2.6. Serum chemistry and histopathology

Blood collected from the inferior vena cava upon sacrifice was subjected to serum chemistry. Assay kits (Wako, Osaka, Japan) were used to measure serum levels of aspartate aminotransferase, ALT, blood urea nitrogen and creatinine, which are biomarkers for hepatic and kidney toxicity. Formalin-fixed liver and kidney samples (#064-00406; Wako) were embedded in Histsec (Merck, Darmstadt, Germany), sliced at 5 μm using a microtome (Leica Microsystems, Wetzlar, Germany) and stained with Carrazzi's hematoxylin and Tissue-Tek eosin solutions (Sakura Finetek USA, Torrance, CA) for histopathological examination. Frozen liver tissues were placed in Tissue-Tek Intermediate cryomolds (#4566; Sakura Finetek USA) filled with precooled Tissue-Tek O.T.C embedding compound (#4583; Sakura Finetek USA) and flash-frozen by immersion in liquid nitrogen. Samples were sliced at 5 μm using a Leica CM1850 (Model 1850-11-1; Leica Biosystems, Wetzlar, Germany) and were air-dried for an hour. The resulting sections were rinsed with distilled water for 30 s and 60% 2-propanol (#03065-35; Nakarai Tesque, Kyoto, Japan) for 60 s. Oil Red O staining stock solution was prepared by dissolving 0.3 g of Oil Red O dye (#154-02072; Wako) in 100 mL of 2-propanol with gentle overnight incubation at 60 °C. Then, 30 mL of stock solution was diluted with 20 mL of distilled water to give a working solution. Samples were stained with this working solution at 37 °C for 15 min, rinsed with 60% 2-propanol and distilled water, and stained with hematoxylin (Gill's Formula) (#H-3401; Vector, Burlingame, CA) solution (25% in PBS) for 2 min at room temperature for histological analysis.

2.7. Statistical analysis

Pharmacological studies were performed with 4–9 mice per treatment group. All data are expressed as means ± SD. $P < 0.05$ was considered to be statistically significant in all cases. Statistical comparisons of results were performed by Dunnett's multiple comparison tests.

3. Results

3.1. Design and physicochemical properties of anti-apoC-III LNA–AON

We first designed AONs targeting apoC-III carrying LNAs (**A301SL**). We placed nine LNAs in the strand, keeping a six natural-nucleotide gap, which is thought to be sufficient for the introduction of RNase H-mediated scission of the mRNA strand (Yamamoto et al., 2012). At the same time, we prepared a corresponding conventional phosphorothioate AON designated **A301S** (Table 1). **A301SL**, **A301S** and 2′-O-methoxyethyl RNA-based apoC-III AON, reported previously by Graham et al. (2013), possess the phosphorothioate backbone, but they have different target sequences. As introduction of 2′-O-methoxyethyl RNAs into conventional phosphorothioate AONs moderately improves mRNA binding and in vivo antisense potency, **A301S** is speculated to have weaker potential than 2′-O-methoxyethyl RNA-based AON. Ideally, the potency and toxicity characteristics of **A301SL** should be compared with those of a corresponding 2′-O-methoxyethyl RNA-containing counterpart; however, as we were unable to obtain their phosphoroamidites, we herein utilized **A301S** as a non-LNA control. Note that the sequence, length and composition of AONs have not been fully optimized. A thermal melting study was carried out and T_m values of **A301SL** and **A301S** with their complementary RNA strands were determined. As expected, **A301SL** showed excellent target affinity when compared with conventional phosphorothioate AON (Table 1).

Table 1
Antisense oligonucleotides used in this study.

	Sequence ID	Sequence[a]	T_m (°C)
1	A301S	5′-tcttatccagctttattagg-3′	48
2	A301SL	5′-TCtTaTCcagcttTaTTaGg-3′	79

[a] Oligonucleotides with LNA (upper case letters) and DNA (lower case letters). All inter nucleotide linkages are phosphorothioated. Melting temperatures (T_m) of 1:1 mixtures of **A301S** and complementary RNA or **A301SL** and complementary RNA.

3.2. Hepatic reduction of apoC-III mRNA expression after systemic administration of LNA–AON

In order to assess the mRNA silencing potency of AONs, we repeatedly administered **A301SL** and **A301S** to C57Bl/6J male mice. After feeding 6-week-old male C57Bl/6J mice a high-fat diet for 2 weeks, mice were subjected to intraperitoneal (i.p.) injection of naked AON at a dosage of 10 and 20 mg/kg/injection five times over 2 weeks. Peripheral blood sampling was performed on day 0 just before the first injection, and on days 8 and 16 post-dose under feed-deprived condition for lipid component analysis and toxicity evaluation. Mice were dissected and their livers were harvested for measurement of gene expression on day 16 post-injection. As shown in Fig. 1, a significant dose-dependent decrease in hepatic apoC-III mRNA levels was only observed in **A301SL**-treated arms. **A301SL** suppressed hepatic apoC-III mRNA expression by ~29% and ~72% on average at a dosage of 10 and 20 mg/kg respectively, while **A301S** failed to achieve any reduction in apoC-III mRNA in the liver, even at the higher dose.

