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ELISA System for Human Endothelial Lipase

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BACKGROUND: Endothelial lipase (EL) regulates the metabolism of HDL cholesterol (HDL-C). However, the role of EL in regulating plasma HDL-C concentrations and EL's potential involvement in atherosclerosis in humans has not been fully investigated due to the lack of reliable assays for EL mass. We developed an ELISA system for serum EL mass.

METHODS: Human recombinant EL proteins, purified from cultured media of human EL–transfected Chinese hamster ovary cells, were used as antigen and calibrator. Two specific monoclonal antibodies were generated in mice against recombinant EL protein for a sandwich ELISA. We measured EL mass in human serum using EL recombinant protein as a calibration standard.

RESULTS: The EL antibodies did not cross-react with lipoprotein lipase and hepatic triglyceride lipase. The detection limit of the ELISA was 20 pg/mL, which is approximately 10 times lower than that of previous ELISA systems. Recovery of spiked EL in serum was 90%–105%. Assay linearity was intact with a >4-fold dilution of serum. Intra- and interassay CVs were <5%. The serum EL mass in 645 human subjects was [mean (SE)] 344.4 (7.7) pg/mL (range 55.2–1387.7 pg/mL). Interestingly, serum EL mass was increased in patients with diagnosed cardiovascular disease and inversely correlated with serum HDL-C concentrations. There was no difference in EL mass between pre- and postheparin plasma samples.

CONCLUSIONS: This ELISA should be useful for clarifying the impact of EL on HDL metabolism and EL's potential role in atherosclerosis.

A large number of studies have established an inverse relationship between HDL cholesterol (HDL-C)[6] and risk for cardiovascular disease (CVD) in humans *(1–5)*, and low HDL-C is considered one of the most important negative risk factors for atherosclerotic CVD *(6)*. Even after LDL cholesterol (LDL-C) is intensively controlled to low concentrations with statin therapy, low HDL-C remains a clinically significant cardiovascular risk factor *(7, 8)*. Furthermore, low HDL-C is frequently accompanied by hypertriglyceridemia, and these lipid disorders synergistically contribute to an increased risk for CVD *(9)*. Increased plasma triglyceride (TG) concentrations and low plasma concentrations of HDL-C have emerged as diagnostic criteria for the metabolic syndrome. Despite the therapeutic potential of HDL in combating CVD, there is a limited therapeutic strategy available for selectively raising HDL-C concentrations. Moreover, because of the multiplicity of HDL metabolism in humans, it is difficult to make an etiological diagnosis for the cause of high or low HDL-C concentrations in clinical settings.

Endothelial lipase (EL), a member of the triacylglyceride lipase family, is synthesized by vascular endothelial cells *(10–13)*. Experiments in engineered mice with a disrupted native EL locus, as well as in mice overexpressing human EL (hEL), have revealed an inverse relationship between plasma HDL-C concentration and EL expression *(11, 14)*. Previous studies have shown that plasma EL mass measured by ELISA is inversely correlated with HDL-C concentrations in humans *(15, 16)*. Association-based human genetic studies have provided evidence that variations in the EL genomic *LIPG* locus such as T111I, G26S, and N396S are linked to differences in circulating HDL-C concentrations or CVD *(17–21)*, although recent studies with a large number of subjects have established associations between *LIPG* single-nucleotide polymorphism

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[6] Nonstandard abbreviations: HDL-C, HDL cholesterol; CVD, cardiovascular disease; LDL-C, LDL cholesterol; TG, triglyceride; EL, endothelial lipase; hEL, human EL; CHO, Chinese hamster ovary; FBS, fetal bovine serum; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase; HRP, horseradish peroxidase.

and HDL-C concentrations, but none with CVD *(21–23)*. Thus, genetic variation in EL can modulate plasma HDL-C concentrations, although the relationship with CVD remains controversial. Although EL is still expected to be an attractive pharmacological target for raising HDL-C concentrations, the clinical significance of plasma EL concentration on plasma HDL-C concentrations and atherosclerosis in humans has not been fully elucidated due to the lack of a standard and reliable assay system. To obtain a better understanding of the association of EL and plasma concentrations of HDL-C in humans, our goal was to establish a new assay system for EL mass measurement using newly generated antibodies.

Materials and Methods

MONOCLONAL ANTIBODY PREPARATION

We transfected Chinese hamster ovary (CHO) cells (American Type Culture Collection) with the human EL-c-myc/pHBAP-3-neo plasmid *(10)* and selected stable transfectants with 500 μg/mL G418 (Invitrogen). We screened EL expression in the stable transfectants using an ELISA system with monoclonal antibodies against EL (clones 11–9B and 2–12E) *(16)* and chose 1 of the high expression clones, referred to as hEL-myc/CHO 53A5, for subsequent experiments. The cells were cultured for 24 h in TIL medium (Immuno-Biological Laboratories) containing 10% fetal bovine serum (FBS) (PAA Laboratories), after which the medium was changed for serum-free TIL medium. After 3 days, the supernatants were collected and measured. We purified EL protein from the concentrated conditioned medium of hEL-myc/CHO 53A5 using an immunoaffinity column containing anti-EL carboxy-terminus antibody (clone 2–12E) *(14, 16)*, emulsified with Freund complete adjuvant, and then immunized into BALB/c mice (Charles River). After a boost with immunogen, we carried out fusion of spleen cells with X63-Ag8.653 myeloma cells (Immuno-Biological Laboratories) with PEG1500 (Roche Applied Science) followed by screening for desired hybridoma reactive only to EL by immunoblotting. We identified 2 monoclonal antibodies that reacted with amino terminus (26A1) and carboxy terminus (48A1) of EL among 15 clones.

PREPARATION OF EL CALIBRATORS AND OTHER LIPASES

To obtain standard full-length EL protein, the concentrated culture medium of hEL-myc/CHO 53A5 was incubated with 70% NH_4SO_4, and then put through immuno-affinity columns containing monoclonal antibodies against amino (clone 5–3B) and carboxy (clone 2–12E) terminus of EL *(14, 16)*. We estimated the purity of the recombinant hEL protein by densitometry using a Multi Gauge (Fujifilm) and determined the concentration of the protein by comparison with BSA (PAA Laboratories) as an indicator after electrophoresis. In addition, we used the culture supernatant of hEL-myc/CHO 53A5 cells as a working standard for the ELISA system.

To test the cross-reactivity of the ELISA with other lipase members, including lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL), we purchased recombinant human LPL protein from BioVendor and generated recombinant human HTGL as follows. Full-length human HTGL cDNA was identified in human liver cDNA (Clontech) by PCR using primers 5′-ATCGGAGAAATGGACAC AAGTCCC-3′ and 5′-CGCTCGAGTCTGATCTTT CGCTTTGATGTTTT-3′. A FLAG-epitope tag was added to the 3′ end of cDNA before subcloning. To generate the plasmid encoding hHTGL-Full, the cDNA was inserted into pcDNA3.1(+) expression vector (Invitrogen). Human HTGL exhibits cell surface binding through the 5 carboxyl-terminal residues (KRKIR) *(24)*. Therefore, to promote secretion to a culture supernatant, we prepared a truncated human HTGL mutant (hHTGL-471) by deleting the 5 carboxyl-terminal residues. We used antisense PCR primer (5′-CGCTCGAGTGATGTTTTAGACTTTATTTCACA-3′) to generate the plasmid encoding hHTGL-471. The PCR product was inserted into pcDNA3.1(+) expression vector after a FLAG-epitope tag was added to the 3′ end.

CHO cells were transfected with the hHTGL-471 plasmid and selected with 500 μg/mL G418 to establish stable transfectants. We screened hHTGL-471 expression in the transfectants by using anti–c-FLAG rabbit IgG (Immuno-Biological Laboratories) and generated 1 of the high-expression clones, referred to as human HTGL-471/CHO 3B1. We purified HTGL protein from the concentrated conditioned medium of human HTGL-471/CHO 3B1 using an anti-FLAG M2 affinity gel (Sigma-Aldrich). We estimated the purity of the recombinant human HTGL protein by densitometry using a Multi Gauge and determined the concentration of the protein by comparison with BSA as an indicator after electrophoresis.

IMMUNOBLOTTING AND IMMUNOPRECIPITATION-IMMUNOBLOTTING

We analyzed the supernatant from hEL-myc/CHO 53A5 cells by SDS-PAGE, followed by staining with Coomassie Brilliant Blue. For immunoblotting, 5 μL conditioned medium of hEL-myc/CHO 53A5 was used for 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was incubated with 2 μg of the 26A1 or 48A1 antibody followed by incubation with secondary antibody conjugated with

horseradish peroxidase (HRP) (1:4000, Immuno-Biological Laboratories). We assessed the reactivity of antibodies by immunoprecipitation-immunoblotting. The supernatant from the hEL-myc/CHO 53A5 cells was incubated with 2 μg 26A1 or 48A1 or mouse IgG (as negative control) and then with added Protein-G Sepharose (GE Healthcare Japan). After further incubation, the supernatant was centrifuged, and the resulting pellet was washed 3 times with 500 μL TNE buffer (10 mmol/L Tris-HCl, pH 7.8, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Nonidet P-40). The pellet was subsequently lysed, and we performed immunoblot analysis using biotinylated anti-EL amino-terminus monoclonal antibody (clone 11–9B) *(14, 16)* followed by HRP-conjugated streptavidin system. We visualized the EL signal by use of an ECL reagent (Amersham Biosciences).

IMMUNOFLUORESCENCE

The hEL-myc/CHO 53A5 and control mock-CHO cells cultured on coverslips were washed with prewarmed PBS (137 mmol/L NaCl, 10 mmol/L phosphate, 2.7 mmol/L KCl, pH 7.4), fixed with 4% paraformaldehyde in PBS for 15 min, and permeabilized with 0.1% Triton X-100 for 60 min. After saturation of unspecific sites with 100 g/L BSA/PBS, the cells were first incubated with the EL 26A1 or 48A1 antibody (1:200) or negative control (mouse mAb IgG Isotype Control, Cell Signaling Technology), and then with fluorescence-labeled secondary antibody (Alexa Fluor594 goat anti-mouse IgG, Invitrogen, 1:200). DAPI was used for nuclear staining. Images were captured by use of the Biozero BZ-8000 microscope (Keyence).

