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Special Report

Background to Discuss Guidelines for Control of Plasma HDL-Cholesterol in Japan*

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A decrease in high density lipoprotein-cholesterol (HDL-C) is a strong risk factor for atherosclerotic disorders in Japan, probably more important than an increase in low density lipoprotein-cholesterol (LDL-C). While there are rational grounds for the argument that elevation of HDL-C leads to decreased risk, there has as yet been no direct evidence of such an effect. If elevation of HDL-C decreases the risk, this effect is expected throughout the normal range of HDL-C or perhaps even higher than that. Simulation based on epidemiological data indicated that it may eventually reduce the incidence of ischemic heart disease by 60-70% in Japan. In the risk management guideline, "low" HDL-C is presently defined as 40 mg/dL or below. While there is no evidence that strongly urges a change in this definition, the results of epidemiological studies support "The higher the HDL-C level, the lower the risk," even in the "normal range". Elevation of the HDL-C level may reduce the risk, probably at least up to 70 mg/dL; however, there are no supportive data for this effect still being obtained over 80 mg/dL. Patients with homozygous CETP deficiency should be followed-up while controlling other risk factors, so as not to dismiss the possibility of a risk increase with an extremely elevated HDL-C level.

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Key words; HDL, LDL, Guidelines, NNT, Prevention

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Clinical Relevance of HDL-C Management

Numbers of epidemiological studies have established that the risk of coronary artery disease increases as plasma HDL-C decreases, and decreases as it increases. In addition, many experimental approaches

have demonstrated that cholesterol is extracted by HDL particles in the culture medium from cultured cells, including macrophages overloaded with cholesterol.

From these two lines of evidence, HDL is believed to be a "preventive factor" against atherosclerosis. This view is strongly associated with the hypothesis that HDL plays a central role in the recovery of cholesterol molecules from tissues and organs, which cannot be catabolized in peripheral cells, and in their transport to the liver for conversion to bile acids. From the viewpoint of public health, many research results suggest that a decrease in HDL-C contributes more than an increase in LDL-C to the development of ischemic heart disease in Japan. In studies conducted at Nagoya City University, for example, narrowing of the coronary artery was more closely related to triglycerides (TG) and HDL-C than to total cholesterol (TC) or LDL-C[1, 2], and this tendency is commonly observed in many other reports. HDL-C is thus suggested to be a strong determinant of atherosclerosis in Japan and perhaps a more important risk factor than LDL-C from a public health point of view.

HDL is smaller (12 nm or less in diameter) than other lipoproteins, abundant in protein and does not contain much TG, so it has a greater hydrated density than other lipoproteins (d=1.063-1.21). Similarly to other plasma lipoproteins, however, HDL functions to transport cholesterol among cells or organs using the flow of blood or extracellular fluid. Cholesterol, an essential molecule for the life of animals, requires a number of steps and plenty of energy for synthesis, and its dietary intake is not always guaranteed; therefore, the animal body has developed systems to use cholesterol sparingly as a precious material. As a result, little cholesterol is converted to energy in its catabolism, and, with the exception of a very small amount used for the production of steroid hormones, most cholesterol is transported to the liver for conversion to bile acids and is recycled an reused in the intestine before excretion. Its steroid backbone is not degraded in the metabolism in the animal body and finally broken down by microorganisms in the environment. Therefore, cholesterol molecules must be released from most somatic cells for metabolic homeostasis, and HDL receives these cholesterol molecules for their transport. Cholesterol is converted to cholesteryl acylester (CE) as a fatty acyl chain and transferred from phosphatidylcholine to its hydroxyl group to form an ester bond, for packing cholesterol molecules into the core of HDL. CE is recovered by the liver directly from HDL by a selective uptake reaction, or as LDL particles after being transferred to apolipoprotein B-containing lipoproteins by CE transfer protein (CETP). As a result of these activities, HDL is considered to exert a preventive effect against atherosclerosis as it interferes with the excessive accumulation of cholesterol in cells from LDL, etc., by extracting it.

No drug has been marketed yet to independently increases HDL-C; therefore, the question of whether increasing HDL-C is effective for preventing and treating atherosclerotic disorders has not been answered. However, researchers have recently directed more attention to HDL and, accordingly, more research results on HDL metabolism have recently accumulated. Much effort to develop drugs targeting HDL has been initiated. On the other hand, some existing drugs are known to increase plasma HDL-C. Drugs that reduce TG generally increase HDL-C, primarily because these drugs reverse low HDL-C induced by high TG through CETP[3]. In addition, fibrates have been suggested to directly increase HDL production[4]. Many clinical studies have also shown that statins elevate HDL-C as well as decreasing LDL-C. Concerning their mechanism, statins have recently been reported to increase HDL synthesis in the liver, unlike their effects in peripheral tissues[5]. The mechanism of the increase in HDL through exercise and alcohol intake has not been sufficiently elucidated. As mentioned below, the question of whether HDL-C increase by inhibiting CETP prevents atherogenesis has been shelved because of the failure to develop a CETP-inhibiting drug, perhaps due to a business-oriented strategy[6].

Position of HDL in Risk-Reducing Strategies

Large-scale clinical studies targeted to high LDL-C and high TG, major risk factors of atherosclerotic diseases, such as ischemic heart disease, have indicated that ischemic heart disease can be prevented by reducing LDL-C and TG and, particularly, that mortality due to the disease can be lowered by controlling the LDL-C level, with a consequent reduction in the total number of deaths in the high-risk group. In addition, based on stratified analysis of the results of many clinical trials, the conclusion has been reached that an increase in HDL-C contributes to the prevention of diseases as a "statistically independent factor". In consideration of the above-stated marked epidemiological contribution of HDL-C as a "negative risk factor" and the significant "indirect evidence" of an increase in HDL-C in the prevention of atherogenesis, the argument that a standard should be set for the control of HDL-C appears to be well grounded. However, it is also true that a consensus concerning

HDL-C management, similar to that in evidence-based quantitative guidelines for the control of LDL-C and the management and treatment of high TG, is difficult to reach at present, when no therapeutic technique specifically targeted to increase HDL-C has reached a practical level and there is no direct evidence concerning the prevention and treatment of atherosclerotic disorders using such a technique. Thus, any therapeutic guideline regarding HDL-C is merely a "proposal" based on indirect circumstantial evidence until the results of a large-scale clinical trial of a technique to specifically increase HDL-C become available.

Recently, some negative implications have been spread regarding the anti-atherosclerotic effect of an increase in HDL-C, inviting some confusion in the discussion. One is the discontinuation of a large-scale clinical study on the prevention of ischemic heart disease by increasing HDL-C, carried out to develop the CETP inhibitor torcetrapib, due to an increase in the mortality rate in the treated group[6]. Another is a large-scale epidemiological study reporting that a mutation to cause dysfunction of ABCA1, a rate-regulating protein of HDL biogenesis, is not likely to be a risk factor of ischemic heart disease[7]. The first report appears to support the contention of researchers arguing that "an increase in HDL-C by CETP inhibition has no anti-atherosclerotic effect," and allowed the generalized assertion that "the HDL-C increasing strategy is a mistake" to emerge; however, these reports do not necessarily mean the failure of CETP inhibitors themselves, and the pressor effect of a particular drug, torcetrapib, is likely to have led to such results. This incidence postponed an answer to the question of whether increasing HDL-C with a CETP inhibitor is a good idea, the most important medical issue, and markedly complicated the strategy for developing HDL-C elevating agents in general. Also, studies on ABCA1 mutation have shown that the maximum decrease in HDL is about 20%, suggesting that this does not necessarily reject the benefit of high HDL-C.