3.3. Serum reduction of apoC-III protein after systemic administration of LNA–AON

Changes in serum apoC-III protein concentration were confirmed by Western blot analysis. Although the quantitative capacity of Western blot analysis is very limited, we found that **A301SL** removed about half of apoC-III protein from sera at a dosage of 20 mg/kg on day 16, while **A301S** showed no significant reductions in apoC-III protein levels, which is consistent with the changes in hepatic apoC-III mRNA expression levels (Fig. 2). Collectively, we

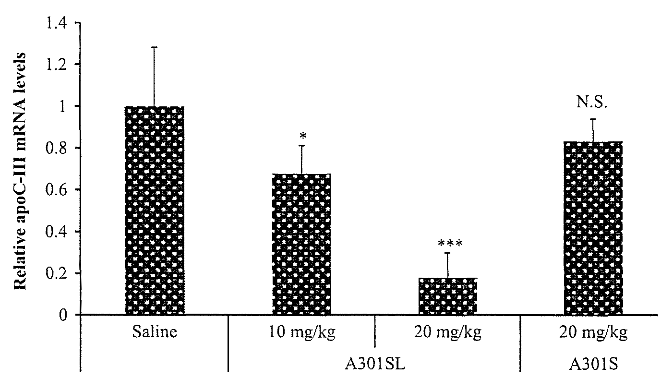

Fig. 1. Hepatic apoC-III mRNA silencing effects of **A301SL** and **A301S**. Western diet-fed mice received intraperitoneal administration of these two AONs at 10 or 20 mg/kg five times over 16 days. Relative hepatic apoC-III mRNA expression levels were determined by means of two-step real-time RT-PCR, and there was a significant reduction in A301SL-treated arms (Dunnett's multiple comparison test, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, N.S.; not significant). Error bars represent group means + SD.

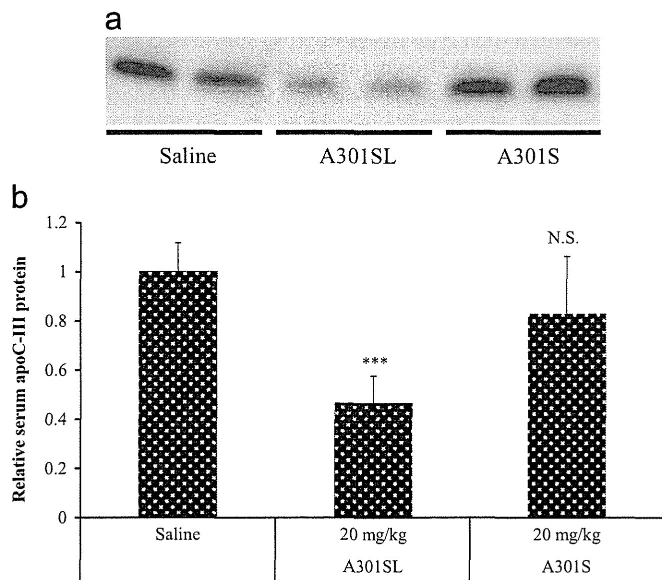

Fig. 2. Effects of **A301SL** and **A301S** on serum apoC-III protein levels. Western diet-fed mice received i.p. administration of these two AONs at 20 mg/kg for five times over 16 days. After completion of dosing, reductions in apoC-III protein level in serum were investigated by Western blotting. (a) Representative images of the membrane, and (b) there was a significant reduction in **A301SL**-treated arms (Dunnett's multiple comparison test, $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$, N.S.; not significant). Error bars represent group means ± S.D.

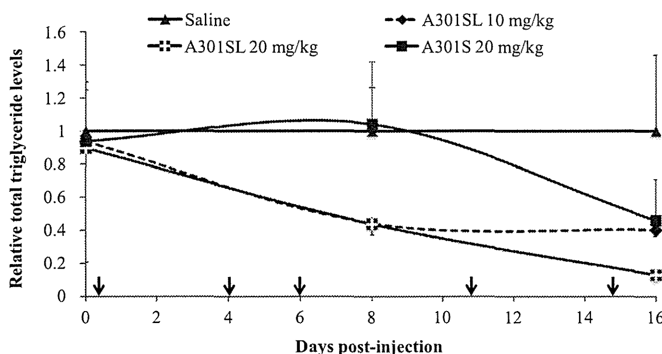

Fig. 3. Effects on serum triglyceride levels over time. Western diet-fed mice received intraperitoneal administration of two AONs, **A301SL** at 10 and 20 mg/kg/injection and **A301S** at 20 mg/kg/injection five times over 16 days. On days 0, 8 and 16, blood samples were collected from tail vein and total triglyceride levels were measured. Dose-dependent reductions were observed in **A301SL** groups, and delayed reductions were seen in the **A301S**-treated arm. Error bars represent group means ± S.D. Arrows indicate the date of administration.

successfully showed that the LNA–AON designed here is a potential inhibitor of apoC-III expression in vivo.

3.4. Serum changes in triglyceride-rich lipoprotein particles concentrations after systemic administration of LNA–AON

To confirm the ability of LNA–AON to modify serum lipids, we assessed the changes in triglyceride contents in fasting peripheral blood collected on days 0, 8 and 16 post-injection. As shown in Fig. 3, **A301SL** was confirmed to reduce serum total triglyceride concentration dose-dependently and more efficiently when compared to **A301S**. Total serum triglyceride levels with a 20 mg/kg/injection of **A301SL** were reduced by ~56% and ~87% over time, as compared to saline-treated controls, whereas **A301S** reduced total serum triglyceride levels by ~54% on day 16. We further conducted HPLC analysis of sera collected on day 8 to determine

Table 2
Serum lipoprotein profiles of hypertriglyceridemic mice on day 8.