EL SANDWICH ELISA PROTOCOL

Microtiter plates (96 wells) were coated by adding 100 μL of 100 mmol/L carbonate buffer (pH 9.5) to each well that also contained 1.0 μg purified 48A1 mouse monoclonal IgG, followed by incubation overnight at 4 °C. The plates were then washed with PBS-T and blocked with 200 μL of 1% (wt/vol) BSA in PBS containing 0.05% NaN_3/well overnight at 4 °C. After two washings with PBS-T, test samples and recombinant EL, as a standard, that had been serially diluted with 1% BSA in PBS-T per 100 μL were added to the wells of the coated microtiter plate in duplicate and incubated at 4 °C overnight. After 4 washes with PBS-T, 100 μL HRP-conjugated 26A1 mouse IgG Fab′ was added to each well and the samples were incubated for 30 min at 4 °C. The wells were washed 5 times with PBS-T, and 100 μL tetramethyl benzidine solution (Kem-En-Tec) was added to each well as a substrate, followed by incubation in the dark for 30 min at room temperature. The reaction was terminated by adding 100 μL of 0.5 mol/L H_2SO_4. We measured absorbance of the solution at 450 nm by means of an ELISA reader (E-Max; Molecular Devices).

To assess the intra- and interassay precision for the ELISA, we established 3 QC samples covering the high, middle, and low range of the calibration curves. We determined intraassay precision by 24 repeated measurements of each QC sample in a plate, and interassay precision by assessing each QC sample across 6 different plates with quintuple wells. Additionally, for assessing the recovery rate in blood samples, different concentrations of recombinant EL added to samples were measured, and the recovery rate was validated as the difference between the measured concentration and the theoretical concentration. The analytical limit of quantification for this kit was determined on the basis of the guidelines provided by CLSI evaluation protocols.

The ELISA assay system was finally designed as a kit (Immuno-Biological Laboratories, code 27182).

PREPARATION OF BLOOD SAMPLES

The investigation conformed to the principles outlined in the Declaration of Helsinki, and the clinical study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine.

We conducted the first set of experiments to determine the effect of heparin administration on plasma EL mass. We collected pre- and postheparin plasma samples from overweight and obese participants in a nutritional research study conducted at the University of California, Davis Clinical and Translational Science Center's Clinical Research Center as described previously *(25)*. The activity of HTGL in the plasma samples was measured as described previously *(26)*.

The second set of experiments was conducted to evaluate EL mass in patients with diagnosed existing CVD. Whole blood was obtained from 645 Japanese patients consecutively admitted to Kobe University Hospital, Kobe, Japan, from April 2008 to March 2011, with written informed consent. Because it has been reported that preheparin EL mass is significantly correlated with postheparin EL mass, blood was collected without administration of heparin in the fasting state, while both pre- and postheparin plasma was obtained from some patients. The sera and plasma were immediately separated and kept frozen at −80 °C until assay. In some preliminary experiments, we obtained plasma and sera of healthy subjects (Veritas). Plasma concentrations of HDL-C, LDL-C, and TG were measured enzymatically.

STATISTICAL ANALYSIS

We conducted statistical analysis with Stat View version 5.0 (SAS Institute). We used Spearman correlation coefficient analysis to assess associations between measured parameters. Results are expressed as mean (SE), and $P < 0.05$ was considered significant.

Results

IDENTIFICATION OF RECOMBINANT hEL AND CHARACTERIZATION OF ANTIBODIES AGAINST EL

We generated a pair of antibodies recognizing the amino terminus (26A1) and carboxy terminus (48A1) that exhibited a highly specific reactivity with the EL protein derived from hEL-myc/CHO 53A5 cells. Immunoblotting revealed a strong signal for 68-kDa mature EL protein (Fig. 1A). To confirm whether the antibodies had the ability to react against native EL in aqueous conditions, we performed an immunoprecipitation-immunoblotting analysis. Both 26A1 and 48A1 antibodies were able to immunoprecipitate EL originated from the conditioned medium of hEL-myc/CHO 53A5 (Fig. 1B). Immunofluorescence revealed that EL expression was abundantly detected in the cytosol of hEL-myc/CHO 53A5 (Fig. 1C). No signal was detected in negative controls, i.e., mock-transfected cells with primary antibodies or hEL-myc/CHO 53A5 cells without primary antibodies. Thus, we were able to identify EL expression in culture medium of hEL-myc/CHO 53A5 cells and confirm the specific reactivity of the 26A1 and 48A1 antibodies with EL protein.

SPECIFICITY, RECOVERY, AND IMPRECISION OF THE ELISA

Because the 26A1 and 48A1 antibodies had a highly specific reactivity with the EL protein, we chose them for establishment of the new sandwich ELISA system. The standard dose–response curve for the EL ELISA system exhibited a linear shape when plotted on a log/log scale over a range from 31 to 2000 pg/mL, and the linearity was excellent ($R^2 = 0.99$) (Fig. 2A). The new ELISA system worked for both serum and EDTA plasma samples equally. Although EL has a 44% and 41% amino acid sequence homology with LPL and HTGL *(10)*, the cross-reactivity of this ELISA against human LPL and HTGL was <0.1% (Fig. 2B). Imprecision was determined with 3 supplemented QC controls (high, middle, and low). The intraassay imprecision exhibited CVs of 1.9% in the high, 2.7% in the middle, and 3.0% in the low controls (Table 1). Additionally, the interassay results for the CVs were 3.7% in the high, 2.0% in the middle, and 2.8% in the low controls. Thus, we considered the ELISA system to be reliable from the standpoint of imprecision. The recoveries were >85.6% for human EDTA plasma samples at 4× dilution, >74.7% for human serum, and nearly 100% for TIL media supplemented with 10% FBS (Table 2). We calculated the assay limit of quantification as 5.7 pg/mL using CLSI protocols.

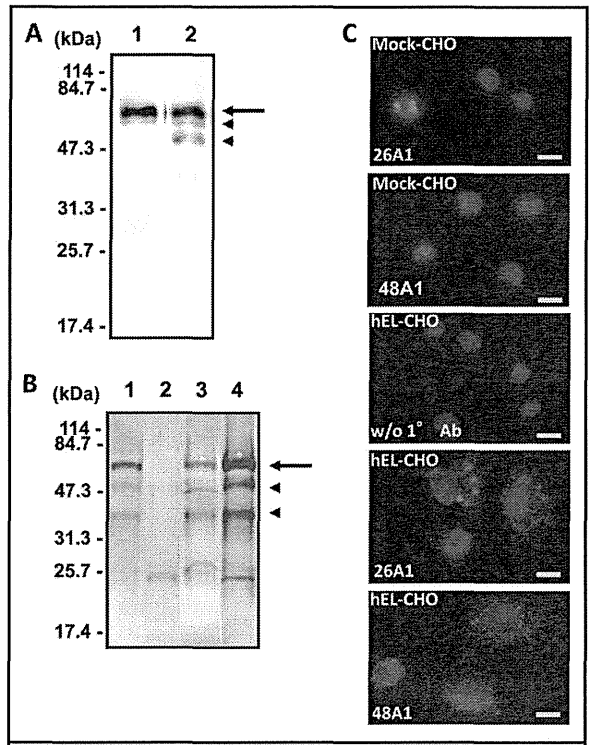

Fig. 1. Immunoblotting of EL protein with anti-EL monoclonal antibodies.

(A), A strong signal of full-length EL (68 kDa) was detected with both 48A1 (*lane 1*) and 26A1 (*lane 2*) anti-EL antibodies, whereas smaller-sized minor bands were also detected with 26A1. (B), Twenty-fold concentrated culture medium of hEL-myc/CHO 53A5 (*lane 1*), EL protein immunoprecipitated from culture supernatant of hEL-myc/CHO 53A5 with mouse IgG (negative control, *lane 2*), with 48A1 (*lane 3*), or with 26A1 (*lane 4*) were detected. (C), Expression of EL (red) in hEL-myc/CHO 53A5 (hEL-CHO) cells was evaluated by immunofluorescence with the 48A1 or 26A1 antibodies. DAPI (blue) is for nuclear stain. Mock-transfected (Mock-CHO) cells treated with the antibodies and hEL-myc/CHO 53A5 in which the primary antibody was replaced by nonspecific IgG (w/o 1° Ab) are shown as negative controls. Scale bar indicates 10 μm.

Because EL has several heparin-binding domains, we investigated the effect of heparin administration on plasma EL mass. Unexpectedly, there was no significant difference in EL mass between pre- and postheparin samples (Fig. 3, A and B), in contrast to the marked heparin-releasable HTGL activity in the same samples

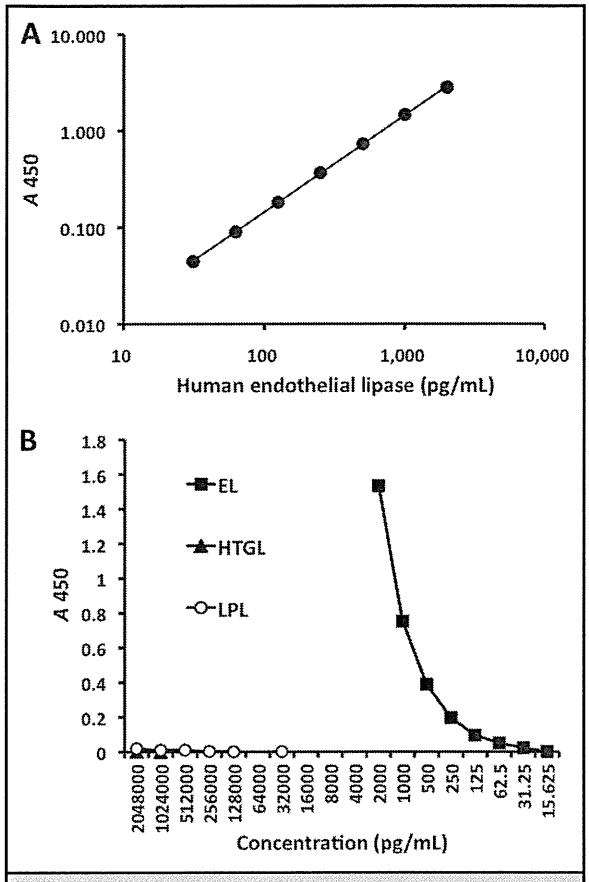

Fig. 2. Characteristic of the hEL ELISA system.
(A), Calibration curve for calculation of EL concentrations. (B), Specificity of the EL ELISA system. *A*, absorbance.