Under these circumstances, the position has not changed that an elevation of HDL-C is an important part of the anti-atherosclerotic strategy, including CETP inhibition. The above discussion may be summarized as follows: 1) a decrease in HDL-C is a strong risk factor for atherosclerotic disorders, 2) there are rational grounds for the supposition that this risk can be reduced by correcting low HDL-C (increasing HDL-C), but 3) no direct evidence has been obtained that increasing HDL-C is effective for the prevention and treatment of atherosclerotic disorders, 4) changes in HDL-C may include changes in the number and size of HDL particles, and the difference in their clinical significance may become a problem in the future.

Simulation of Atherosclerosis Prevention by Increasing HDL-C

There are qualitative scientific grounds for lowering the LDL-C level to reduce the risk of atherosclerotic disorders or, more specifically from an evidence-based viewpoint, to reduce the probability of the occurrence of ischemic heart disease; however, to prepare specific guidelines for diagnosis and treatment, quantitative criteria are considered indispensable. This is a problem with the concept in setting therapeutic goals for target groups. A quantitative profile of increases in the risk associated with elevations of the LDL-C level is necessary, and, if possible, results directly showing that the treatment reverses this curve of increasing risk must be presented. It is not impossible to set medical goals according to this parameter alone, but how criteria are set markedly affects the cost-effectiveness of treatment depending on the distribution of the HDL-C level and demographic composition of the target population; therefore, simulation involving these factors is one of the tasks that must be implemented to devise guidelines.

Fig. 1B shows the relationship between the LDL-C level and incidence (per 1,000 people) of myocardial infarction (lethal/non-lethal) in the JLIT, a cohort study that followed up a simvastatin-treated group for 5 years[8]. From this graph, the distribution of the HDL-C level in Japanese of corresponding ages (**Fig. 1A**)[9], and the population composition of the Japanese by age, the number of people needed to treat (NNT) and number of patients in whom the disease is prevented can be calculated when the control target is fulfilled 100% by reducing LDL-C (**Fig. 1C**). According to this calculation, the primary prevention efficacy, expressed as the inverse of NNT, is high at a target LDL-C level of 140 mg/dL but begins to fall rapidly as it is reduced to 120 mg/dL. Reflecting this, the incidence of myocardial infarction shows no further decrease when the target control level is set lower than 140 mg/dL. According to this analysis, roughly 140 mg/dL is considered to be medically and medico-economically appropriate as the target control level of LDL-C for primary prevention, at least on the basis of the results of the JLIT. In this case, the maximum preventive effect is 30-35% for myocardial infarction, which is in close agreement with the results of the MEGA study, the only large-scale interventional study of ischemic heart disease conducted in Japan using a statin[10].

Fig. 2B shows the decreases in the risk of ischemic heart disease associated with elevations of the HDL-C level in 3 epidemiological studies with prospective risk evaluation carried out in Japan including the JLIT[8, 11, 12]. While it is difficult to directly compare the incidences because the clinical definition of the endpoint varied among the studies, the peak decrease of the risk associated with increased HDL-C is less notable than that associated with the change of LDL-C in all studies. In other words, HDL-C-dependent decreases in the risk were observed even at HDL-C exceeding 60 mg/dL in all 3 studies. **Fig. 2C** shows the results of simulation similar to that of LDL-C performed using the results of the JLIT, which analyzed the therapeutic outcomes, on the basis of the HDL-C distribution curve in Japanese (**Fig. 2A**)[9] and the population composition. Since decreases in the risk associated with increases in HDL-C have not been directly demonstrated, the simulation was based on the hypothesis that increases in the risk associated with decreases in HDL can be reversed by increasing HDL-C. In contrast with the results concerning LDL, little decrease or peaking of the preventive efficacy associated with increased HDL-C was observed with an HDL-C level over 60 mg/dL. Reflecting this, the preventive effect against myocardial infarction could still be increased by raising the HDL-C level beyond 60 mg/dL. These results suggest that, under the hypothesis that the risk of myocardial infarction is reversibly reduced by elevating HDL-C, myocardial infarction can be prevented in 60-70% of the Japanese population at risk.

As far as these results are concerned, it can be concluded that the criterion of a "low HDL-C level" is unnecessary in guidelines for the control of HDL-C, and that the higher the HDL-C the better; however, according to the results in **Fig. 2A**, some studies have shown relatively large increases in the risk associated with decreases in HDL-C at about 50 mg/dL or below and, particularly, below 40 mg/dL; therefore, it may be reasonable to set a "caution level" around here. On the other hand, views on high HDL-C are divided. First, there is no epidemiological evidence indicating that higher HDL-C is better, even when it exceeds 60 mg/dL. This is probably because the population falling in this category is small (even though high HDL-C is relatively frequent in Japan) and cardiovascular incidence is low, making it difficult to obtain significant results.

In addition, the controversy is further complicated by the inclusion in this category of cases of homozygous CETP-deficient patients, in which elevations of HDL may not be considered to decrease the

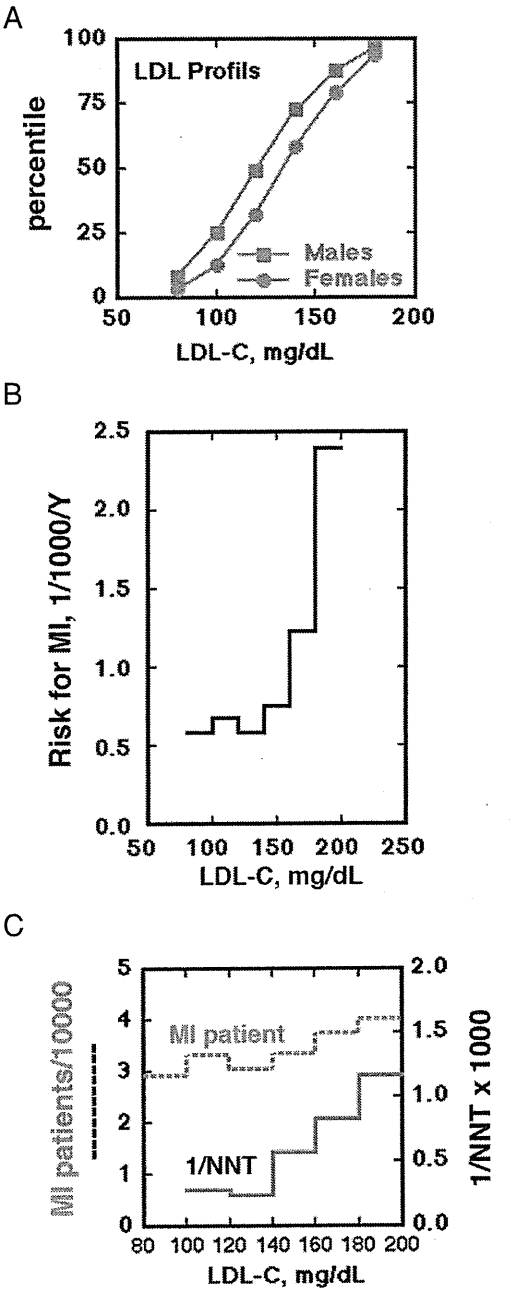

Fig. 1. Prevention of ischemic heart disease in Japanese by reducing LDL.