	20 mg/kg		
	Saline	A301SL	A301S
Triglyceride [mg/dL]			
Total TG	54.9 ± 13.0	22.1 ± 5.7^b	51.5 ± 13.1
Chylomicron	0.9 ± 0.5	0.2 ± 0.1^b	0.5 ± 0.2
Large VLDL	30.3 ± 9.9	5.2 ± 2.3^a	24.1 ± 7.2
Medium VLDL	10.8 ± 1.7	4.9 ± 1.5^a	10.4 ± 2.5
Small VLDL	2.9 ± 0.3	2.0 ± 0.5^c	3.1 ± 0.6
Large LDL	3.4 ± 0.3	2.7 ± 0.7	4.0 ± 0.8
Medium LDL	2.5 ± 0.2	2.3 ± 0.6	3.4 ± 0.8
Small LDL	1.4 ± 0.2	1.3 ± 0.4	1.9 ± 0.4
Very small LDL	1.1 ± 0.2	1.0 ± 0.3	1.3 ± 0.3
Very large HDL	0.28 ± 0.06	0.19 ± 0.05	0.29 ± 0.08
Large HDL	0.26 ± 0.04	0.27 ± 0.11	0.34 ± 0.09
Medium HDL	0.24 ± 0.04	0.49 ± 0.28	0.48 ± 0.18
Small HDL	0.11 ± 0.01	0.59 ± 0.40^c	0.48 ± 0.23
Very small HDL	0.74 ± 0.09	1.17 ± 0.43	1.13 ± 0.21
Cholesterol [mg/dL]			
TC	133.4 ± 15.6	109.5 ± 11.7*	125.1 ± 14.9
Chylomicron	0.13 ± 0.05	0.04 ± 0.02^a	0.07 ± 0.02^b
Large VLDL	6.3 ± 1.8	1.3 ± 0.5^a	3.9 ± 0.7^a
Medium VLDL	4.7 ± 0.9	2.6 ± 0.6^a	2.9 ± 0.4^a
Small VLDL	3.0 ± 0.5	2.7 ± 0.8	2.4 ± 0.4
Large LDL	5.0 ± 0.6	5.0 ± 1.4	4.8 ± 0.7
Medium LDL	4.9 ± 0.6	5.2 ± 1.6	5.9 ± 0.8
Small LDL	3.4 ± 0.4	3.6 ± 1.1	4.4 ± 0.7
Very small LDL	6.1 ± 2.3	5.6 ± 2.0	11.7 ± 3.8^c
Very large HDL	7.5 ± 2.3	7.2 ± 2.3	9.1 ± 1.7
Large HDL	32.1 ± 5.2	27.9 ± 3.3	29.4 ± 3.3
Medium HDL	35.3 ± 3.4	28.6 ± 1.1^b	29.9 ± 3.2^c
Small HDL	15.7 ± 1.0	12.1 ± 0.2^a	12.5 ± 1.7^b
Very small HDL	9.1 ± 0.8	7.6 ± 0.5^c	8.0 ± 1.0

TG; triglyceride, TC; total cholesterol. Data are means ± S.D.
[a] $P<0.001$ vs. saline group.
[b] $P<0.01$ vs. saline group.
[c] $P<0.05$ vs. saline group.

the precise serum lipid profile. HPLC analysis revealed that **A301SL** markedly reduced VLDL-triglycerides, and larger VLDL-triglycerides were preferentially removed (Table 2). Moreover, substantial reductions in VLDL- and HDL-cholesterol were also observed in the **A301SL**-treated arm, and a much milder but similar trend was seen in the **A301S**-treated arm. These trends were particularly evident on day 16 (Fig. 4), and are consistent with the slight but not significant reductions in hepatic apoC-III mRNA and serum apoC-III protein levels, as shown in Figs. 1 and 2 on day 16.

3.5. Histopathological analysis of murine liver and kidneys

Pharmacological and toxicological characteristics of **A301SL** upon dosing were estimated by histopathological analysis. While all individuals in the saline group showed fat accumulation in the liver, induced by the Western diet, no such findings were observed in the **A301S**- and **A301SL**-treated arms (Fig. 5 and Table 3). We further visualized and compared fat drops in the livers by direct lipid staining with Oil Red O. As shown in Fig. 5, LNA–AON markedly reduced hepatic fat accumulation. Histopathologically, no severe cellular damage was noted, even at the highest doses in the centrilobular and perilobular hepatocytes, which were frequently seen after toxicological insult. On the other hand, moderate granulomas and granular degeneration were observed in the liver. Serum chemistry profiles showed slight increases in serum