(Fig. 3A). Although there are heparin-binding sites in the carboxy-terminal end of EL, we confirmed that heparin does not block the binding sites of the amino-terminus (26A1) or carboxy-terminus (48A1) antibody.

Table 1. Intra- and interassay imprecision.

QC	Measured value, pg/mL	SD, pg/mL	CV, %	n
Intraassay imprecision				
High	1126.9	20.85	1.9	24
Middle	285.9	7.71	2.7	24
Low	90.8	2.69	3.0	24
Interassay imprecision				
High	1100.7	40.81	3.7	6
Middle	282.7	5.58	2.0	6
Low	91.4	2.54	2.8	6

Table 2. Recovery validation.

Sample	Theoretical value, pg/mL	Measured value, pg/mL	% Recovery
Human plasma	1147.9	982.7	85.6
	647.9	557.9	86.1
	397.9	357.4	89.8
Human serum	1098.6	820.6	74.7
	598.6	449.3	75.1
	348.6	274.3	78.7
TIL media supplemented with 10% FBS	1000.0	941.1	94.1
	500.0	483.0	96.6
	250.0	245.7	98.3

INVERSE CORRELATION OF SERUM EL MASS AND HDL-C LEVELS IN CVD

The serum EL mass in 645 consecutive human subjects was 344.4 (7.7) pg/mL, and ranged from 55.2 to 1387.7 pg/mL. No patients had an EL concentration below the limit of quantification. The distribution of EL mass was skewed to the left (Fig. 4A). The EL mass was not correlated with serum HDL-C (Fig. 4B and Supplemental Fig. 1B, which accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue12) or LDL-C concentrations (data not shown) in this population. Because our previous study showed that EL mass was associated with plasma HDL-C concentrations in patients with CVD *(16)*, we next investigated the serum EL mass in patients having atherosclerotic CVD. The EL mass concentration in these 228 patients with CVD was 395.8 (15.1) (range 57.7–1387.7) pg/mL, which was significantly higher than that in the 645 consecutive patients ($P < 0.001$), and the EL distribution was again skewed to the left (Fig. 5A). Concomitantly, the patients with CVD had significantly lower concentrations of serum HDL-C than those without CVD {46.2.0 (1.0) vs 52.0 (0.6) mg/dL [1.20 (0.03) vs 1.35 (0.02) mmol/L]}, $P < 0.001$). When serum EL concentration was compared to the lipid profile in the CVD patients, it was inversely correlated with plasma HDL-C concentrations ($R = -0.250$, $P < 0.001$) (Fig. 5B and online Supplemental Fig. 2B), but not with LDL-C ($R = -0.055$, NS), or triglyceride ($R = 0.078$, NS) concentrations.

Discussion

We generated specific EL monoclonal antibodies against recombinant EL that reacted with the amino (26A1) and carboxy (48A1) terminus. Both antibodies had strong reactivity with native EL protein and en-

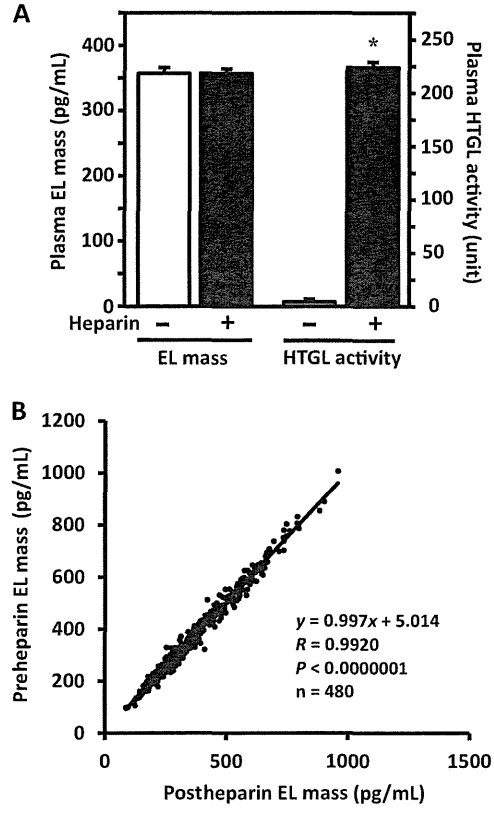

Fig. 3. Effect of heparin administration on plasma EL mass.

(A), Administration of heparin did not affect plasma EL mass but markedly increased plasma HTGL activity in the same pre- and postheparin samples (n = 480). *$P < 0.001$ vs. without heparin. (B), Scatter plot showing association between pre- and postheparin EL mass.

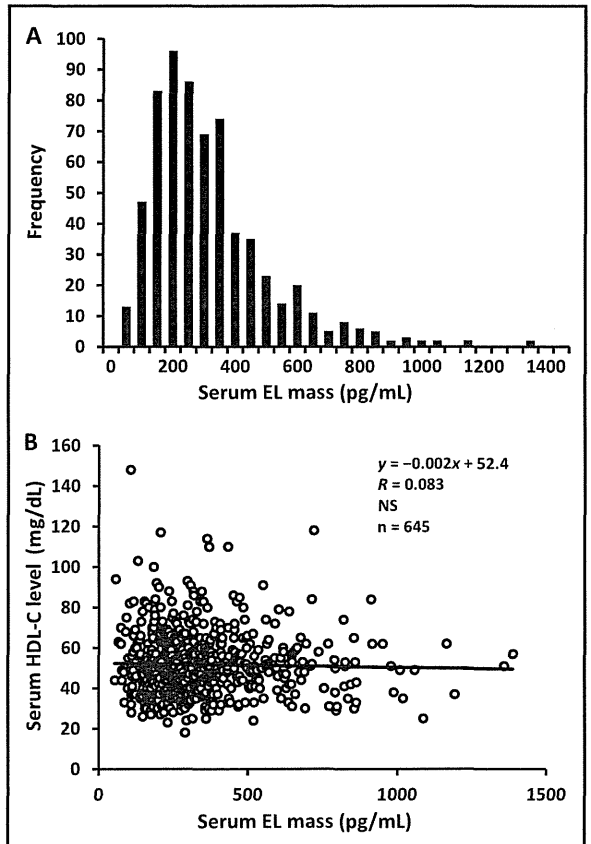

Fig. 4. Serum EL mass and HDL-C concentrations in all patients.

(A), Histogram showing the skewed distribution of EL mass (n = 645). (B), Lack of correlation of serum EL mass with serum HDL-C concentrations. NS, not statistically significant.

abled the detection of full-length EL by the sandwich ELISA. The limit of quantification of 5.7 pg/mL for hEL is much lower than that of previous ELISA systems, probably because the new antibodies are more specific for full-length EL protein than the old ones, which were generated against peptide fractions of EL *(16)*. The serum EL mass in preheparin plasma was approximately 70–1000 pg/mL in the present study. Previous studies by our group and another group reported that the concentrations of EL mass in preheparin plasma were approximately 10–1000 μg/L, concentrations approximately 1000-fold higher than measured with the new assay *(15, 16)*. These differences have resulted in confusion as to whether the plasma concentration of EL is really higher than the concentrations of LPL and HTGL without heparin infusion. The concentrations of other lipases, including LPL and HTGL, in preheparin plasma have been reported to be <30~100 μg/L *(27, 28)*. When compared with the low plasma concentrations of other lipase members, we speculate that the range of EL concentrations determined by the new ELISA is more reasonable than those by the previous one. We consider the specificity of the antibodies used for this assay to be suitable for determining specific EL mass concentrations in human plasma, which showed different reactivity from the antibodies generated against peptide fractions in EL *(16)*. The new ELISA may also be useful for identifying cases of genetic deficiency of EL in humans.

In the present study, a modest but significant inverse correlation between serum EL and HDL-C concentrations in patients with CVD was noted, whereas the relationship was not observed in all patients. The EL concentration was not correlated with serum LDL-C or TG concentrations. These findings confirmed the previous notion that EL is a determinant of

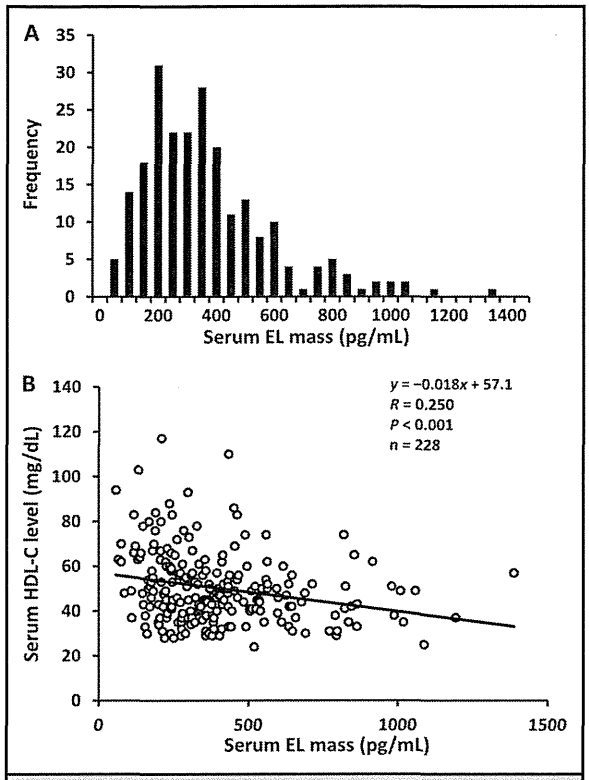

Fig. 5. Serum EL mass and HDL-C concentration in patients with cardiovascular disease.

