC (17), and Ins1159A (18). We found C478T homozygous muta-

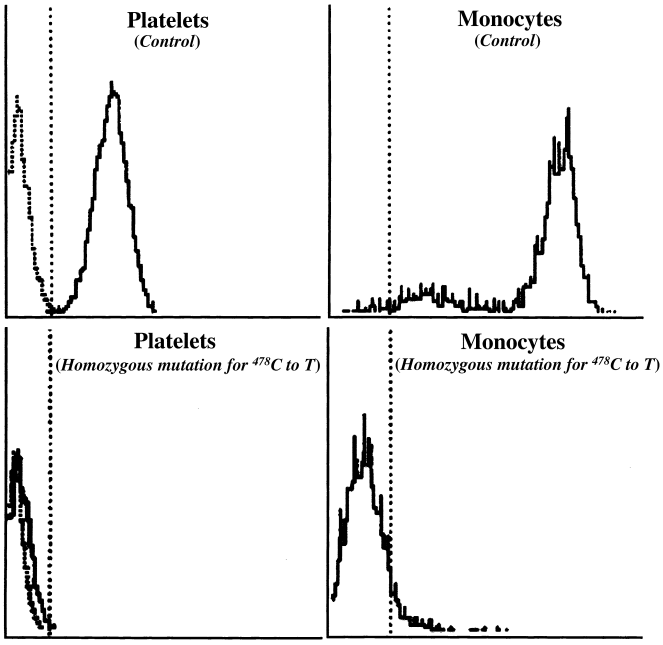

Fig. 2. Flow cytometry analysis of FAT/CD36 expression. Expression of FAT/CD36 on platelets and monocytes was performed using flow cytometry as described in Materials and Methods. Positive fluorescence intensity is demonstrated for both platelets and monocytes from a normal (control) subject who has the wild-type *FAT/CD36* gene (upper panel), whereas no fluorescent signals are detected on either platelets or monocytes in the patient with the *FAT/CD36* mutation (homozygote of C478T; Case 8 in Table 2) (lower panel). Abscissa, logarithmic scale of fluorescence; Ordinate, cell number.

tion in 24 cases (Cases 1–24 in Table 2), combinations of two respective mutations, C478T and Del539AC, in 8 cases (Cases 25–32), C478T and Ins1159A in 6 cases (Cases 33–38), and Del539AC and Ins1159A in 1 case (Case 39). In 8 patients (Cases 40–47), one heterozygous mutation, C478T, Del539AC, or Ins1159A, was detected.

To search mutations in all 47 patients further, we screened each exon, from exon III to exon XIV of the *FAT/CD36* gene by PCR-PHFA. In every patient from Cases 1 to 39, PCR-PHFA revealed mutations identical to those detected by PCR-RFLP but no other mutations.

In the remaining cases in which one of three reported mutations was detected by the PCR-RFLP, PCR-PHFA detected mutated exons in 6 patients (Cases 40–42 and 44–46). Cloning and sequencing of these mutated exons revealed four distinct mutations in the *FAT/CD36* gene as follows: exon VI, a single nucleotide insertion of T at nucleotide position 770 (Ins770T), in Case 46; exon IX, a base substitution T to C at nucleotide position 970, in Case 40; exon XIII, a 12-base deletion at nucleotide position 1438 (12bp-Del 1438), in Cases 41, 44, and 45; and exon XIII, a 43-base duplication at nucleotide position 1414 (43bp-Dupl 1414), in Case 42.

In the remaining 2 patients (Cases 43 and 47), neither PCR-RFLP nor PCR-PHFA detected any other mutation except the single mutation, Ins1159A (Case 43) and C478T (Case 47). Thus, we carried out RT-PCR of the total RNA isolated from the blood of two patients. The yield of RT-PCR products with the NAK5(+)-NAK4(−) primers encompassing the entire coding region of the *FAT/CD36* mRNA was quite low. Therefore, we re-amplified these PCR products into five overlapping, smaller fragments, using five pairs of the primers described in Subjects and Methods. In Case 43, sequencing of cloned cDNA fragment produced by CMP9–CMP12 primers revealed a skipping of exon XIII. To investigate what mutation in the *FAT/CD36* gene might induce the exon XIII skipping, we carried out PCR of the genomic DNA using IN12F–IN13R primers, and cloned the PCR products. Sequencing of three out of 9 cloned plasmids revealed a 10-base deletion of TATTACAG/AG at the boundary region of intron XII and exon XIII, in which G/A denotes the boundary. Six other clones showed the wild-type sequence. It is therefore concluded that the deletion of the 10 bases at the boundary causes skipping of exon XIII. As the original upstream primer used for amplifying exon XIII, CD36-13U contains a part of the deleted bases at the 3′ end, the genomic DNA of Skip exon XIII was not amplified and thus not detected by PCR-PHFA. Accordingly, we repeated PCR-PHFA using a new set of primers, CD36-13U3 and CD36-13L2, which corresponded to the upstream regions of deleted sequences for all DNA samples; we did not find the mutation except in Case 43.