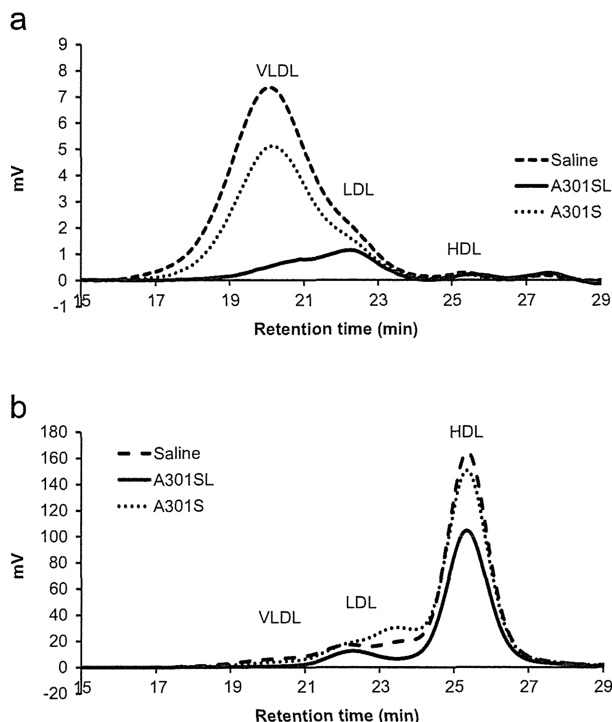

Fig. 4. Representative HPLC lipoprotein profiles of western diet-fed C57BL/6J mice received intraperitoneal administration of saline (dashed line), **A301SL** (solid line) at 20 mg/kg/injection or **A301S** (dotted line) at 20 mg/kg/injection five times over 16 days. Five saline-, **A301SL**- and **A301S**-treated mice were analyzed and the data from one representative individual mouse were presented. Corresponding (a) triglyceride and (b) cholesterol profiles were obtained from one identical mouse in each arm.

transaminases and slight decreases in blood urea nitrogen (Table 4). Elevations in transaminases may be due to the granular degeneration of hepatocytes. There were no significant changes in serum creatinine levels in each group.

4. Discussion

We have scarcely obtained selective inhibitors of apoC-III, which is thought to be a potential drug for the treatment of dyslipidemia, diabetes and cardiovascular diseases, as well as a useful tool for elucidation of the physiological roles of apoC-III. To develop a selective inhibitor of apoC-III, we designed an LNA-based 20-mer phosphorothioated AON (**A301SL**), in which LNAs are expected to greatly help with the target binding for the usage in vivo. As expected, **A301SL** achieved efficient dose-dependent reductions in hepatic apoC-III mRNA and decreased serum apoC-III protein concentration, which could be associated with the observation of efficient dose-dependent reductions in serum triglyceride concentration and attenuation of fat in the liver. *One limitation is that serum change of apoC-III protein was here confirmed by semiquantitative Western blot analysis.* For further study, we moved onto a precise lipoprotein profiling analysis of sera using HPLC methodology. Through this analysis, we found that serum reductions in triglycerides and cholesterol levels were largely a result of decreases in VLDL-triglycerides and VLDL-cholesterol from sera. It is also noteworthy that larger-sized VLDL was more susceptible to removal from blood, resulting in a shift of particle size distribution to smaller diameters (Table 2 and Fig. 4). Generally, large triglyceride-rich VLDL-1 are preferentially converted into atherogenic small, dense LDL, through a process mediated principally by cholesteryl ester transfer protein, lipoprotein lipase and hepatic lipase (Millar and Packard, 1998). Lipoprotein lipase activity is known to be modified by apoC-III protein and lipoprotein lipase preferentially hydrolyzes larger triglycerides-rich VLDL subfractions than smaller particles (Fisher et al., 1995). Thus, preferential removal of triglycerides from larger VLDL particles observed here can be explained as a result of derepression of lipoprotein lipase activity *via* successful silencing of apoC-III with LNA–AON. Combined with previous observations that, among triglycerides-rich lipoprotein subfractions in combined hyperlipidemia patients such as type IIb, VLDL-1 has the highest potential to induce accumulation of triglycerides and cholesterol in macrophages and foam cell formation (Milosavljevic et al., 2001), selective apoC-III inhibitors would possibly show anti-atherogenic phenotype.

Both apoC-III-null subjects and apoC-III-deficient mice generally possess reduced plasma total cholesterol levels, as well as total triglycerides, when compared to those of normal controls (Gerritsen et al., 2005; Jong et al., 2001; Pollin et al., 2008; Takahashi et al., 2003). We also observed a 33% reduction in total cholesterol levels along with apoC-III attenuation by the LNA–AON. This decrease in plasma cholesterol levels was reflected in both apolipoprotein B-containing and HDL fractions (Table 2 and Fig. 4). However, the mechanistic background for the reduction of plasma cholesterol upon apoC-III attenuation is controversial. A previous study showed that apoC-III deficiency in apolipoprotein E-knockout mice accelerated the kinetics of uptake of cholesterol ester, which is related to the function of hepatic lipase (Jong et al., 2001). In addition, hepatic lipase transgenic rabbits and hepatic lipase transgenic and adenovirus-transduced mice were reported to reduce plasma triglycerides and apolipoprotein B-containing lipoprotein cholesterols as well as HDL cholesterol (Applebaum-Bowden et al., 1996; Busch et al., 1994; Dichek et al., 1998; Fan et al., 1994). As our findings are in line with these previous observations, we speculate that activation of hepatic lipase resulting from apoC-III attenuation by the LNA–AON caused a reduction in plasma cholesterol levels. In contrast, Old Order Amish individuals with an *APOC3*-null mutation have higher plasma HDL cholesterol concentrations, as well as lower levels of triglycerides and non-HDL cholesterol than those of normal subjects (Pollin et al., 2008). In addition, knockout effects of apoC-III on plasma cholesterol levels also vary between genetic backgrounds of mice and experimental conditions (Jong et al., 2001; Takahashi et al., 2003). There are only a small number of reports focusing on the relationship between cholesterol metabolism and apoC-III (Kinnunen and Ehnholm, 1976). To determine the true effects of apoC-III modulation on cholesterol metabolism, further experimental data is necessary.