(A), Histogram showing the skewed distribution of EL mass (n = 228). (B), Inverse correlation of serum EL mass with serum HDL-C concentrations.

plasma HDL-C concentrations, particularly in patients with CVD. It has been reported that EL expression is highly regulated by a variety of factors including inflammatory cytokines, biomechanical forces, lipopolysaccharide, angiotensin II, and oxidized LDL *(29–31)*. In fact, plasma EL mass was increased in experimental endotoxemia in humans and correlated with inflammatory markers such as C-reactive protein, interleukin-6, and secretory phospholipase A2-IIa *(32, 33)*. In contrast, statins reduce EL expression and plasma EL mass *(16, 34)*, which is accompanied by increased plasma HDL-C concentrations in humans *(16)*. These findings suggest that a change in EL expression associated with inflammatory states may at least in part account for the variation of HDL-C concentrations in CVD patients.

Cell culture experiments revealed that cytokine-stimulated EL expression was concomitant with an increase in EL activities *(35)*. However, EL activity is partly regulated through posttranscriptional mechanisms. It has been reported that EL forms a head-to-tail dimer in the human plasma, and the homodimer formation is essential for the maintenance of EL activity *(36)*, as is the case with LPL and HTGL. In addition, EL is proteolytically processed into 40- and 28-kDa fragments and inactivated by proprotein convertases *(37)*. In this regard, our sandwich ELISA system can recognize the dimer of full-length EL with the intact enzymatic activity. On the other hand, angiopoietin-like 3 is known to act as an endogenous EL inhibitor *(38)*. In addition, human heat-inactivated serum inhibited EL phospholipase activity *(39)*, indicating the existence of some endogenous EL inhibitor in human serum. Furthermore, a naturally occurring variant in the EL gene (*LIPG*), glycine-26 to serine, which is associated with increased HDL, exhibits impaired synthesis *(20)*.

It has been reported that EL has several heparin-binding domains and binds to heparan sulfate proteoglycans on the vascular endothelium *(12, 15, 40)*. Therefore, EL should be released into plasma by heparin treatment *(15)*. In the present study, however, there was no difference in EL mass between pre- and postheparin plasma samples, whereas control HTGL activity was markedly increased by the heparin administration. When we evaluated EL mass by our previous ELISA system *(16)*, we confirmed that the administration of heparin did not affect plasma the EL protein. Thus, the interaction of EL with heparan sulfate proteoglycans needs to be determined by further studies.

In conclusion, we developed a sandwich ELISA using newly generated monoclonal antibodies specific to human plasma EL. The limit of quantification, range of linearity, and imprecision for EL quantification are suitable for both experimental and clinical use. From the preliminary study of a healthy reference range, we found that reference-range EL concentrations were between approximately 50 and 1400 pg/mL in human plasma. This range is much lower and, we suggest, more reasonable than the range measured with previous EL assays. It is possible that patients with low (EL deficiency) and high serum EL concentrations associated with high or low HDL-C concentrations will be identified by use of this new assay for EL.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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References

1. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S, Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels: the Framingham Study. JAMA 1986;256:2835–8.
2. Assmann G, Schulte H, von Eckardstein A, Huang Y. High-density lipoprotein cholesterol as a predictor of coronary heart disease risk: the PROCAM experience and pathophysiological implications for reverse cholesterol transport. Atherosclerosis 1996;124 Suppl:S11–20.
3. Curb JD, Abbott RD, Rodriguez BL, Masaki K, Chen R, Sharp DS, et al. A prospective study of HDL-C and cholesteryl ester transfer protein gene mutations and the risk of coronary heart disease in the elderly. J Lipid Res 2004;45:948–53.
4. Sharrett AR, Ballantyne CM, Coady SA, Heiss G, Sorlie PD, Catellier D, et al. Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density sub-fractions: the Atherosclerosis Risk in Communities (ARIC) study. Circulation 2001;104:1108–13.
5. Turner RC, Millns H, Neil HA, Stratton IM, Manley SE, Matthews DR, et al. Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS:23). BMJ 1998;316:823–8.
6. Grundy SM, Cleeman JI, Merz CN, Brewer HB Jr., Clark LT, Hunninghake DB, et al. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. Circulation 2004;110:227–39.
7. Barter P, Gotto AM, LaRosa JC, Maroni J, Szarek M, Grundy SM, et al. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. N Engl J Med 2007;357:1301–10.
8. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005;366:1267–78.
9. Davis CE, Gordon D, LaRosa J, Wood PD, Halperin M. Correlations of plasma high-density lipoprotein cholesterol levels with other plasma lipid and lipoprotein concentrations. Circulation 1980;62:IV24–30.
10. Hirata K, Dichek HL, Cioffi JA, Choi SY, Leeper NJ, Quintana L, et al. Cloning of a unique lipase from endothelial cells extends the lipase gene family. J Biol Chem 1999;274:14170–5.
11. Jaye M, Lynch KJ, Krawiec J, Marchadier D, Maugeais C, Doan K, et al. A novel endothelial-derived lipase that modulates HDL metabolism. Nat Genet 1999;21:424–8.
12. Choi SY, Hirata K, Ishida T, Quertermous T, Cooper AD. Endothelial lipase: a new lipase on the block. J Lipid Res 2002;43:1763–9.
13. Yasuda T, Ishida T, Rader DJ. Update on the role of endothelial lipase in HDL metabolism, reverse cholesterol transport, and atherosclerosis. Circ J 2010;74:2263–70.
14. Ishida T, Choi S, Kundu RK, Hirata K, Rubin EM, Cooper AD, et al. Endothelial lipase is a major determinant of HDL level. J Clin Invest 2003;111:347–55.
15. Badellino KO, Wolfe ML, Reilly MP, Rader DJ. Endothelial lipase concentrations are increased in metabolic syndrome and associated with coronary atherosclerosis. PLoS Med 2006;3:e22.
16. Kojima Y, Ishida T, Sun L, Yasuda T, Toh T, Rikitake Y, et al. Pitavastatin decreases expression of endothelial lipase both in vitro and in vivo. Cardiovasc Res 2010;87:385–93.
17. Edmondson AC, Brown RJ, Kathiresan S, Cupples LA, Demissie S, Manning AK, et al. Loss-of-function variants in endothelial lipase are a cause of elevated HDL cholesterol in humans. J Clin Invest 2009;119:1042–50.
18. Shimizu M, Kanazawa K, Hirata K, Ishida T, Hiraoka E, Matsuda Y, et al. Endothelial lipase gene polymorphism is associated with acute myocardial infarction, independently of high-density lipoprotein-cholesterol levels. Circ J 2007;71:842–6.
19. Tang NP, Wang LS, Yang L, Zhou B, Gu HJ, Sun QM, et al. Protective effect of an endothelial lipase gene variant on coronary artery disease in a Chinese population. J Lipid Res 2008;49:369–75.
20. Brown RJ, Edmondson AC, Griffon N, Hill TB, Fuki IV, Badellino KO, et al. A naturally occurring variant of endothelial lipase associated with elevated HDL exhibits impaired synthesis. J Lipid Res 2009;50:1910–6.
21. Vergeer M, Cohn DM, Boekholdt SM, Sandhu MS, Prins HM, Ricketts SL, et al. Lack of association between common genetic variation in endothelial lipase (LIPG) and the risk for CAD and DVT. Atherosclerosis 2010;211:558–64.
22. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, et al. Plasma HDL cholesterol and risk of myocardial infarction: a Mendelian randomisation study. Lancet 2012;380:572–80.
23. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature 2010;466:707–13.
24. Brown RJ, Schultz JR, Ko KW, Hill JS, Ramsamy TA, White AL, et al. The amino acid sequences of the carboxyl termini of human and mouse hepatic lipase influence cell surface association. J Lipid Res 2003;44:1306–14.
25. Stanhope KL, Schwarz JM, Keim NL, Griffen SC, Bremer AA, Graham JL, et al. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. J Clin Invest 2009;119:1322–34.
26. Imamura S, Kobayashi J, Sakasegawa S, Nohara A, Nakajima K, Kawashiri M, et al. A novel method for measuring human hepatic lipase activity in postheparin plasma. J Lipid Res 2007;48:453–7.
27. Kotani K, Adachi S, Tsuzaki K, Sakane N. Relationship between serum preheparin lipoprotein lipase mass, plasma glucose and metabolic syndrome in older subjects. Age Ageing 2009;38:123–6.
28. Tornvall P, Olivecrona G, Karpe F, Hamsten A, Olivecrona T. Lipoprotein lipase mass and activity in plasma and their increase after heparin are separate parameters with different relations to plasma lipoproteins. Arterioscler Thromb Vasc Biol 1995;15:1086–93.
29. Hirata K, Ishida T, Matsushita H, Tsao PS, Quertermous T. Regulated expression of endothelial cell-derived lipase. Biochem Biophys Res Commun 2000;272:90–3.
30. Kojma Y, Hirata K, Ishida T, Shimokawa Y, Inoue N, Kawashima S, et al. Endothelial lipase modulates monocyte adhesion to the vessel wall: a potential role in inflammation. J Biol Chem 2004;279:54032–8.
31. Hara T, Ishida T, Kojima Y, Tanaka H, Yasuda T, Shinohara M, et al. Targeted deletion of endothelial lipase increases HDL particles with anti-inflammatory properties both in vitro and in vivo. J Lipid Res 2011;52:57–67.
32. Paradis ME, Badellino KO, Rader DJ, Deshaies Y, Couture P, Archer WR, et al. Endothelial lipase is associated with inflammation in humans. J Lipid Res 2006;47:2808–13.
33. Badellino KO, Wolfe ML, Reilly MP, Rader DJ. Endothelial lipase is increased in vivo by inflammation