A: Distribution curve of the plasma LDL-C level in Japanese[9]. B: Relationship between the plasma LDL-C level and risk of "myocardial infarction" observed in the JLIT[8]. C: Simulation of the prevention of "myocardial infarction" based on Graphs A and B and demographic data for Japanese. Solid lines represent the inverse of NNT (·1,000) as an indicator of the treatment efficacy for managing lipoproteins to a target. The value of each horizontal segment is the efficacy when reaching a target LDL-C value at the left end of the segment in all Japanese at ages covered by the JLIT. Each horizontal segment of broken lines represents the number of MI patients when LDL is reduced to or lower than the level of the right end of the segment.

risk. The argument that increased HDL does not necessarily contribute to decreased risk is supported by the absence of a further decrease in the risk when the HDL-C increases above 70 mg/dL and the increased risk in patients with a homozygous CETP defect[13]; however, HDL-C is usually 80 mg/dL or higher and often reaches 100-200 mg/dL or even higher in patients with a homozygous CETP defect[13-16], and such high HDL-C should be considered separately from regular high HDL-C. Still, researchers are not in agreement concerning the increase in risk. In this sense, the differentiation of homozygous CETP deficiency is necessary in patients showing HDL-C exceeding 80 mg/dL, and there is no clinical or experimental evidence pointing to any conclusion about whether HDL-C should be maintained above this level. Nevertheless, the high prevalence of CETP deficiency among Japanese (1/20 for D442G and 1/100 for I14A) may have a limited but significant impact on the association between high HDL and atherogenesis in Japanese.

Proposal of Standards for Management of the HDL-C Level

On the basis of the above discussion, this article summarizes a proposal for the management of the HDL-C level as follows:

1) The evidence status is summarized as (1) A decrease in HDL-C is a strong risk factor for atherosclerotic disorders, particularly in Japan and, from the viewpoint of public health, it may be a more important risk factor than an increase in LDL-C; (2) While there are rational grounds for the argument that elevated HDL-C leads to a decreased risk, (3) there is as yet no direct evidence that elevating HDL-C is effective for the prevention and treatment of atherosclerotic disorders.

2) If elevations of HDL-C through interventional measures cause reversible decreases in the risk, this effect is expected, at least, up to 60 mg/dL or higher, and a simulation indicated that it eventually reduce the incidence of ischemic heart disease in Japan by 60-70%.

3) In risk management, high HDL-C is presently defined as 40 mg/dL or below. While there is no evidence that strongly urges a change in this definition, the results of epidemiological studies support "the higher the HDL-C level, the lower the risk," even in the "normal range" so that elevation of HDL-C may reduce the risk probably at least up to 70 mg/dL; however, there are no supportive data for this effect still being obtained over 80 mg/dL. Patients with a

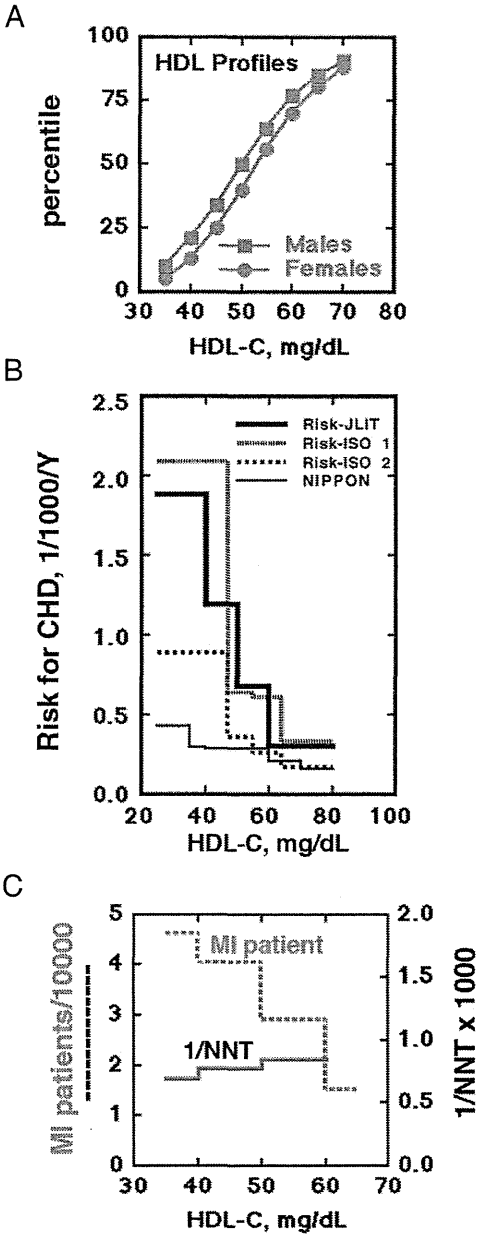

Fig. 2. Prevention of ischemic heart disease in Japanese by increasing HDL-C.

A: Distribution curve of the plasma HDL-C level in Japanese[9]. B: Relationship between the plasma HDL-CH level and risk of ischemic heart disease in Japanese. "Myocardial infarction" in the JLIT[8], "coronary artery disease" and "definitive diagnosis of myocardial infarction" by Kitamura, Iso, *et al.*[11], and "deaths due to cardiovascular diseases" according to NIPPON DATA[12]. C: Simulation for prevention of "myocardial infarction" based on Graphs A and B and demographic data of Japanese. Solid lines represent the inverse of NNT (x 1000) as an indicator of the treatment efficacy for managing lipoproteins to a target. The value of each horizontal segment is the efficacy when reaching a target HDL level at the right end of the horizontal segment in all Japanese at ages covered by the JLIT. Each horizontal segment of broken lines represents the number of MI patients when HDL is raised to the left end of the segment.

homozygous CETP deficiency should be followed-up while controlling other risk factors, not to dismiss the possibility of the risk increase with an extremely elevated HDL-C level. A gender-dependent strategy for HDL-C management should be discussed when further epidemiological and clinical evidence becomes available.