The toxicological characteristics of **A301SL** and **A301S** were estimated based on serum biochemistry characteristics and histopathological analysis. As phosphorothioated AONs accumulate mainly in the kidney and liver, hepatotoxicity and/or nephrotoxicity are primary concerns. Our experiments found only moderate hepatotoxicity for **A301SL** and **A301S,** as shown in the moderate increases in liver transaminases and decreases in blood urea nitrogen, while no significant changes in serum creatinine levels were noted. Histopathological observations supported these data (Fig. 5, Tables 3 and 4). Similar hepatotoxicity attributable to LNA-modified phosphorothioated AONs, which was avoidable by substituting 2′,4′-BNA^{NC} chemistry for LNA, has been reported (Prakash et al., 2010; Yamamoto et al., 2012). Dose-related hepatotoxicity could be tolerable based on the systemic AON recently approved by the US Food and Drug Administration (FDA) named "Kynamro", which also shows serum elevation of transaminases, specifically alanine aminotransferase (ALT) (http://www.kynamro.com/). However, it is necessary to determine how AONs trigger toxicity in order to resolve this issue (Levin, 1999). Therefore, we further conducted Oil Red O staining of liver samples. The results

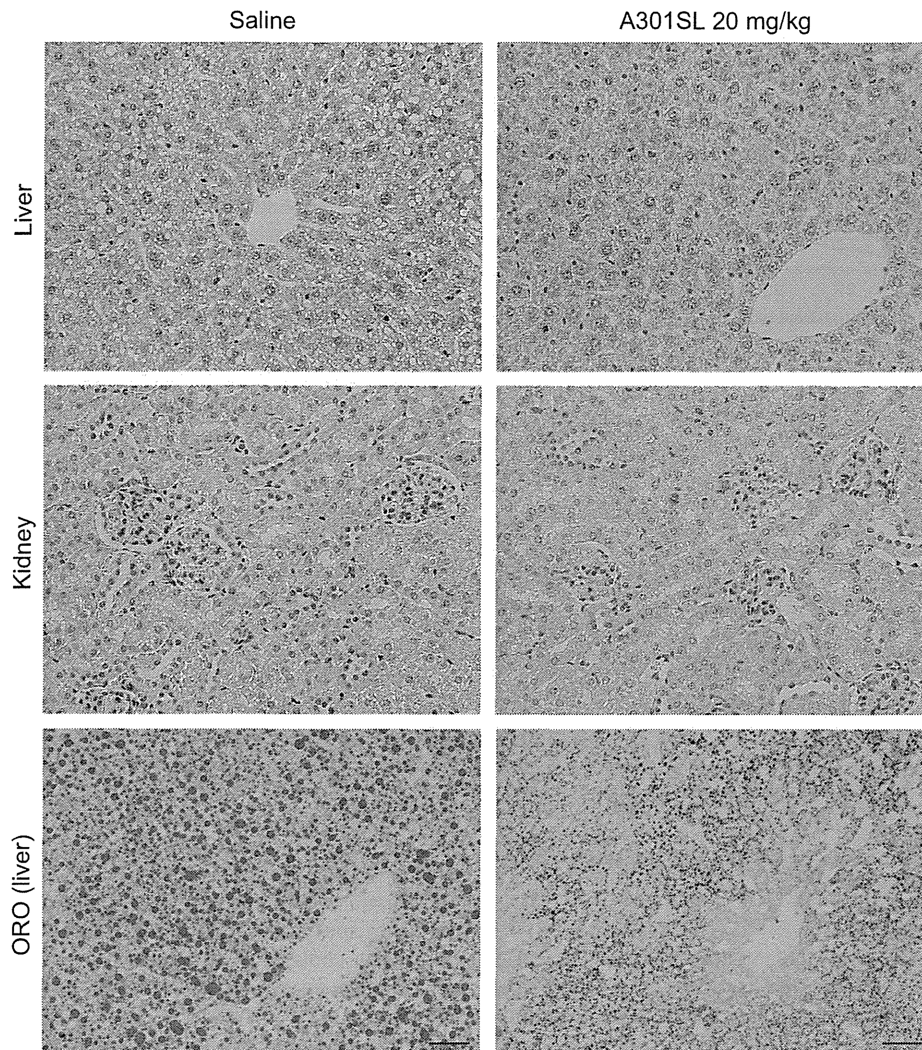

Fig. 5. Representative histopathological changes in livers and kidneys subjected to 16 days of saline (left) and **A301SL** (right) dosing were assessed by H&E or Oil Red O staining (× 200 magnification). Peripheral fatty changes were observed in the liver of the saline-treated mice (top and bottom), while periportal granular degeneration were seen in the highest dose of **A301SL**-treated mice with complete loss of fatty changes (top). No significant changes were observed in kidneys (middle).

Table 3
Histopathological findings.