Case 47 was analyzed by the same procedure as described for Case 43. Skipping of exon XII (Skip exon XII) was detected by sequencing of the RT-PCR product using CMP9–CMP12 primers. The PCR product of the genomic DNA using CD36-11U-CD36-13L primers was cloned. Sequencing of three out of five cloned plasmids showed a seven-base deletion of TTTAG/AT at the boundary region of intron XI and exon XII, in which G/A denotes the boundary. Two other clones showed the wild-type sequence. We confirmed by PCR-PHFA, using a new set of primers, CD36-12U int and CD36-12L2, that none of the 47 patients, except for Case 47, had the mutation.

Thus, a total of nine mutations, including six newly identified mutations, were detected in this study. All the new mutations were confirmed by direct sequencing, and new mutations that created restriction sites were analyzed by PCR-RFLP (data not shown). **Figure 3** shows the mutation map of the *FAT/CD36* gene, and Table 2 summarizes the genotypes of patients. All the patients demonstrated either a homozygous mutation of C478T or one of diverse combinations of two mutations. In the latter case, we did not attempt to identify the allelic locations.

To confirm that mutations of the gene are specifically associated with a defect in uptake of myocardial ^{123}I-BMIPP, we performed PCR-RFLP and PCR-PHFA, as described above, of the genomic DNA from all the control subjects who showed both normal uptake of myocardial ^{123}I-BMIPP and expression of FAT/CD36 on both platelet and monocyte membranes. We did not find any mutations in the genomic DNA. These results suggest the causal relationship between mutations of the *FAT/CD36* gene and the uptake defect of myocardial ^{123}I-BMIPP.

DISCUSSION

Cardiomyocytes utilize a family of LCFAs as a preferential energy substrate. However, so far, mechanisms underlying myocardial LCFA uptake have not been fully elucidated. Since ^{123}I-BMIPP, an LCFA analogue, is normally taken up by cardiomyocytes, and its methyl group prevents ready catabolism through β-oxidation (20), the myocardial uptake of LCFAs could be evaluated using ^{123}I-BMIPP. All the patients enrolled in this study showed a total defect in myocardial ^{123}I-BMIPP accumulation, and none of them exhibited a global myocardial perfusion defect, as reported previously by us (10, 12, 21) and others (22–25). Therefore, myocardial perfusion disorder is unlikely to explain the pathogenesis of the defect in myocardial ^{123}I-BMIPP uptake demonstrated in these patients.

Several molecules have been proposed to function as transporters for LCFAs. Among them, FAT/CD36 appears to be the most potential candidate in the heart, as proposed previously (10, 12, 13, 21). Indeed, the knock-out mouse of the *FAT/CD36* gene demonstrated a significant defect in myocardial ^{123}I-BMIPP uptake (26). However, the link between the defect in myocardial LCFA uptake and FAT/CD36 abnormality has not been fully established in humans. In this report, we analyzed protein expression and mutations of the *FAT/CD36* gene from 47 patients with the total defect in myocardial ^{123}I-BMIPP uptake. Analysis of the *FAT/CD36* gene was thoroughly performed by PCR-RFLP, PCR-PHFA, and DNA sequencing. The PCR-PHFA method can detect not only known mutations, but also unknown mutations in a PCR amplicon shorter than about 300 bp. Because all the exons of the gene are relatively short (at most, 188 bp in exon XIV), the sensitive PCR-PHFA method was extremely useful in finding mutations that are not detected by the PCR-RFLP method. Our analysis revealed a total of nine segregated mutations, three known and six new. All the patients carried a combination of these two mutations.

Although allelic locations of the two mutations of the *FAT/CD36* gene found in each patient have not yet been fully determined, except for the C478T homozygote (Cases 1–24), we argue that each of the two mutations should be located in the respective alleles. If the two mutations are located in the same allele, the other allele must be the wild-type expressing the wild-type FAT/CD36 protein that can be detected by flow cytometric analysis. Indeed, our preliminary results show that 26 heterozygous individuals carrying the wild-type and one mutated allele (C478T, Del539AC, or Ins1159A) express the FAT/CD36 protein on platelets and monocytes at approximately 50% of the level of the wild-type (unpublished data). In contrast, we did not detect the protein on platelets or monocytes from any of the patients carrying two mutations in this study. These results strongly support our argument that each of the two mutations locates in the respective alleles. However, we cannot formally eliminate the possibility that the protein product of one mutated allele might interfere with the expression of other wild-type alleles, and that some mutations cannot be detected by PCR-RFLP or PCR-PHFA analysis.