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Molecular genetic epidemiology of homozygous familial hypercholesterolemia in the Hokuriku district of Japan

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ABSTRACT

Aim: Familial hypercholesterolemia (FH) is caused by mutations of FH genes, i.e. LDL-receptor (LDLR), PCSK9 and apolipoprotein B (ApoB) gene. We evaluated the usefulness of DNA analysis for the diagnosis of homozygous FH (homo-FH), and studied the frequency of FH in the Hokuriku district of Japan.
Methods: Twenty-five homo-FH patients were recruited. LDLR mutations were identified using the Invader assay method. Mutations in PCSK9 were detected by PCR-SSCP followed by direct sequence analysis.
Results: We confirmed 15 true homozygotes and 10 compound heterozygotes for LDLR mutations. Three types of double heterozygotes for LDLR and PCSK9 were found. No FH patients due to ApoB mutations were found. The incidences of homo-FH and hetero-FH in the Hokuriku district were 1/171,167 and 1/208, respectively.
Conclusions: Our observations underlined the value of FH gene analysis in diagnosing homo-FH and confirmed extraordinarily high frequency of FH in the Hokuriku district of Japan.

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disease characterized by the triad of (1) hypercholesterolemia due to a high level of plasma low-density-lipoprotein (LDL), (2) tendon xanthomas and (3) premature coronary heart disease [1]. Patients with homozygous FH (homo-FH) have two mutant alleles of either of three FH-associated genes (FH genes), which are as follows: LDL-receptor (LDLR), proprotein convertase subtilisin/kexin type 9 (PCSK9) and apolipoprotein B (ApoB) gene [2]. Homo-FH patients are likely to be identified in early childhood because of the early appearance of xanthomatosis associated with an exceptionally high plasma cholesterol levels (exceeding 15.6 mmol/l), reflecting an extreme increase in LDL concentration. Use of the classical diagnosis of homo-FH and hetero-FH has led to an estimate of the prevalence of homo- and hetero-FH is 1 in 1,000,000 and 1 in 500 persons, respectively throughout the world [1]. The clinical phenotype of FH is highly variable and depends on the FH genes mutations present. Patients with a mild phenotype of homozygous FH often show a heterozygous phenotype of FH. Our aims of the present study were two. First we evaluated the molecular genetic epidemiology of homo-FH and then studied the frequency of hetero- and homo-FH in the Hokuriku district of Japan.

2. Patients and methods

2.1. Diagnostic criteria of FH [3,4]

I. Hetero-FH
   a) Clinical diagnostic criteria are hypercholesterolemia with tendon xanthomas, or hypercholesterolemia in the first- or second-degree relative of FH patients.
   b) Genetic diagnostic criteria are mutations of FH genes.
II. Homo-FH
   a) Clinical diagnostic criteria are juvenile xanthomatosis with plasma cholesterol level about twice that of parents or other family members with hetero-FH.
   b) Genetic diagnostic criteria are true homozygotes, compound heterozygotes and double heterozygotes for FH genes.

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Table 1
Clinical characteristics of 25 homo-FH patients in Hokuriku district of Japan.

	Case no.	Family no.	Name	FH gene mutations	Sex	Age (years)	TC (mmol/L)	TG (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	ATT (mm)	Father/mother	Consanguineous marriage	Outcome
True homozygote	1	1	M.K.	Ex2-3del/Ex2-3del	F	40	14.3	1.61	12.9	0.7	8	?/Hetero	+	D
	2	2	H.Y.	Ex2-3del/Ex2-3del	F	34	16.4	0.91	13.3	1.5	27	?/Hetero	+	A (LDL-a)
	3	3	T.T.	Ex2-3del/Ex2-3del	M	49	14.5	4.38				?/Hetero	+	D
	4	3	Y.I.	Ex2-3del/Ex2-3del	F	52	16.2	3.56	13.7	0.8	31	?/Hetero	+	A (med)
	5	4	M.I.	K790X/K790X	F	20	19.0	3.08				Hetero/hetero	?	D
	6	5	S.S.	K790X/K790X	F	26	26.1	8.85			20	Hetero/hetero	–	D
	7	5	Y.S.	K790X/K790X	F	23	13.9	1.11	12.9	0.5	20	Hetero/hetero	–	A (LDL-a)
	8	6	S.Y.	D280Y/D280Y	M	24	13.9	0.55	12.9	0.8	42	Hetero/hetero	+	A (LDL-a)
	9	6	K.Y.	D280Y/D280Y	F	27	15.8	1.42			13	Hetero/hetero	+	D
	10	6	K.M.	D280Y/D280Y	F	40	15.9	2.03			25	Hetero/hetero	+	D
	11	7	M.N.	R395W/R395W	F	73	15.6	1.21	14.4	0.6	11	?/?	?	D
	12	8	A.Y.	V502M/V502M	M	28	13.0	1.30			17	Hetero/hetero	–	A (med)
	13	9	Y.E.	IVS15-3C>A/IVS15-3C>A	M	11	23.6	3.39				Hetero/hetero	–	D
	14	10	S.Y.	PCSK9 E32K/PCSK9 E32K	F	49	10.9	1.73	8.2	1.3	9	?/?	?	A (med)
	15	11	Y.G.	PCSK9 E32K/PCSK9 E32K	F	44	8.4	1.95	6.1	1.4		Hetero/hetero	–	A (med)
Compound heterozygote	16	13	A.T.	K790X/P664L	M	5	17.8	3.05	15.1	0.7	6	Hetero/hetero	–	A (LDL-a)
	17	13	M.T.	K790X/P664L	M	15	14.1	2.73	11.4	0.8	9	Hetero/hetero	–	A (LDL-a)
	18	14	N.Y.	K790X/?	F	36	11.1	1.05	9.5	1.0	14	Hetero/hetero	–	A (med)
	19	15	K.C.	C163R/?	F	21	12.8	0.80	11.1	1.4	10	Hetero/hetero	–	A (med)
	20	16	Y.T.	Exon3-6dup/?	M	48	15.1	8.67		0.8	30	?/?	?	A (med)
	21	17	M.M.	W23X/?	M	36	12.0	2.42	9.8	1.0	28	Hetero/hetero	–	A (LDL-a)
	22	18	H.T.	R94H/W159X	M	59	12.8	1.45	11.1	1.0	22	?/?	–	A (med)
	23	19	Y.G.	IVS15-3C>A/PCSK9 E32K	M	45	12.0	2.51	9.6	1.3	15	?/hetero	–	A (med)
	24	20	T.K.	K790X/PCSK9 E32K	M	1	9.0	2.55	6.4	1.4	–	Hetero/hetero	–	A (med)
	25	21	M.K.	C183S/PCSK9E32K	M	3	16.4	1.25	14.2	0.7	-	Hetero/hetero	–	A (med)

Data are shown in mmol/L; F: female, M: male; ATT: achilles tendon thickness; Hetero: hetero-FH; D; deceased; A; alive; LDL-a: LDL-apheresis; med: medication.

2.2. Patients

Twenty-five clinically or genetically diagnosed homo-FH patients were selected for the study of genetic epidemiology of homo-FH and the calculation of the incidence of FH in the Hokuriku district. Written informed consent was obtained from each of the subjects prior to participation in the study.

2.3. Laboratory measurements

Blood samples for assays were drawn after overnight fasting. Concentrations of plasma total cholesterol (TC), triglyceride (TG) and high-density-lipoprotein-cholesterol (HDL-C) were determined at accredited clinical laboratories using routine clinical methods. LDL-cholesterol (LDL-C) concentrations were calculated using the Friedewald equation [5].