		Saline	A301SL		A301S
	Dose	–	10 mg/kg	20 mg/kg	20 mg/kg
	Number of mice examined–	9	4	5	5
Organ Liver	Findings				
	Normal	0	3	0	2
	Fatty change, periportal	9	0	0	0
	Granuloma	0	1	1	2
	Granular degeneration, periportal	0	0	5	0
Kidney(s)					
	Normal	9	5	5	4
	Hemorrhage	0	0	0	1

All lesions showed a moderate grade.

Table 4
Effects on serum chemistry.

		AST (IU/L)	ALT (IU/L)	BUN (mg/dL)	Cre (mg/dL)
Saline		17.6 ± 2.8	9.1 ± 3.2	29.7 ± 6.4	0.2 ± 0.05
A301SL	10 mg/kg	22.5 ± 4.6	20 ± 9.2^{b}	30.1 ± 4	0.1 ± 0.05
	20 mg/kg	44.2 ± 13.8^{b}	14.5 ± 3.3	20.4 ± 3.3^{b}	0.1 ± 0.00
A301S	20 mg/kg	22 ± 2	7.5 ± 0.9	18.4 ± 2.6^{b}	0.1 ± 0.00

AST; aspartate aminotransferase, ALT; alanine aminotransferase, BUN; blood urea nitrogen, Cre; serum creatinine. Data are presented as means ± S.D.
$^{a}P < 0.001$, $^{c}P < 0.05$ vs. saline group.
^{b} $P < 0.01$ vs. saline group.

showed that our AON does not induce steatosis, which is a typical feature of drug-induced hepatotoxicity (Begriche et al., 2011). Instead, we observed drastic regression of steatohepatitis in **A301SL**-treated arms (Fig. 5), which is presumably an on-target-based pharmacological effect.

In conclusion, we successfully developed an anti-apoC-III LNA-AON. Although this selective apoC-III inhibitor of **A301SL** shows improved potency and safety, it will nevertheless be of help to further elucidate the molecular biology and molecular physiology of apoC-III that other non-selective inhibitors of apoC-III and have failed to reveal.

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□ CASE REPORT □

Familial Hypercholesterolemia with Multiple Large Tendinous Xanthomas and Advanced Coronary Artery Atherosclerosis

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Abstract

We herein report the case of a 53-year-old man with severe coronary ischemia who underwent successful coronary artery bypass surgery. Of note, he had hypercholesterolemia and presented with multiple large tendinous xanthomas and thickened Achilles tendons that had been present for more than two decades. Together with a family history of dyslipidemia, the patient was diagnosed as having familial hypercholesterolemia. Irrespective of an extensive search for possible mutations in the genes presumably involved in the patient's pathophysiology, including low-density lipoprotein receptor (LDLR), proprotein convertase subtilisin/kexin type 9 (PCSK9), autosomal recessive hypercholesterolemia (ARH) and apolipoprotein B (APOB), we were not able to identify the gene mutations responsible for the phenotype observed in the present case.

Key words: familial hypercholesterolemia, tendinous xanthomas, coronary artery disease

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Introduction

Familial hypercholesterolemia (FH) is characterized by increased levels of serum low-density lipoprotein (LDL) cholesterol with a high prevalence of coronary atherosclerosis. It may be inherited as an autosomal dominant trait, and the frequencies of homo- and heterozygotes are estimated to be $1/1\times10^6$ and 1/500, respectively, in the general population. Establishing a diagnosis in these patients is important because lipid-lowering therapy not only slows the progression of atherosclerosis, but also may achieve regression of atherosclerotic vascular lesions. Genetically determining the presence of FH is feasible, although testing is not yet available for routine use. In a large portion of patients, the diagnosis of heterozygous FH is based on laboratory and clinical criteria (1, 2).

Case Report

A 33-year-old man was diagnosed as having hypercholesterolemia more than two decades ago. The lipid profile of his mother at 72 years of age was as follows: total cholesterol, 257 mg/dL; LDL cholesterol, 167 mg/dL; high density lipoprotein (HDL) cholesterol, 72 mg/dL; and triglycerides 138 mg/dL. She was not taking any lipid-lowering drugs nor did she have xanthomas. We could not obtain information regarding abnormal lipid metabolism from other family members. Since then, multiple subcutaneous nodular tumorous lesions began to develop in the present patient and grew larger on the sites of the bilateral Achilles tendons, the ulnar side of the right wrist, the dorsum of the right hand and the