in humans. Circulation 2008;117:678–85.
34. Qiu G, Hill JS. Atorvastatin decreases lipoprotein lipase and endothelial lipase expression in human THP-1 macrophages. J Lipid Res 2007;48:2112–22.
35. Jin W, Sun GS, Marchadier D, Octtaviani E, Glick JM, Rader DJ. Endothelial cells secrete triglyceride lipase and phospholipase activities in response to cytokines as a result of endothelial lipase. Circ Res 2003;92:644–50.
36. Griffon N, Jin W, Petty TJ, Millar J, Badellino KO, Saven JG, et al. Identification of the active form of endothelial lipase, a homodimer in a head-to-tail conformation. J Biol Chem 2009;284:23322–30.
37. Jin W, Fuki IV, Seidah NG, Benjannet S, Glick JM, Rader DJ. Proprotein convertases are responsible for proteolysis and inactivation of endothelial lipase. J Biol Chem 2005;280:36551–9.
38. Shimamura M, Matsuda M, Yasumo H, Okazaki M, Fujimoto K, Kono K, et al. Angiopoietin-like protein3 regulates plasma HDL cholesterol through suppression of endothelial lipase. Arterioscler Thromb Vasc Biol 2007;27:366–72.
39. McCoy MG, Sun GS, Marchadier D, Maugeais C, Glick JM, Rader DJ. Characterization of the lipolytic activity of endothelial lipase. J Lipid Res 2002;43:921–9.
40. Fuki IV, Blanchard N, Jin W, Marchadier DH, Millar JS, Glick JM, Rader DJ. Endogenously produced endothelial lipase enhances binding and cellular processing of plasma lipoproteins via heparan sulfate proteoglycan-mediated pathway. J Biol Chem 2003;278:34331–8.

Original Article

Multicenter Study to Determine the Diagnosis Criteria of Heterozygous Familial Hypercholesterolemia in Japan

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Aim: Heterozygous patients of familial hypercholesterolemia (FH) are known to have a high risk of coronary artery disease (CAD). Early diagnosis and prompt treatment are necessary to prevent their CAD. In this study we tried to amend the Japanese diagnostic criteria of FH for general practitioners by examining each component of the current criteria.
Methods: A multicenter study was performed, which included 1356 dyslipidemic patients at 6 centers. Pretreatment demographic information including LDL-cholesterol (LDL-C), Achilles tendon thickness (ATT), family history of FH and premature CAD and the result of genetic analysis were analyzed.
Results: Of 1356 patients, 419 were diagnosed with FH by criteria in 1988, which were used as a golden standard. We tried to define FH according to 3 conventional major items, i.e., 1) LDL-C, 2) ATT and/or cutaneous nodular xanthomas (CX), 3) family history of FH and/or family history of premature CAD. We then determined the cutoff of LDL-C using the new criteria. When we used 180 mg/dL as the cutoff of LDL-C, 94.3% of FH patients and 0.85% of non-FH satisfied 2 or more criteria. When we used 190 mg/dL, 92.1% of FH and 0.85% of non-FH satisfied 2 or more criteria; therefore, we chose 180 mg/dL for the cutoff of LDL-C in the new criteria and proposed that the diagnosis of definite FH can be made if 2 or more criteria are satisfied.
Conclusions: We examined each component for the diagnosis of heterozygous FH in a multicenter study in Japan.

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Key words; Diagnosis criteria, Familial hypercholesterolemia, LDL cholesterol, Achilles tendon thickness, LDL receptor

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Introduction

Familial hypercholesterolemia (FH) is a genetic disease caused by a mutation in genes related to low-density lipoprotein (LDL) metabolism. Heterozygous FH patients manifest high LDL cholesterol (LDL-C)

levels, skin and/or tendon xanthomas, and increased risk of premature coronary artery disease (CAD)[1]. High LDL-C levels are the first symptom that appears even from birth, while xanthomas on the Achilles tendon usually appear during or after the late 10s and CAD that determines the prognosis of FH patients appears during or after the third decade of life in men and the fifth decade in women[2-4]. Because morbidity and mortality of CAD in heterozygous FH are much higher than in the general population[1, 5-7], special attention should be paid to screen these patients and to prevent their atherosclerotic complications. For the diagnosis of FH, several criteria have been published throughout the world, including ours, reported in 1988[8]; however, appropriate diagnosis of FH by primary care physicians is not performed in general practice in Japan[9]. Therefore, it is very important to establish useful diagnostic criteria for primary care physicians to diagnose FH with high specificity and sensitivity.

Because FH patients are estimated to be more than 250,000, primary care physicians need to take care of most of them; therefore, the criteria should be as simple as possible for clinical usefulness and have high sensitivity and specificity. We have used diagnosis criteria for FH published in 1988 in Japan[8], which include hypercholesterolemia, presence of skin/tendon xanthoma and reduced LDL receptor activity as major items; however, it is difficult to measure LDL receptor activity in routine clinical practice and even lipid specialists do not measure its activity. Furthermore, it is not covered by Japan's health insurance system; therefore, it is necessary to make the current diagnostic criteria easy to use for general practitioners. Toward this end, we performed a multicenter collaborative study of 1397 patients with dyslipidemia.

Methods

Subjects

A total of 1397 patients with dyslipidemia, referred to the outpatient clinic of 6 hospitals (Kyoto University Hospital, Osaka University Hospital, Nippon Medical School Hospital, Chiba University Hospital, Kanazawa University Hospital, and National Cerebral and Cardiovascular Center Hospital), were the subjects to this study. Among these patients, 41 were excluded due to missing data. Consequently, 1356 patients with dyslipidemia were eligible for the present study. Most had been diagnosed with or without FH by lipid specialists at each hospital according to the criteria for FH reported in 1988, and genetic analysis was performed in 223 patients, some of whom were diagnosed with FH based only on mutations of the LDL receptor or PCSK9. The criteria were as follows: Major items included 3 items, (1) IIa or IIb phenotype at serum cholesterol level of 260 mg/dL or above; (2) Tendinous xanthoma or xanthoma tuberosum is present; (3) Reduced or abnormal receptor activity. Minor items included 3 items: (1) Xanthoma palpebratum; (2) Arcus juvenalis (<50 years); (3) Juvenile (<50 years) ischemic heart disease.

Table 1. Clinical characteristics of non-FH and FH patients in this cohort

	non-FH	FH	*p*
N	937	419	
Male (n, %)	453 (48.3%)	170 (42.7%)	<0.01
Age (y.o.)	58.3±16.3	52.9±18.6	<0.01
TC (mg/dL)	236±53	339±72	<0.01
LDL-C (mg/dL)	146±46	257±67	<0.01

Determination of Conventional Criteria for FH

In this study we tried to amend the current criteria. For the primary care setting, three major items, i.e. serum level of LDL-C, family history and specific physical findings of FH, were chosen as diagnostic items because all are easily assessed by general practitioners. Family history and specific physical findings were also separated in more detail. Finally, we set 5 items, (1) LDL-C, (2) specific physical findings: a) ATT, b) cutaneous nodular xanthomas (CX), (3) family history: a) family history of FH in 1st or 2nd degree relatives, b) family history of premature CAD in 1st or 2nd degree relatives. A family history of premature CAD was defined as having CAD before the age of 55 in males and 65 in females. First, we assessed the prediction for FH by the combination of physical findings and family history, and then we determined the cutoff point of LDL-C with the combination of the above-mentioned two items. LDL-C levels were calculated by the Friedewald formula. ATT levels were measured by X-ray according to the method previously described and determined as positive with 9 mm or more[10].

The data in the medical records of the patients were sent to the National Cerebral and Cardiovascular Center and examined. The study protocol was approved by the ethics committee of the National Cerebral and Cardiovascular Center (D#M20-25-2 for the multicenter trial and ID#M17-56-4 for the genetic analysis). The ethics committee of each hospital also approved the study protocol.