2.4. FH genes analysis

Detailed methods of FH genes analysis were described in our previous paper [6], and are described in brief here. Genomic DNA was prepared from white blood cells using a Genomic DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA). Primers covering all of the exons and exon–intron boundary sequence of LDLR and PCSK9 were designed using Primer3 online software (http://frodo.wi.mit.edu/). LDLR mutations were identified using the Invader assay method (Third Wave Technologies, Inc., Madison, WI, USA) for point mutations previously identified in Japan [7]. The multiplex ligation-dependent probe amplification (MLPA) method for large rearrangements was performed using a P062B LDLR MLPA kit (MRC Holland, Amsterdam, Netherlands) and DNA sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) for the other mutations. ApoB mutations were screened by the methods reported in our previous paper [8]. Mutations in PCSK9 were detected by polymerase chain reaction (PCR) single-strand conformational polymorphism (SSCP) followed by direct sequence analysis.

2.5. Statistical analysis

Plasma lipid concentrations were compared among FH groups using Student's *t*-test. All data in the text are expressed as mean ± SD. JMP 5.1.2 software (SAS Institute, Cary, NC, USA) was used for statistical analyses. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Genotypic analyses of homo-FH patients

A DNA study of FH gene mutations was performed in all 25 clinically and genetically diagnosed homo-FH patients. Thirteen LDL-R mutants were found in these homo-FH patients, and one PCSK9 mutant (PCSK9 E32K) was found in five patients (Table 1). True homo-FH was confirmed in 15 patients (Table 1, Fig. 1), and six true homozygotes (13 patients) were for LDLR mutations, and one (2 patients) was true homozygote for PCSK9 E32K. Four true homozygotes of Ex2-3del in three families and three homozygotes of D280Y in one family were born to consanguineous marriage. The geographical distributions of these homo-FH patients are shown in Fig. 1. Nine types of compound LDLR mutations were found in 10 homo-FH patients. Three types of LDLR and PCSK9 double heterozygotes were found. No case of FH patients due to ApoB mutation was

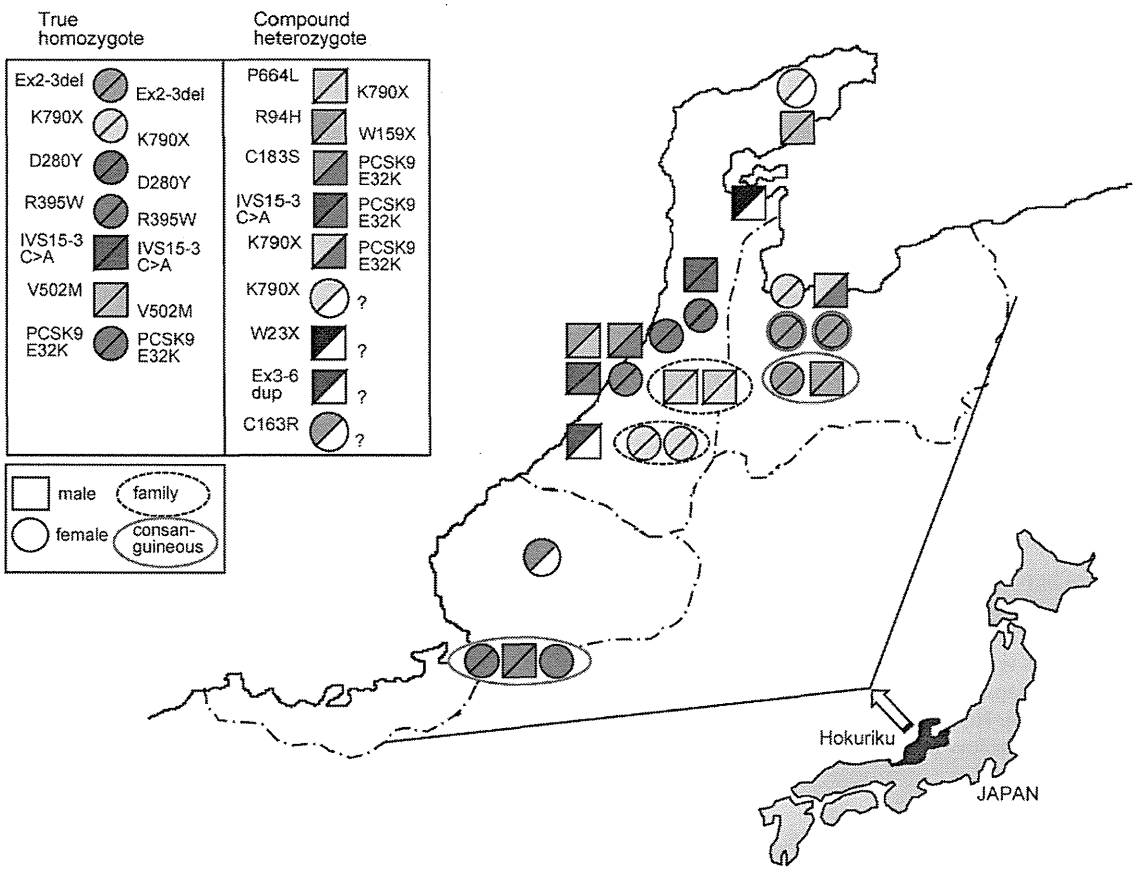

Fig. 1. Geographical location of 25 homo-FH patients with FH gene mutations in the Hokuriku district of Japan.

found. No consanguineous marriage was found in these compound or double heterozygote families (Table 1, Fig. 1).

Thirteen cases diagnosed by DNA analysis showed less than 14.3 mmol/L of TC. Mean (±SD) TC levels (15.7 ± 4.0 mmol/L) in homo-FH patients confirmed by DNA analysis were significantly lower than those (18.5 ± 4.1 mmol/L) diagnosed by clinical criteria ($p < 0.0442$) [9]. Two patients who were true homozygotes due to a mutation in the PCSK9 gene were newly found among our homozygotes. PCSK9 E32K produced relatively mild homo-FH phenotypes. The mean (±SD) plasma cholesterol level in homozygotes for LDLR gene mutations (15.7 ± 3.8 mmol/L) was significantly higher than that (11.3 ± 3.2 mmol/L) in true homozygotes or double heterozygotes for PCSK9 mutations ($p < 0.0276$).

The outcomes of the patients are shown in Table 1. Seven patients died of cardiac death and one (M.K.) died of leukemia. Seventeen patients are alive, and 6 patients have been treated with LDL-apheresis, and 11 patients with medications (Table 1).

3.2. Frequency of FH calculated using the Hardy–Weinberg equilibrium

Among 11 true homo-FH families consanguineous marriage was detected in four. In the remaining seven families, there were no consanguineous marriages. In nine families of compound heterozygotes no consanguineous marriage was found (Table 1, Fig. 1). The Hardy–Weinberg equilibrium was used to calculate the frequency of hetero-FH. The frequencies of homo-FH, hetero-FH and unaffected persons are p^2, $2pq$ and q^2, respectively (where $p+q=1$) and the general population in the Hokuriku district was 3,081,000. If the 7 patients from consanguineous marriage were ignored, p^2 could be 18/3,081,000 = 1/171,167 and then $p = 1/414$. As $q = 1 - p = 413/414$, the frequency of the hetero-FH ($2pq$) is $2 \times 1/414 \times 413/414 = 1/208$. Therefore, frequencies of the homo-FH and the hetero-FH were 1/171,167 and 1/208, respectively.