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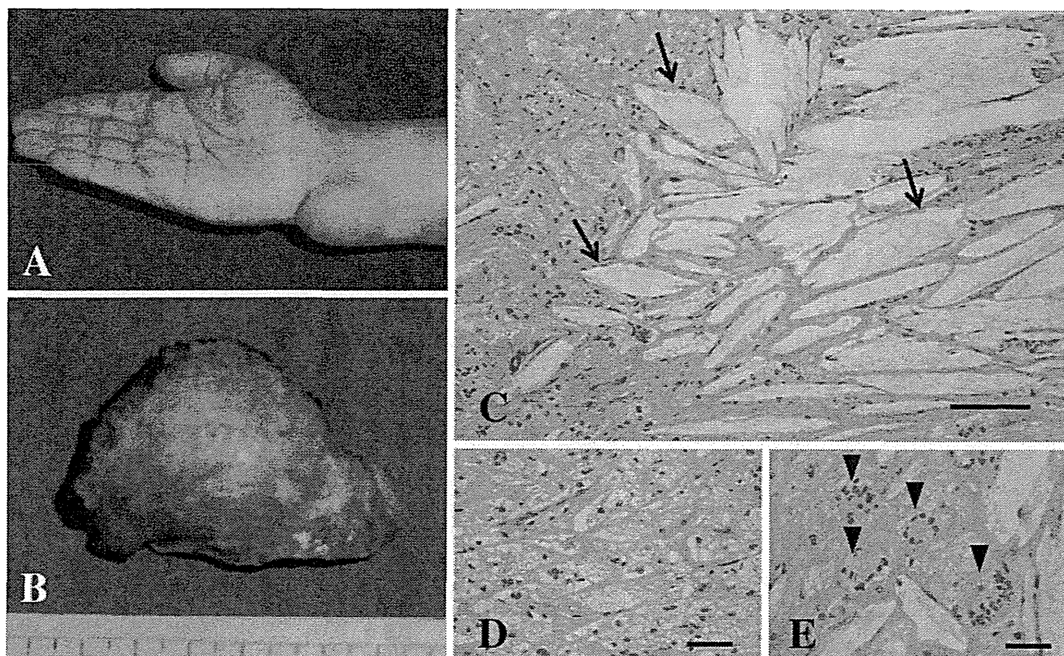

Figure 1. A nodular tumor on the ulnar side of the right wrist observed in 1999 (A). The resected tumor measured 6.0 cm × 3.5 cm in size and was yellowish and lustrous (B). A microscopic examination of the resected tumor revealed numerous extracellular cholesterol deposits (cholesterol clefts) (arrows in C) and diffuse sheets of foamy cells (xanthoma cells) (D) within fibrous tissue. Multinucleated giant cells were also conspicuous (arrowheads in E). The scale bar indicates 100 μm in C (magnification: ×40) and 50 μm in D, E (magnification: ×100).

left fingers. These subcutaneous tumors grew up to the size of a ping-pong ball or an egg. In 1999, at 41 years of age, the patient was admitted to the orthopedics department where some of the subcutaneous tumorous lesions were surgically resected (Fig. 1A, B). A histological examination of the resected specimens revealed numerous extracellular cholesterol (cholesterol clefts) and diffuse sheets of foamy cells (xanthoma cells) interspersed with inflammatory cells within fibrous tissue. Multinucleated giant cells were also conspicuous (Fig. 1C-E). A diagnosis of tendinous xanthomas was then made. The patient's serum LDL cholesterol level was 193 mg/dL at that time, and statin therapy was started thereafter. Meanwhile, however, he did not continue to visit the hospital regularly; therefore, the statin therapy was discontinued 12 years prior to the patient's admission to our hospital in 2010.

In 2007, at 50 years of age, the patient was again diagnosed as having hypercholesterolemia and diabetes mellitus at a routine health check-up; however, medical treatment was not started at that time. In October 2009, he began to experience chest oppression on exertion. The chest pain was associated with cold sweating and subsided when the patient was at rest for five minutes. In May 2010, the duration of chest pain became prolonged up to 20 to 30 minutes and the frequency increased up to three to four times per day. The patient visited a regional hospital where electrocardiogram showed ST-T segment changes suggestive of the presence of unstable angina pectoris. He was immediately hospitalized and an intravenous infusion of heparin sodium and isosorbide dinitrate was started, which markedly, but not completely, relieved his symptoms. He was then transferred to our hospital for further evaluation and treatment.

On admission, the patient's consciousness was clear, his body temperature was 36.8 degrees and his blood pressure was 108/72 mmHg. His heart sounds were normal and no abnormal heart murmurs were audible. No abnormal neurological findings were noted. Chest X-ray and electrocardiogram showed no significant abnormalities on admission. Multiple nodular subcutaneous tumors were observed on the dorsum of the bilateral hands, the bilateral wrists, the soles of the bilateral feet and the bilateral Achilles tendons (Fig. 2A, B). Severe thickening of the bilateral Achilles tendons (right side: 40 mm, left side: 30 mm) was confirmed on an X-ray examination (Fig. 2C, D). Echocardiography demonstrated that the aortic valve had three cusps, while valvular calcification was not significant.

The laboratory data obtained on admission are shown in the Table. At the time of admission, the patient began taking lipid- and glucose-lowering drugs, including rosuvastatin calcium (5.0 mg/d), metformin hydrochloride (750 mg/d), pioglitazone hydrochloride (15 mg/d) and glimepiride (3.0 mg/d). He is a former smoker with a Brinkman index of 390. The serum levels of sitosterol and campesterol measured by means of gas chromatography (SRL, Co., Ltd., Tokyo, Japan) were 1.2 μg/mL and 1.9 μg/mL, respectively, neither of which were elevated.