Except in rare cases, direct examination of the myocardial expression of FAT/CD36 is hampered because endomyocardial biopsy to obtain a myocardial specimen is not conducted routinely. Instead, we investigated the expression of FAT/CD36 on platelets and monocytes in this study. It is noteworthy that the FAT/CD36 protein was not

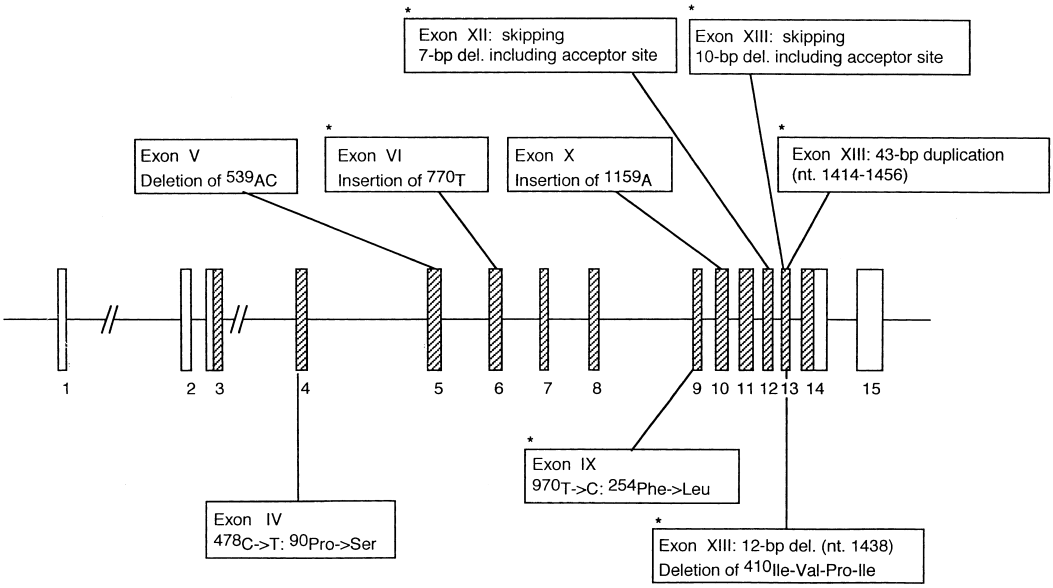

Fig. 3. Distribution of mutations found in human *FAT/CD36* gene. Mutations identified by polymerase chain reaction (PCR)-restriction fragment polymorphism, PCR-preferential homoduplex formation assay, and DNA sequencing were mapped. Mutations producing frameshift are shown in boxes above the bar; mutations producing amino acid substitution and deletion are shown in boxes below the bar. Exons are depicted as boxes numbered 1–15. Shaded boxes represent coding exons, and open boxes represent untranslated regions. Asterisk indicates mutations newly identified in this study.

detected on either platelet or monocyte membranes in any of these patients by the flow cytometrical analysis, using a commercially available antibody (OKM5) that interacts with epitopes within the domain between amino acid positions 155–183 (27). The single amino acid substitution mutation Pro90Ser (C478T) was reported to cause abnormal post-translational processing, which led to shortening the half-life of the protein (16). It is possible that the single amino acid substitution Phe254Leu (T970C) and four amino acid deletions 410Ile-Val-Pro-Ile (12bp-Del 1438), which were discovered in this study, may also affect the protein expression or cause conformation changes of the protein. Del539AC and Ins1159A were reported to produce the respective in-frame shift mutations that prematurely terminate the translation at amino acid positions 131 and 351, respectively (16–18). The mutations identified in this study, Ins770T, Skip exon XII, 43 bp-Dupl 1414, and Skip exon XIII, also cause in-frame shift and premature termination at amino acid positions 212, 391, 415, and 430, respectively, to produce truncated proteins. Although the influence of these mutations on expression and conformation is not known at present, we speculate that these mutated proteins could be unstable. Furthermore, the mRNA encoding these mutated FAT/CD36 proteins could be expressed in a reduced amount as compared with the wild-type mRNA. A similar result was observed in the expression of other genes, such as insulin receptor and β-globin (28–32). Regardless of our speculation, we did not detect the expression of these mutated FAT/CD36 proteins on either platelet or monocyte membranes. It is therefore probable that these mutated FAT/CD36 proteins are neither present in the membrane of cardiomyocytes nor functional, which may cause a total lack of myocardial LCFA uptake. Note that all 47 patients showed the apparently normal hepatic accumulation of ^{123}I-BMIPP, despite the lack of myocardial uptake of ^{123}I-BMIPP. *FAT/CD36* mRNA was detected in the heart, skeletal muscle, and adipocyte, but not in the liver (4, 33). Thus, hepatic uptake of LCFAs would not be performed by FAT/CD36 and would not be affected by the mutations of the gene. This phenomenon was also observed in the knock-out mouse of the gene (26).