4. Discussion

It has been thought that homo-FH is easily diagnosed. However, it is sometimes difficult to differentiate severely affected heterozygotes from mild-type homozygotes without DNA analysis of FH genes. Recently, analysis of FH gene mutations has made it possible to identify presymptomatic homo-FH phenotype. DNA analysis of FH-associated genes shows true homozygote of identical heterozygous mutant, compound heterozygote of different heterozygous mutant of the same gene, and double heterozygote of different FH gene mutations. True homozygotes often came from a consanguineous marriage, whereas compound or double heterozygotes almost never did so. True homozygotes and compound heterozygotes for PCSK9 E32K show mild phenotypic homo-FH, compared with LDLR mutant homo-FH patients [6]. Currently, the diagnosis of FH involves clinical assessment and biochemical tests (lipid profile), but DNA-based testing will play a greater role in the identification and management of FH [10].

The prevalence of hetero-FH among the general population has been estimated to be at least, 1 in 500 among Caucasians, ranging from 1 in 200 to 1 in 1000 [11,12]. In 1978, we summarized 51 homozygous patients with FH in Japan, and estimated the frequency of homo-FH in Japan as 1 in 1.45 millions, and that of hetero-FH as 1 in 500 [9]. However, the low incidence of FH in most countries is due to the low frequency of homo-FH diagnosed by classical diagnostic criteria based on physical signs, laboratory findings and data from the parents. In the present study genetic diagnosis detected unexpectedly mild phenotypic cases of homo-FH, and several new cases of homo-FH were discovered. As FH is an autosomal dominant genetic disease, the frequency of hetero-FH has been calculated by the incidence of homo-FH in the district using the Hardy–Weinberg equilibrium. Here we found an extraordinarily high incidence of FH in this district of Japan.

A remarkably high prevalence of FH has been reported in several areas of the world, owing to founder effects. In Lebanon, the estimated prevalence of homozygotes and heterozygotes is 1 in 10,000 and 1 in 171, respectively [13]. In the Hokuriku district of Japan, the most frequent mutation in hetero-FH was the K790X LDLR mutation, with a frequency of 31.7% [7]. However, this mutant was less frequent in the Osaka district (5.4%) in Japan. No historical immigration records have been maintained in this district, and thus the K790X mutant of LDLR should not be classified as a founder gene in Japan, but only as a historically old mutant that prevailed locally and widely over a long period of time. High frequency of FH gene mutations might have produced true homozygotes without consanguineous marriage.

These observations underline the value of gene analysis in diagnosing hetero- and homo-FH patients and the extraordinarily high frequency of FH in this district of Japan.

Conflict of interest

There is no conflict of interest to disclosure.

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Short communication

A novel type of familial hypercholesterolemia: Double heterozygous mutations in LDL receptor and LDL receptor adaptor protein 1 gene

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ABSTRACT

Background: Autosomal recessive hypercholesterolemia (ARH) is an extremely rare inherited hypercholesterolemia, the cause of which is mutations in low-density lipoprotein (LDL) receptor adaptor protein 1 (LDLRAP1) gene.
Methods: A total of 146 heterozygous familial hypercholesterolemic (FH) patients with a mutation in LDLR gene were screened for genes encoding proprotein convertase subtilisin/kexin type 9 (PCSK9) and LDLRAP1.
Results: Among the 146 subjects, we identified a 79-year-old Japanese female with double mutations in LDLR gene (c.2431A>T) and LDLRAP1 gene (c.606dup). Two other relatives with double mutations in those genes in her family were also identified. Although the proband exhibited massive Achilles tendon xanthoma and coronary and aortic valvular disease, serum LDL-C level of subjects with double mutations was similar with that of subjects with single LDLR mutation (284.0 ± 43.5 versus 265.1 ± 57.4 mg/dl).
Conclusion: Additional mutation in LDLRAP1 may account for severer phenotype in terms of xanthoma and atherosclerotic cardiovascular disease in FH patients.

1. Introduction

Familial hypercholesterolemia (FH) is an inherited disease characterized by the triad of (1) hypercholesterolemia due to a high level of plasma LDL, (2) tendon xanthomas and (3) premature coronary artery disease [1]. Patients with homozygous FH have been defined as who have two mutant alleles of either of three following FH-associated genes: LDLR, apolipoprotein B (ApoB) gene and proprotein convertase subtilisin/kexin type 9 (PCSK9) [2]. Previously, we identified several homozygous FH patients who possessed double heterozygous mutations in LDLR gene and PCSK9 gene in relatively mild phenotypic patients compared with those with double mutations in LDLR gene [3]. In addition to autosomal dominant types of FH, recessive form of FH-associated gene was identified in 1992 [4]. The null mutations in the LDL receptor adaptor protein 1 (LDLRAP1) gene, which serves as an adaptor for LDLR endocytosis in the liver, causes autosomal recessive hypercholesterolemia (ARH) [5]. It is described that several heterozygous LDLRAP1 mutation carrier showed elevated LDL-C levels [6,7]. However, there is no data on clinical significance of adding a mutation in LDLRAP1 gene onto single LDLR gene mutation.

2. Methods

2.1. Study subjects

This study was approved by the Ethics Committee of Graduate School of Medical Science, Kanazawa University, and all study subjects gave their written informed consent to participate. We examined consecutive unrelated 146 subjects with a single mutation in the LDLR gene (male = 96, mean age = 56.5 ± 16.0, mean LDL-C = 265.6 ± 57.7 mg/dl) since 2003 to 2008. All the participants were free from unstable or acute cardiovascular diseases. All the lipid-lowering therapy had been transiently suspended for one to three months to diagnose lipid disorders correctly. Although it has been described the existence of the rebound effect after transient suspension of statin therapy [8], it is also reported that short-term suspension of statins is safe for at least patients with stable

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Table 1
Characteristics of the screened FH subjects.

Age (year)	56.5 ± 16.0
Sex (male/female)	96/50
BMI (kg/m^2)	23.2 ± 3.8
ATT (mm)	12.5 ± 3.5
TC (mg/dl)	330.1 ± 43.1
TG (mg/dl)	114.6 ± 35.1
HDL-C (mg/dl)	42.3 ± 8.7
LDL-C (mg/dl)	265.6 ± 57.7
ApoA-I (mg/dl)	121.8 ± 29.4
ApoB (mg/dl)	189.6 ± 25.8

Values are mean ± SD.

cardiovascular disease [9]. Complications related to this short-term suspension of lipid-lowering therapy have not been observed so far in our institute. The characteristics of the study subjects were listed in Table 1 and Supplementary Table S1.