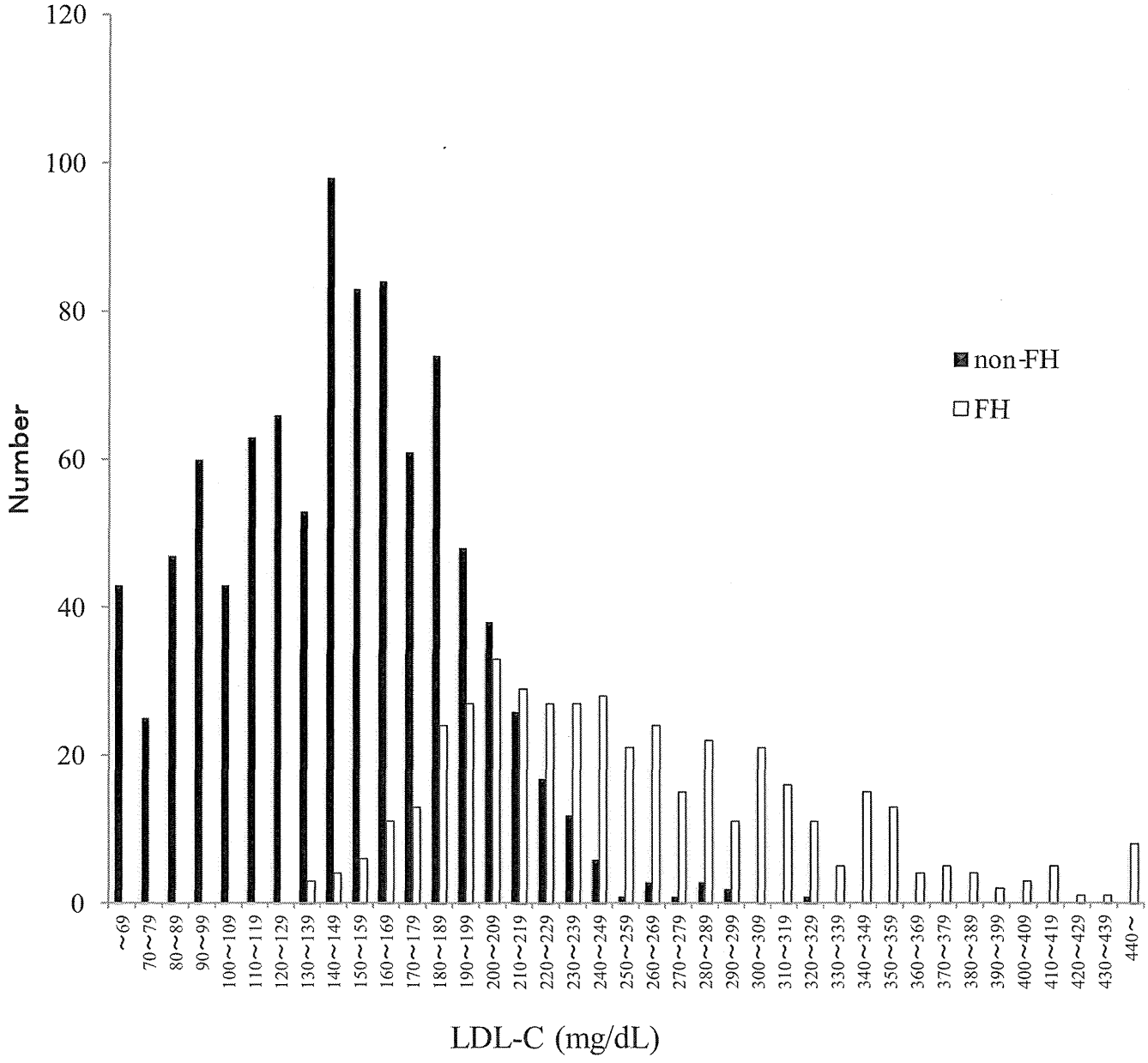

Fig. 1. Distribution of LDL-C levels before treatment in FH and non-FH patients. LDL-C levels were calculated by the Freidewald formula in patients with dyslipidemia diagnosed with FH or non-FH by specialists.

Statistical Analyses

Continuous variables are presented as the means ± SD. Categorical data are presented as numbers and percentages. Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) were used to assess differences between groups in continuous variables. Differences in categorical variables were assessed by the χ^2 test.

Results

Among 1356 patients, 419 had been diagnosed with FH, while 937 with non-FH. Patient demographic data are shown in **Table 1**. FH patients were younger than non-FH patients. TC and LDL-C levels were 339 and 257 mg/dL in FH patients, respectively, and were significantly higher than in non-FH patients. The distribution of LDL-C levels in both groups is shown in **Fig. 1**. FH patients were divided into 3

Table 2. LDL-C levels in FH patients with or without genetic data

LDL-C (mg/dL)	FH (Total)	FH (Mut+)	FH (Mut−)	FH (no genetic data)	*p*-value
N	419	224	41	173	
Mean	257.4	266.2*	229.0*	252.9	
SD	67.39	69.85	60.14	63.70	
MEDIAN	244	253	216	241	0.003
IQ					
25%	205	213	189	203	
75%	300	308	244	295	

FH (Mut+): mutations in the LDL receptor or PCSK9, FH (Mut−): no mutations found, FH (no genetic data): no genetic analysis
*$p<0.005$ by Bonferroni

Table 3. Sensitivity and specificity in screening FH by physical findings and family history

	Specificity	Sensitivity
Physical findings		
ATT (+) (%)	98.6	64.1
CX (+) (%)	99.6	9.4
ATT (+) or CX (+) (%)	98.6	64.6
ATT(+) and CX(−)	99.6	11.7
Family history		
Family history of FH (+) (%)	93.6	98.2
Family history of CAD (+) (%)	96.3	28.3
Family history of FH (+) or CAD (+) (%)	91.7	98.7
Family history of FH (+) and CAD (+) (%)	98.2	27.4

ATT: Achilles tendon thickness, CX: Cutaneous nodular xanthomas
FH ($n=224$) was diagnosed by mutations in the LDL receptor and/or PCSK9. Non-FH ($n=937$) was diagnosed by specialists.

groups depending on their genetic data: FH with mutation(s) in LDL receptor or PCSK9, FH with no mutation(s) and FH with no genetic data. The mean and median of LDL-C along with SD and interquartile range of each group are shown in **Table 2**. LDL-C levels in FH with mutations were higher than those in FH without mutations.

We tried to define FH according to the screening standards as 3 major items, i.e., 1) LDL-C, 2) ATT and/or cutaneous nodular xanthomas (CX), 3) family history of FH and/or family history of premature CAD. We used LDL-C instead of total cholesterol, because LDL-C should better reflect the activity of the LDL receptor and is used for the goal of lipid management in the current Japanese guideline[8]. We incorporated "family history" as a major item because general practitioners were able to find FH by a family history of FH and/or premature CAD instead of LDL receptor activity. Sensitivity and specificity in screening FH by physical findings and family history are listed in **Table 3**. Based on these data, we decided to use 1) ATT or CX, and 2) family history of FH or CAD as 2 major items in addition to high LDL-C levels.

Next we tried to determine the cutoff levels of LDL-C. The percentage of the patients who satisfied each criterion according to LDL-C levels is listed in **Table 4**. Levels of 180 or 190 mg/dL are suggested as candidate cutoff levels. Therefore, the criteria for model 1 were set as those who satisfy 2 or more of the 3 criteria: 1) LDL-C 180 mg/dL or higher, 2) ATT (+) or CX (+), 3) Family history of FH or CAD, and for model 2, for which the cutoff point of LDL-C was changed to 190 mg/dl or higher, their sensitivity, specificity, and false positive and false negative rates were compared (**Table 5**). When we compared model 1 with model 2, higher sensitivity in model 1 than model 2 was obtained without any change in specificity, suggesting that 180 mg/dL is a better cutoff for LDL-C. The percentages were quite similar in FH with mutation (s) in LDL receptor or PCSK9, FH with no mutation (s) and FH with no genetic data. The diagnostic criteria of FH were then determined

Table 4. Percent satisfying each LDL-C level in non-FH and FH patients

	non FH	FH (All)	FH (Mut+)	FH (Mut−)	FH (No genetic data)
N	937	419	223	41	155
LDL-C ≥ 170 mg/dL (%)	30.5	94.5	96.0	85.4	94.8
LDL-C ≥ 180 mg/dL (%)	24.3	94.3	94.6	82.9	92.9
LDL-C ≥ 190 mg/dL (%)	16.6	92.1	93.7	75.6	89.7
LDL-C ≥ 200 mg/dL (%)	11.6	80.0	84.3	63.4	78.1

FH(Mut+): mutations in the LDL receptor or PCSK9, FH(−): no mutations found, FH (no genetic data): no genetic analysis

Table 5. Accuracy metrics of FH criteria using LDL-C cutoff levels of 180 or 190 mg/dL

	Sensitivity (%)	Specificity (%)	False positive (%)	False negative (%)
Model 1: Satisfying 2 or more of the following criteria: 1) LDL-C ≥ 180 mg/dL, 2) ATT(+) or CX(+), 3) Family history of FH or CAD	94.5	99.1	0.85	5.5
Model 2: Satisfying 2 or more of the following criteria: 1) LDL-C ≥ 190 mg/dL, 2) ATT(+) or CX(+), 3) Family history of FH or CAD	92.1	99.1	0.85	7.9

Table 6. Diagnostic criteria for adult (15 years or older) heterozygous FH

1	Hyper-LDL-cholesterolemia (LDL-C level before treatment: 180 mg/dL or more)
2	Tendon xanthoma (tendon xanthoma of the dorsal hands, elbows, and knees, or Achilles tendon thickening) or nodular xanthoma of the skin
3	Family history (relatives in the second degree): FH or premature CAD

-A diagnosis should be made after ruling out the possibility of secondary hyperlipidemia.
-Patients meeting 2 criteria should be regarded as having FH. Concerning those meeting 1 criterion, refer to Fig. 4. When FH is suspected, gene tests should be conducted to make a diagnosis.
-Nodular xanthoma of the skin does not include palpebral xanthoma.
-Patients with Achilles tendon thickening (9 mm or more) on radiography should be regarded as having xanthoma.
-When the LDL-C level is 250 mg/dL or more, FH should be strongly suspected.
-During drug therapy, the pretreatment lipid level should be employed as a reference value.
- CAD in males younger than 55 years old and females younger than 65 years old is defined as premature CAD.
-When a diagnosis of FH is made, the patient's family should also be investigated.
- LDL-C may be decreased after surgery, myocardial infarction, severe inflammation and so on. In these cases, LDL-C values before the diseases should be requested to give a diagnosis.
- To diagnose patients who have already been treated with statins, pretreatment levels of LDL-C should be requested; however, termination of statin treatment is not recommended to obtain pretreatment levels of LDL-C, even if the data are not available.

and are shown in **Table 6.**

Discussion

FH has the highest prevalence in genetic metabolic diseases, being heterozygous in one in 500 of the general population[1, 11]. Most young heterozygous FH patients have no symptoms other than high LDL-C levels, and those who have Achilles tendon thickness have no symptoms. The reason for undiagnosed FH patients to go to a clinic may be mainly divided into the following 4 situations: 1) a chance visit to a primary care physician due to flu or gastritis, etc., 2) recommendation of further medical examination due to high cholesterol at a health checkup, 3) transportation to the emergency room due to the development of acute coronary syndrome, 4) recommendation of medical consultation due to the presence of FH in his/

her family. The diagnostic criteria should be applied to these patients. Accordingly, conventional criteria are needed for the primary care setting.

Heterozygous FH patients show high levels of LDL-C, cutaneous and tendon xanthomas, and are complicated with myocardial infarction at young age by atherosclerosis due to intravascular exposure to high levels of LDL-C for many years. Because early diagnosis and treatment are recommended for these patients, the diagnostic criteria for FH have been reported in many countries including Japan[8, 12-17]. While some criteria give a satisfactory diagnosis of FH using specific items, others are adopting a scoring system. The Japanese criteria reported in 1988[8] were as follows. Major items included the following 3 items: (1) the patient shows the IIa or IIb phenotype at a serum cholesterol level of 260 mg/dL or above, in principle; (2) Tendinous xanthoma or xanthoma tuberosum is present; (3) Reduced or abnormal receptor activity is noted by LDL receptor analysis; however, for LDL receptor activity, even lipid specialists do not routinely measure activity. It would be even more difficult for primary care physicians to measure activity for the diagnosis of FH.