From the screening of 6,970 patients, we found that 33 patients (0.47%: 33/6,970) had the total defect in myocardial accumulation of ^{123}I-BMIPP. Among them, 28 blood samples were available for this study. All 28 of these patients (0.40%: 28/6,970) carried two mutations in the *FAT/CD36* gene and showed no expression of CD36 protein on either platelet or monocyte membranes. It was reported that 0.3–0.5% of the Japanese population show no expression of CD36 protein on either platelet or monocyte membranes, which is referred to as type I CD36 deficiency (17). Thus, the prevalence of type I CD36 deficiency in our patients is within the range reported for the entire Japanese population.

Our main goal in this work was to investigate the link between *FAT/CD36* mutations and total defect in cardiac LCFA uptake. One could argue that the total myocardial LCFA uptake defect presented in this study may be secondary to heart disease, since it is well known that the uptake and utilization of LCFAs in the heart is significantly altered in a variety of cardiovascular diseases (34). However, the data presented in this work and other evidence strongly support our conclusion that mutations of the *FAT/CD36* gene cause a defect in LCFA uptake in the heart, as supported by the following: 1) Defect in myocardial LCFA uptake perfectly correlates to mutations of the *FAT/CD36* gene. 2) A causal link between defective myocardial LCFA uptake and FAT/CD36 deficiency has been recently confirmed (26) by the knock-out mouse of the gene. 3) No mutations of the gene were found in all the control subjects who demonstrated normal uptake of myocardial ^{123}I-BMIPP. 4) We previously reported that two healthy blood donors who had two mutations in the gene revealed total defect in ^{123}I-BMIPP accumulation (13). This evidence suggests that defect in myocardial ^{123}I-BMIPP accumulation is not due to underlying heart disorders but to *FAT/CD36* mutation. Unfortunately, we could not study the relationship between partial defect in ^{123}I-BMIPP uptake and mutations of the *FAT/CD36* gene; the results of such a study would have further supported our conclusions, but quantitative measurement of myocardial ^{123}I-BMIPP accumulation was not available at the medical centers where blood samples were collected.

Since LCFAs are important energy sources for the heart, it is conceivable that a disturbance in LCFA uptake could cause pathophysiological problems. Van der Vusse, van Bilsen, and Glatz (35) proposed that alterations in the tissue content of fatty acid transport proteins, including FAT/CD36, may give rise to myocardial diseases such as cardiac hypertrophy and diabetic heart. Furthermore, given the central importance of LCFAs in particular conditions such as fasting, infection, stress, and vigorous exercise, it is evident that defects in the transport of LCFAs will result in the disruption of normal adaptation. Treem (36) speculated that cumulative episodes of metabolic maladaptation may provoke some cardiac disorders. We previously proposed that FAT/CD36 deficiency could be one etiology of hypertrophic cardiomyopothy (HCM), because we observed cardiac hypertrophy by chemical intervention of FAT/CD36 in rats (37) and found mutations of the *FAT/CD36* gene in approximately 40% of HCM patients (11).

In the present study, *FAT/CD36* mutations were found in a variety of heart diseases including HCM, dilated cardiomyopathy (DCM), coronary artery disease (CAD), hypertension, and valvular heart disease. We think that such nonspecific disease relevance is the result of the following phenomenon; one type of heart disease could transform to another type, and/or one clinically obvious manifestation of the disease could mask a different, subclinical one. For example, it is known that some types of HCM may transform to DCM (dilated HCM) (38). Maron and Mitchell (39) reported that the standard screening procedure for identifying HCM appeared to be limited to and, indeed, failed to identify 47 out of 48 HCM patients. Additionally, HCM shows a broad spectrum of clinical manifestations, with variability in clinical symptoms and different degrees of hypertrophy expression (40), even in a single

family cohort with the same genetic defect (41). Therefore, it is possible that other unknown factors in addition to FAT/CD36 deficiency play a role in these heterogeneous clinical manifestations. Taken together, we propose that an impaired myocardial LCFA uptake that links strongly to mutations of the *FAT/CD36* gene could contribute to human susceptibility to some heart diseases, and pathophysiological consequences might be dependent on other factors as well.

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