2.2. Biochemical analysis

Serum concentrations of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically. LDL-C concentrations were derived using the Friedewald formula. Apolipoprotein E (ApoE) phenotype was separated by isoelectric focusing and detected by Western blot with apoE polyclonal antibody (phenotyping apoE IEF system, JOKOH, Tokyo, Japan). Plasma cholesteryl ester transfer protein (CETP) levels were determined by a specific ELISA [10].

2.3. Genetic analysis

Genomic DNA was isolated from peripheral blood white blood cells according to standard procedures and was used for PCR. Primers for the study were as used previously [3,11]; PCR products were purified by Microcon (Millipore Corp., Bedford, MA) and used as templates for direct sequencing. DNA sequencing was carried out according to the manufacturer's instructions using a dye terminator method (ABI PRISM™ 310 Genetic Analyzer (PerkinElmer Biosystems, Waltham, MA). We screened the study subjects for all coding region of PCSK9 and LDLRAP1 genes as candidate genes that could affect their lipid profile and clinical phenotype. In addition, we analyzed the two common mutations of the CETP gene (c.1321 + 1G > A, previously described as Int14A and c.1376A > G, previously described as D442G) among Japanese population as previously described [12].

3. Results

3.1. Biochemical analysis

Serum lipids and apolipoproteins in the proband and her pedigree are presented in Table 2.

3.2. Sequence of LDLR gene

Mutation in LDLR gene of the proband (c.2431A > T) was one of the most common mutations in Japan [13] (Supplementary Fig. S1A).

3.3. Sequence analysis of candidate genes for inherited hypercholesterolemia

Although there was no genetic abnormality in her PCSK9 gene, we identified another heterozygous mutation in her LDLRAP1 gene (c.606dup, Supplementary Fig. S1B).

3.4. Clinical course of the proband

At the age of 67, she was diagnosed as FH due to severe hypercholesterolemia with Achilles' tendon thickness (Supplementary Fig. S2). Initial levels of TC, TG, and HDL-C concentrations were 367, 108, and 46 mg/dl, respectively under statin therapy (pravastatin 20 mg daily). She underwent coronary artery bypass graft surgery at the age of 70 due to angina pectoris. The more intensive cholesterol lowering therapy using atorvastatin 20 mg daily was introduced for secondary prevention of cardiovascular disease. She was referred to our hospital for further examination of her hypercholesterolemia and coronary artery disease at the age of 78. Although her coronary atherosclerosis including bypass grafts did not progress substantially during 8 years (Supplementary Fig. S3), severe aortic valve stenosis developed causing her chest pain (Supplementary Fig. S4). Although aortic valve replacement surgery was recommended, she refused due to potential complications derived from extreme high age (Fig. 1).

3.5. Family study

Family study was performed as intensively as possible to find another family member with LDLR or LDLRAP1 mutation. We identified two other relatives with double mutations, and one obligate carrier who died suddenly probably due to cardiac event in his forties (Fig. 2).

Table 2
Clinical data of the pedigree.

Subject (gender)	I-1 (female)	II-1 (male)	II-2 (male)	III-1 (female)	IV-1 (male)	IV-2 (male)
LDLR genotype	W/M1	W/W	W/M1	W/M1	W/W	W/M1
LDLRAP1 genotype	W/M2	W/W	W/M2	W/M2	W/W	W/W
Age (year)	79	51	45	32	3	2
ATT (mm)	24	n.d.	n.d.	13	n.d.	n.d.
TC (mg/dl)	393	224	365	392	166	286
TG (mg/dl)	165	46	63	60	39	92
HDL-C (mg/dl)	42	97	96	61	59	62
LDL-C (mg/dl)	318	118	235	299	99	205
ApoA-I (mg/dl)	114	n.d.	n.d.	136	136	141
ApoB (mg/dl)	232	n.d.	n.d.	174	68	129
ApoE phenotype	3/3	3/3	3/3	3/3	3/3	3/3
CETP (μg/ml)	4.2	2.0	3.2	2.6	n.d.	n.d.

LDLR genotype: W = wild type, M1 = c.2431A > T; LDLRAP1 genotype: W = wild type, M2 = c.606dup.

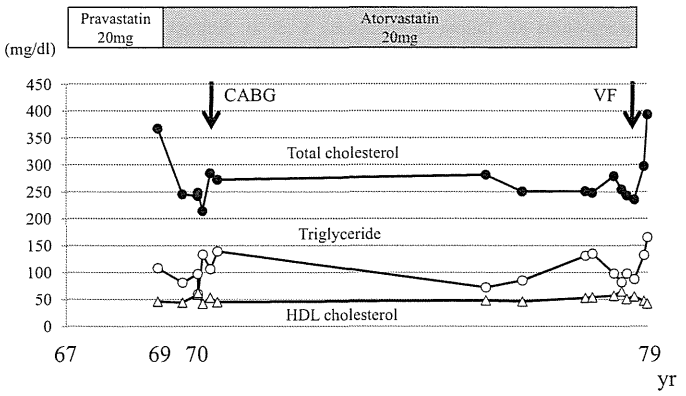

Fig. 1. Clinical course of the proband. Plasma concentration of the total cholesterol (solid circle), triglyceride (open circle), and HDL-C (open triangle) in the proband, and the major clinical events were illustrated. CABG; coronary artery bypass grafting, VF; ventricular fibrillation.

3.6. Genetic analysis for CETP gene

There was no carrier for both of common CETP gene mutation in this family.

4. Discussion

Patients with homozygous FH have two mutant alleles of either of three FH-associated genes (FH genes), namely LDLR, apolipoprotein B-100 and PCSK9 genes. In addition to those dominant form inherited gene mutation recessive form of null mutations in LDLRAP1 gene also causes FH (autosomal recessive hypercholesterolemia:ARH). There are few published data about the clinical characteristics of LDLRAP1 heterozygous mutation carriers because of rarity of this disorder. Previously, we have shown that c.606dup mutation carriers in LDLRAP1 gene had elevated LDL-C concentrations compared with non-carrier family members [14], suggesting that "autosomal recessive hypercholesterolemia" is not necessarily a correct term.

In this paper, we report the first family which exhibit double mutations in LDLR and LDLRAP1 gene with severe xanthomas and coronary artery disease as well as the episode of ventricular fibrillation due to aortic valve stenosis. Besides the proband, we found two other relatives in her family with the same double mutations in LDLR and LDLRAP1 gene.

Some of the pedigrees, including double mutation carriers exhibit relatively high HDL-C level. Previously, we reported that the CETP gene mutations causing higher HDL-C levels are common in Japan [12]. However, there was no carrier of two common CETP gene mutations (c.1321 + 1G > A and c.1376A > G) among this family member. The plasma levels of CETP of this family member were within normal limit, suggesting absence of CETP deficiency. It has been reported that the causes of high HDL-C level were quite heterogeneous [15]. Thus, we cannot exclude the possibility that unknown genetic factors may be involved in their high HDL-C levels. Another possibility of higher HDL-C is their excessive alcohol drinking. The pedigrees whose HDL-C levels were more than 90 mg/dl (II-1 and II-2) were both heavy drinkers (ethanol > 120 g/day).