The cutoff level of serum cholesterol used in the first criterion in the criteria published in 1988 was 260 mg/dL; however, LDL-C is directly affected by dysfunction of the LDL receptor and is routinely measured in clinics by the direct method or Friedewald formula; therefore, we tried to use LDL-C as a cutoff level instead of total cholesterol. The presence of tendon and/or cutaneous nodular xanthomas was also used because of its convenience, high sensitivity and specificity. A family history of FH or premature CAD in 1st or 2nd degree relatives was proposed for the third criterion instead of measuring LDL receptor activity in the new diagnostic criteria. A family history of FH showed high sensitivity and specificity; however, primary care physicians may have difficulty obtaining this because it was not easy for them to reach a diagnosis of FH with the previous criteria. In the present study, accurate diagnosis of a family history of FH seemed to have been given because lipid specialists made the diagnosis at all the hospitals; however, the same result may not be applied to primary care physicians. Therefore, a family history of CAD, which may be easier to obtain, was added to the criteria. It should be noted that the sensitivity of a "family history of FH or CAD" was slightly higher than that of a "family history of FH". Accordingly, we chose a "family history of FH or premature CAD in 1st or 2nd degree relatives" as the third criterion.

The cutoff level of LDL-C for the diagnosis of FH should be set by its sensitivity and specificity in different cutoff points. The cutoff level of LDL-C for the diagnosis of FH was reported to be 190 mg/dL in Simon Broome[17], NICE[15] and 205 mg/dL in MEDPED[16]. In this study, 180 mg/dL was selected as the cutoff level together with the presence of xanthoma and the family history as the criteria for the diagnosis of FH because of its high sensitivity and specificity.

Reduced LDL receptor activity is direct evidence of FH and was used as one of the criteria in the previous version. Usually, LDL receptor activity is determined by the binding of fluorescent-labeled LDL to lymphocytes. The procedure of measuring LDLR activity is cumbersome and it is difficult to measure in routine clinical settings. Further, few companies can measure LDLR activity. Indeed, the specialists involved in this study measured LDLR activity only in 7 of 419 patients of FH, showing the sensitivity of the previous criteria as 60.9%. Therefore, in order to determine criteria sensitive enough to give a diagnosis of FH, the third item was changed from LDLR activity to family history.

There are some limitations in the present study. First, the patients analyzed in this study may have different characteristics from those followed by primary care physicians, because the physicians in this study are taking care of many FH patients and information about family history can be obtained more easily than by primary care physicians. Second, it is sometimes difficult for primary care physicians to take a complete family history, especially FH, and to diagnose ATT and/or the presence of CX, about which information can be missed in the primary care setting. Third, FH has been reported to have mutations in LDL receptor, PCSK9 and apolipoprotein B. Because mutations in PCSK9 may cause milder forms of FH, the sensitivity of the criteria may be reduced in these patients. Further study is required to address the applicability of the criteria for the primary care setting.

In conclusion, we have determined the cutoff of LDL-C for the diagnosis of FH by a multicenter study and proposed conventional diagnostic criteria by using high LDL-C, ATT and/or the presence of CX, and a family history of FH and/or CAD for primary care settings.

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- Novo Nordisk Pharma Ltd. – Research Grants
- Pfizer Co. Ltd. – Research Grants
- Dainippon Sumitomo Pharma Co. Ltd. – Research Grants
- Kyowa Hakko Kirin Co. Ltd. – Research Grants
- Astellas Pharma Inc. – Research Grants
- Shionogi & Co. Ltd. – Research Grants

Dr. Nohara
- MSD – Research Grants
- Kaneka Medix Co. Ltd. – Research Grants
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Dr. Okada
- None

Dr. Ohta
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Dr. Bujo
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Dr. Oikawa
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Dr. Yamashita
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- Skylight Biotech Inc. – Advisory Board
- Astellas Pharma Inc. – Research Grants
- MSD – Research Grants
- Kissei Co. Ltd. – Research Grants
- Otsuka Co. Ltd. – Research Grants
- Shionogi & Co. Ltd. – Research Grants

References

1) Goldstein JL HH, Brown MS: Familial hypercholesterolemia. Edited by Scriver CR BA, Sly WS, Valle D, pp2863-2913, McGraw-Hill, New York, 2001
2) Bujo H, Takahashi K, Saito Y, Maruyama T, Yamashita S, Matsuzawa Y, Ishibashi S, Shionoiri F, Yamada N, Kita T: Clinical features of familial hypercholesterolemia in Japan in a database from 1996-1998 by the research committee of the ministry of health, labour and welfare of Japan. J Atheroscler Thromb, 2004; 11: 146-151
3) Mabuchi H, Koizumi J, Shimizu M, Takeda R: Development of coronary heart disease in familial hypercholesterolemia. Circulation, 1989; 79: 225-232
4) Harada-Shiba M, Sugisawa T, Makino H, Abe M, Tsushima M, Yoshimasa Y, Yamashita T, Miyamoto Y, Yamamoto A, Tomoike H, Yokoyama S: Impact of statin treatment on the clinical fate of heterozygous familial hypercholesterolemia. J Atheroscler Thromb, 17: 667-674
5) Mabuchi H, Miyamoto S, Ueda K, Oota M, Takegoshi T, Wakasugi T, Takeda R: Causes of death in patients with familial hypercholesterolemia. Atherosclerosis, 1986; 61: 1-6
6) Jensen J, Blankenhorn DH, Kornerup V: Coronary disease in familial hypercholesterolemia. Circulation, 1967; 36: 77-82
7) Stone NJ, Levy RI, Fredrickson DS, Verter J: Coronary artery disease in 116 kindred with familial type II hyperlipoproteinemia. Circulation, 1974; 49: 476-488
8) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirobe K, Funahashi T, Yokote K, Yokode M: Diagnostic criteria for dyslipidemia. Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese. Journal of atherosclerosis and thrombosis, 2007; 14: 155-158

9) Teramoto T: Status of lipid-lowering therapy prescribed on recommendations in the 2002 report of the Japan Atherosclerosis Society Guideline for Diagnosis and Treatment of Hyperlipidemia in Japanese Adults: A study of the Japan Lipid Assessment Program (J-LAP). Current Therapeutic Research, 2005; 66: 80-95
10) Mabuchi H, Ito S, Haba T, Ueda K, Ueda R: Discrimination of familial hypercholesterolemia and secondary hypercholesterolemia by Achilles' tendon thickness. Atherosclerosis, 1977; 28: 61-68
11) Mabuchi H, Nohara A, Noguchi T, Kobayashi J, Kawashiri MA, Tada H, Nakanishi C, Mori M, Yamagishi M, Inazu A, Koizumi J: Molecular genetic epidemiology of homozygous familial hypercholesterolemia in the Hokuriku district of Japan. Atherosclerosis, 2011; 214: 404-407
12) Watts GF, Sullivan DR, Poplawski N, van Bockxmeer F, Hamilton-Craig I, Clifton PM, O'Brien R, Bishop W, George P, Barter PJ, Bates T, Burnett JR, Coakley J, Davidson P, Emery J, Martin A, Farid W, Freeman L, Geelhoed E, Juniper A, Kidd A, Kostner K, Krass I, Livingston M, Maxwell S, O'Leary P, Owaimrin A, Redgrave TG, Reid N, Southwell L, Suthers G, Tonkin A, Towler S, Trent R: Familial hypercholesterolaemia: a model of care for Australasia. Atheroscler Suppl, 2011; 12: 221-263
13) Catapano AL, Reiner Z, De Backer G, Graham I, Taskinen MR, Wiklund O, Agewall S, Alegria E, Chapman MJ, Durrington P, Erdine S, Halcox J, Hobbs R, Kjekshus J, Perrone Filardi P, Riccardi G, Storey RF, Wood D: ESC/EAS Guidelines for the management of dyslipidaemias: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). Atherosclerosis, 2011; 217 Suppl 1: S1-44
14) Damgaard D, Larsen ML, Nissen PH, Jensen JM, Jensen HK, Soerensen VR, Jensen LG, Faergeman O: The relationship of molecular genetic to clinical diagnosis of familial hypercholesterolemia in a Danish population. Atherosclerosis, 2005; 180: 155-160
15) Wierzbicki AS, Humphries SE, Minhas R: Familial hypercholesterolaemia: summary of NICE guidance. BMJ, 2008; 337: a1095
16) Williams RR, Hunt SC, Schumacher MC, Hegele RA, Leppert MF, Ludwig EH, Hopkins PN: Diagnosing heterozygous familial hypercholesterolemia using new practical criteria validated by molecular genetics. Am J Cardiol, 1993; 72: 171-176
17) Risk of fatal coronary heart disease in familial hypercholesterolaemia. Scientific Steering Committee on behalf of the Simon Broome Register Group. BMJ, 1991; 303: 893-896

Special Report

Guidelines for the Management of Familial Hypercholesterolemia

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Familial hypercholesterolemia (FH) is a highly prevalent autosomal dominant hereditary disease, generally characterized by three major signs, hyper-low-density-lipoprotein (LDL) cholesterolemia, tendon/skin xanthomas and premature coronary artery disease (CAD). Because the risk of CAD is very high in these patients, they should be identified at an early stage of their lives and started on intensive treatment to control LDL-cholesterol. We here introduce a new guideline for the management of FH patients in Japan intending to achieve better control to prevent CAD. Diagnostic criteria for heterozygous FH are 2 or more of 1) LDL-cholesterol ≥ 180 mg/dL, 2) tendon/skin xanthoma(s), and 3) family history of FH or premature CAD within second degree relatives, for adults; and to have both 1) LDL-cholesterol ≥ 140 mg/dL and 2) family history of FH or premature CAD within second degree relatives, for children. For the treatment of adult heterozygous FH, intensive lipid control with statins and other drugs is necessary. Other risks of CAD, such as smoking, diabetes mellitus, hypertension etc., should also be controlled strictly. Atherosclerosis in coronary, carotid, or peripheral arteries, the aorta and aortic valve should be screened periodically. FH in children, pregnant women, and women who wish to bear a child should be referred to specialists. For homozygotes and severe heterozygotes resistant to drug therapies, LDL apheresis should be performed. The treatment cost of homozygous FH is authorized to be covered under the program of Research on Measures against Intractable Diseases by the Japanese Ministry of Health, Labour, and Welfare.

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Key words; LDL cholesterol, LDL receptor, Achilles tendon thickness, Coronary artery disease, Diagnosis criteria

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Introduction

Familial hypercholesterolemia (FH) is a genetic disorder caused by mutations in the genes related to the low-density lipoprotein (LDL) receptor pathway, and is transmitted by autosomal dominant inheri-

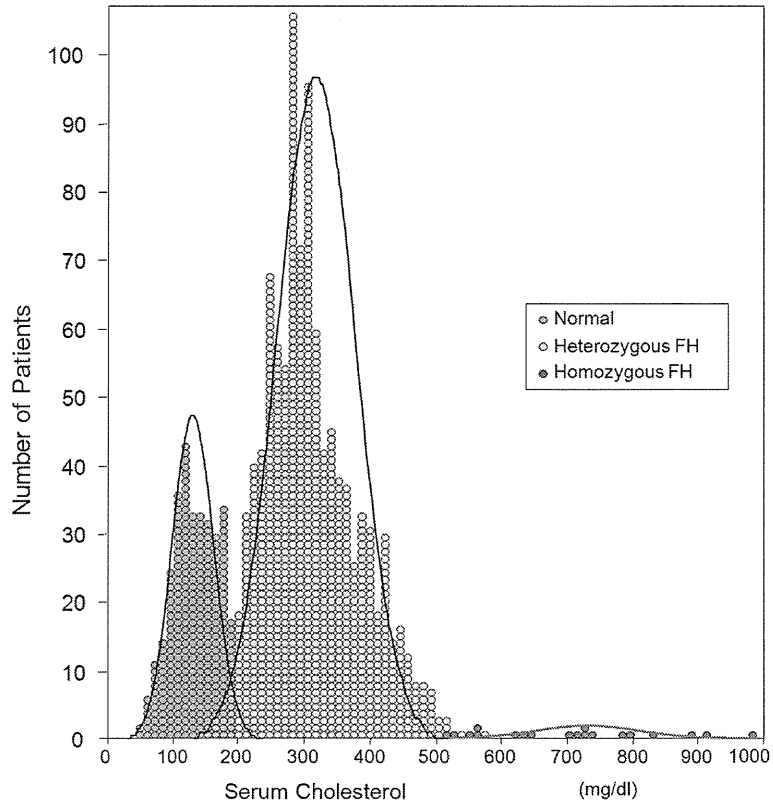

Fig. 1. Distribution of serum total cholesterol levels in normal subjects, and heterozygous and homozygous patients with familial hypercholesterolemia (modified by adding patients to Reference 85).

tance. Heterozygous patients are observed in 1 in every 500, and homozygous patients in approximately 1 in every 1 million people in Japan. The total number of FH patients has been estimated to be 250,000 or higher in Japan. Accordingly, FH is the most frequent hereditary metabolic disorder and is often encountered in daily practice. Primary manifestations of FH are hypercholesterolemia, tendon xanthoma, and premature coronary artery disease (CAD). Since atherosclerosis progresses faster and is accompanied by more severe organ disorders in FH patients than in hyperlipoproteinemic patients without a genetic background, prompt diagnosis and treatment are mandatory.

The effectiveness of reducing LDL-cholesterol (LDL-C) for primary and secondary prevention has been reported by many large-scale clinical studies using statins in high-risk groups of hypercholesterolemia and CAD. On the basis of these reports, guidelines for the prevention of atherosclerosis mainly targeting LDL-C have also been established in Japan; however, the subjects of large-scale clinical studies are not only patients with marked hypercholesterolemia, such as those with FH. In addition, as FH patients are exposed to hypercholesterolemia for a long period of time from their infancy, they exhibit more advanced atherosclerosis and more severe organ disorders than patients with typical hypercholesterolemia without a genetic background, necessitating particular caution in their diagnosis and treatment. Guidelines for the prevention of atherosclerosis classify FH patients into a high-risk group of CAD and stress the importance of early diagnosis and treatment. However, the guidelines for FH have not been sufficiently established in Japan, and no consensus has been reached; therefore, the paper proposes a guideline specific for the diagnosis and treatment of FH for the Japanese population.

Clinical Features and Gene Mutations of Familial Hypercholesterolemia

Familial hypercholesterolemia (FH) is an autosomal dominant hereditary disease characterized by three major signs: 1) hyper- LDL cholesterolemia, 2) tendon/skin xanthoma and 3) premature coronary artery disease (CAD).

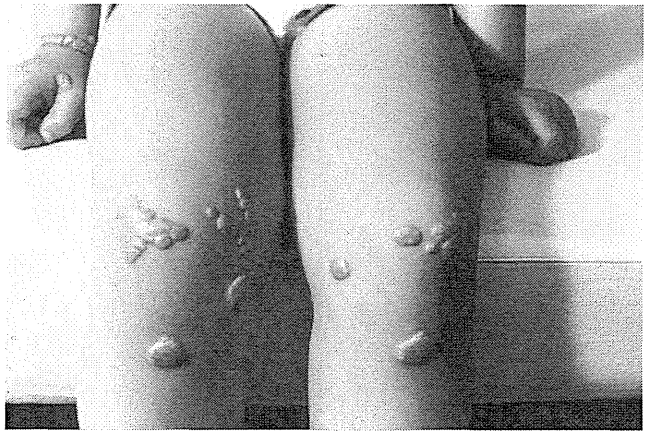

Fig. 2. Xanthoma observed in homozygous FH patients.

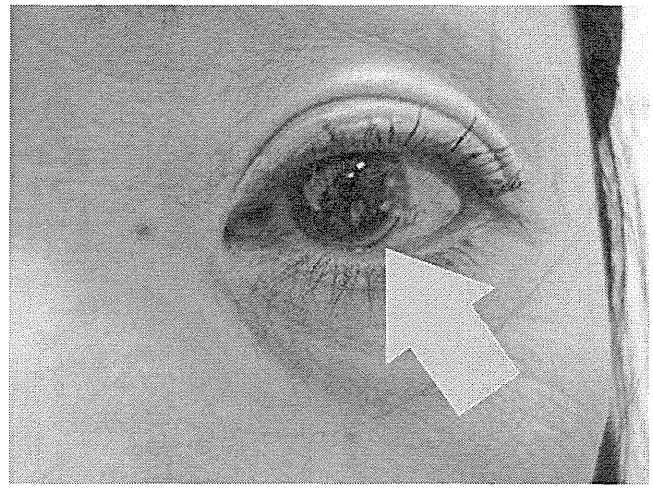

Fig. 3. Corneal arcus in FH patients.

In patients with FH, hyper-LDL cholesterolemia persists after birth, and Achilles tendon xanthoma may appear as early as in their 10s and is found in 50% of patients by the age of 30. Coronary atherosclerosis also develops at a young age, and the CAD risk is very high in FH patients. CAD events, such as myocardial infarction, which determine the prognosis of FH patients, may appear from 30 to 50 years of age in men and 50 to 70 years of age in women[1].

FH is one of the most frequently encountered hereditary diseases that may cause cardiovascular diseases (CVD) in general clinical practice. It should also be emphasized that the contribution of FH to CAD is higher in Japanese public health because of the low prevalence of CAD among the general Japanese population; however, neither diagnosis nor treatment is adequately performed for these patients. According to the literature, identification of FH may be only 20% or less in Europe, the United States and Japan[2, 3]. Health care professionals must understand that FH is a highly prevalent autosomal dominant hereditary disease and therefore surveying family members is important for the identification and treatment of patients.

Clinical Features of FH

Hyper-LDL Cholesterolemia

A patient with a mutation in one of the alleles in the gene involved in LDL metabolism, typically the LDL receptor, is termed heterozygous FH, and a patient with gene mutations in both alleles is termed homozygous FH. Serum total cholesterol (TC) in FH patients and their families shows a trimodal pattern. The mean serum TC in normal adults, heterozygous FH patients, and homozygous FH patients was 179 ± 26, 338 ± 63, and 713 ± 122 (mean ± SD) mg/dL, respectively; the mean value was 2 times higher in heterozygous FH patients and 4 times higher in homozygous FH than in normal adults[4]. There were overlaps between the normal and heterozygous FH, as well as between heterozygous and homozygous FH patients. It is sometimes difficult to differentiate these conditions based on the serum lipid level alone (**Fig. 1**). According to Bujo *et al.*, the mean LDL-C before treatment was 248 mg/dL in 641 Japanese patients with heterozygous FH (296 males and 345 females, mean age: 51 years)[5]. Although there was no gender difference in the LDL-C, triglyceride was significantly higher and HDL-cholesterol (HDL-C) was significantly lower in males than females.

Tendon/Skin Xanthoma

Physical findings such as tendon/skin xanthoma are important signs for the clinical diagnosis of FH. In homozygous FH patients, xanthoma becomes more marked than in heterozygous FH (**Fig. 2**). Xanthoma of the skin frequently develops at sites under regular mechanical stimuli, such as the extensor sides of the elbows/knees and wrist/gluteal regions. Tendon xanthoma appears as Achilles tendon thickening in many cases. It can be diagnosed based on inspection and palpation. Some FH patients may complain of inflammation-related pain of the Achilles tendon; however, FH should not be ruled out based on the absence of xanthoma. In those with a definitive diagnosis of FH by genetic analysis, xanthoma is absent in 20 to 30% of patients[5]. When xanthoma is absent, family surveys and genetic diagnosis are important. Xanthoma may be absent especially before 20 years of age, but becomes more prominent with aging. It should be noted that evaluation of xanthoma is difficult in the