In conclusion, we report the first family with double mutation in LDLR and LDLRAP1 genes associated with autosomal dominant and recessive form of hypercholesterolemia. Although the proband exhibited massive Achilles tendon xanthoma and severe coronary and aortic valvular disease, serum LDL-C level of subjects with double mutations was similar with that of subjects with single LDLR mutation. We suggest that an additional mutation in LDLRAP1 may account for severer phenotype in terms of xanthoma and atherosclerotic cardiovascular disease in FH patients.

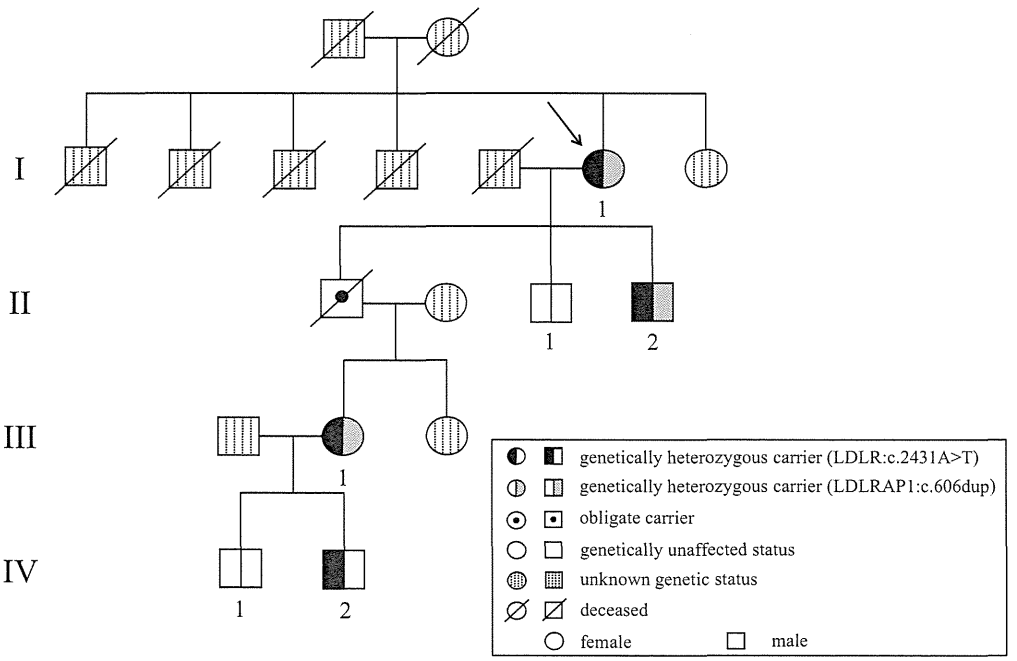

Fig. 2. Pedigree of the proband. Half-filled by black squares or circles indicate the heterozygous mutation carrier in LDLR (c.2431A > T). Half-filled by brown squares or circles indicate the heterozygous mutation carrier in LDLRAP1 (c.606dup). Square with a dot indicates the obligate carrier. Open squares or circles indicate unaffected subjects. Hatched squares or circles indicate the genetically unknown subjects.

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None declared.

Conflict of interest statement

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.08.004.

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Altered Metabolism of Low-Density Lipoprotein and Very-Low-Density Lipoprotein Remnant in Autosomal Recessive Hypercholesterolemia : Results From Stable Isotope Kinetic Study In Vivo

Hayato Tada, Masa-aki Kawashiri, Katsunori Ikewaki, Yoshio Terao, Tohru Noguchi, Chiaki Nakanishi, Masayuki Tsuchida, Mutsuko Takata, Kenji Miwa, Tetsuo Konno, Kenshi Hayashi, Atsushi Nohara, Akihiro Inazu, Junji Kobayashi, Hiroshi Mabuchi and Masakazu Yamagishi

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Altered Metabolism of Low-Density Lipoprotein and Very-Low-Density Lipoprotein Remnant in Autosomal Recessive Hypercholesterolemia

Results From Stable Isotope Kinetic Study In Vivo

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Background—Autosomal recessive hypercholesterolemia (ARH) exhibits different responsiveness to statins compared with that in homozygous familial hypercholesterolemia (FH). However, few data exist regarding lipoprotein metabolism of ARH. Therefore, we aimed to clarify lipoprotein metabolism, especially the remnant lipoprotein fractions of ARH before and after statin therapy.

Methods and Results—We performed a lipoprotein kinetic study in an ARH patient and 7 normal control subjects, using stable isotope methodology (10 mg/kg of [$^2H_3$]-leucine). These studies were performed at baseline and after the 20 mg daily dose of atorvastatin. Tracer/tracee ratio of apolipoprotein B (apoB) was determined by gas chromatography/mass spectrometry and fractional catabolic rates (FCR) were determined by multicompartmental modeling, including remnant lipoprotein fractions. FCR of low-density lipoprotein (LDL) apoB of ARH was significantly lower than those of control subjects (0.109 versus 0.450±0.122 1/day). In contrast, the direct removal of very-low-density lipoprotein remnant was significantly greater in ARH than those in control subjects (47.5 versus 2±2%). Interestingly, FCR of LDL apoB in ARH dramatically increased to 0.464 1/day, accompanying reduction of LDL cholesterol levels from 8.63 to 4.22 mmol/L after treatment with atorvastatin of 20 mg/d for 3 months.

Conclusions—These results demonstrate that ARH exhibits decreased LDL clearance associated with decreased FCR of LDL apoB and increased clearance for very-low-density lipoprotein remnant. We suggest that increased clearance of remnant lipoprotein fractions could contribute to the great responsiveness to statins, providing new insights into the lipoprotein metabolism of ARH and the novel pharmacological target for LDLRAP1. (***Circ Cardiovasc Genet.* 2012;5:35-41.**)

Key Words: lipoproteins ■ ARH ■ genetics ■ metabolism ■ LDLRAP1

Familial hypercholesterolemia (FH) is a common inherited disorder of plasma lipoprotein metabolism, characterized by an elevated level of low-density lipoprotein cholesterol (LDL-C), tendon xanthomas, and premature coronary artery disease.[1] Genetic causes of FH involve gene mutations such as LDL receptor (LDLR), apolipoprotein B-100 (apoB-100), and proprotein convertase subtilisin/kexin type 9 (PCSK9).[2] In contrast, there was a report of autosomal recessive inherited cases, who showed elevation of LDL-C, large xanthomas, and premature coronary artery disease typical of homozygous FH but in whom the fibroblasts had normal LDLR function.[3] Subsequently, Garcia et al[4] showed that this disorder was caused by a recessive form of null mutations in the LDLR adaptor protein 1 (LDLRAP1).

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Since then, evidence has been accumulating that it was not linked to mutations in the LDLR gene.[5,6] The N-terminal domain of LDLRAP1 contains a phosphotyrosine-binding (PTB) domain, which binds to the internalization sequence (FDNPVY) in the cytoplasmic tail of the LDLR.[7] LDLRAP1 protein serves as an adaptor for LDLR endocytosis in the liver

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