Coronary angiography showed multivessel coronary artery stenosis with 99% stenosis in the right coronary artery

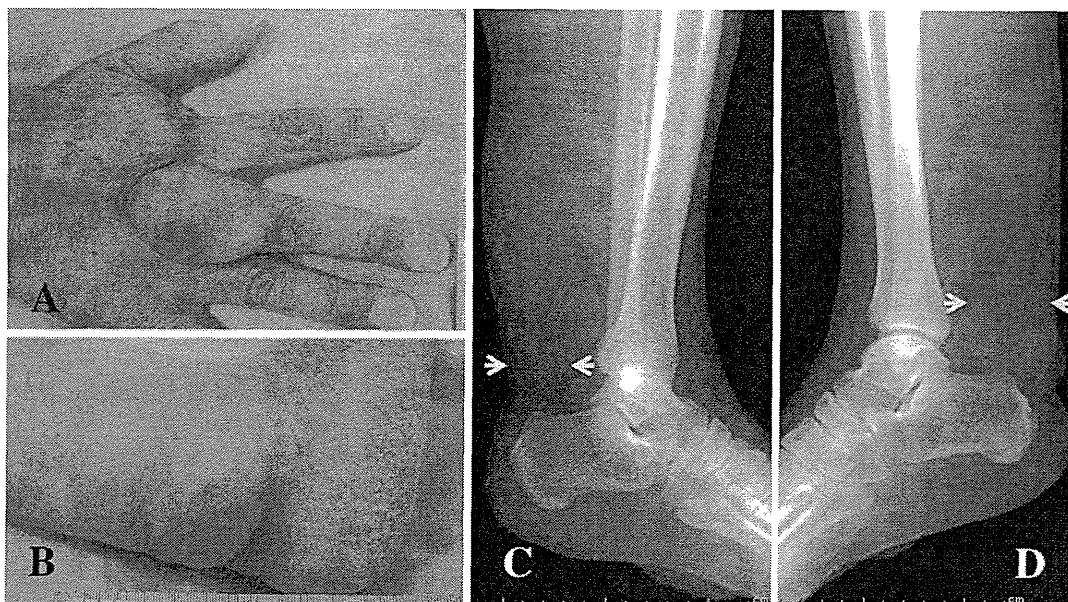

Figure 2. **Large nodular tumors on the back side of the right hand (A) and the right Achilles tendon (B) are presented. Radiography of the Achilles tendons showed severe thickening of the bilateral Achilles tendons [left side (arrows in C): 30 mm, right side (arrows in D): 40 mm].**

Table. **Results of Laboratory Tests on Admission and 1 Month after the Treatment with a Statin and Anti-hyperglycemic Agents**

	before	after	normal range
Total Cholesterol	282 mg/dL	137 mg/dL	(130-219)
LDL- Cholesterol	208 mg/dL	71 mg/dL	(70-139)
HDL- Cholesterol	45 mg/dL	41 mg/dL	(40-77)
Triglyceride	143 mg/dL	124 mg/dL	(30-149)
Apolipoprotein B	NE	79 mg/dL	(73-109)
Lipoprotein (a)	NE	23 mg/dL	(< 30)
Glucose	232 mg/dL	96 mg/dL	(60-109)
HbA1c (JDS)	9.3 %	6.2 %	(4.3-5.8)

NE: not examined, JDS: Japan Diabetes Society

(RCA), 90% stenosis in the left anterior descending artery (LAD) and 99% stenosis in the left circumflex artery (LCX) (Fig. 3A, B). No significant luminal stenosis was seen near the ostial lesions of the right or left coronary arteries. The motion of the left ventricle was within normal limits with an ejection fraction of 65.5%. Coronary artery bypass graft surgery was performed on the 14th hospital day. The left internal thoracic artery was anastomosed to the LAD and saphenous vein grafts were anastomosed to the LCX and RCA, which relieved the patient's chest symptoms on exertion.

The results of the activity and gene analysis (BML Co., Ltd., Saitama, Japan) of the LDL receptor (LDLR) were as follows: the LDL-receptor activity in lymphocytes was 137% (normal rage: >80%) and none of the six common LDLR gene mutations of E119K, C317S, 1847T/C, L547V, P664L and K790X were detected.

In addition, DNA sequencing of all of the exons of LDLR and proprotein convertase subtilisin/kexin type 9 (PCSK9) did not show any gene mutations. On the other hand, sequencing of low-density lipoprotein receptor adaptor protein 1 (LDLRAP 1), which is also known as autosomal recessive hypercholesterolemia (ARH), revealed two mutations. A new DNA sequence variation in LDLRAP1 was found, as follows: 604T > C and 654A > G. The frequency of 604T > C was 0.48/0.52 and that of 654A > G was 0.50/0.50 among 31 normal control subjects. The patient had the C/C variation in 604 and the G/G variation in 654. They were both considered to be single nucleotide polymorphisms. We also sequenced the apolipoprotein B (APOB) gene. The G to A mutation at nucleotide 10,708, the major cause of mutations in familial defective APOB, was not detected using asymmetric polymerase chain reaction (PCR) (3) in this patient. The nucleotides 10,564 to 10,884 on Exon 26 of the APOB gene were sequenced in this patient and found to be normal. The sequences of the primers used in this study for sequencing LDLRAP1 (4), LDLR, PCSK9 and APOB are described in the supplementary file.

Discussion

Currently, more than 1,100 mutations occurring at different sites on the LDLR gene in patients with FH have been reported (5). It has been reported that LDLR gene mutations can be identified in 67.8% of FH patients in the Japanese population (6). In the present case, on the other hand, we were unable to identify LDLR gene mutations by screening six commonly observed sites. Patients with these six mutations comprise approximately 30% of the total number of FH heterozygotes investigated in the Japanese population (6). Taking into consideration the finding that the LDLR activity in lymphocytes was within the normal range in this patient, a nonLDLR-mediated mechanism may underlie the pathophysiology in this patient, although the possibil-

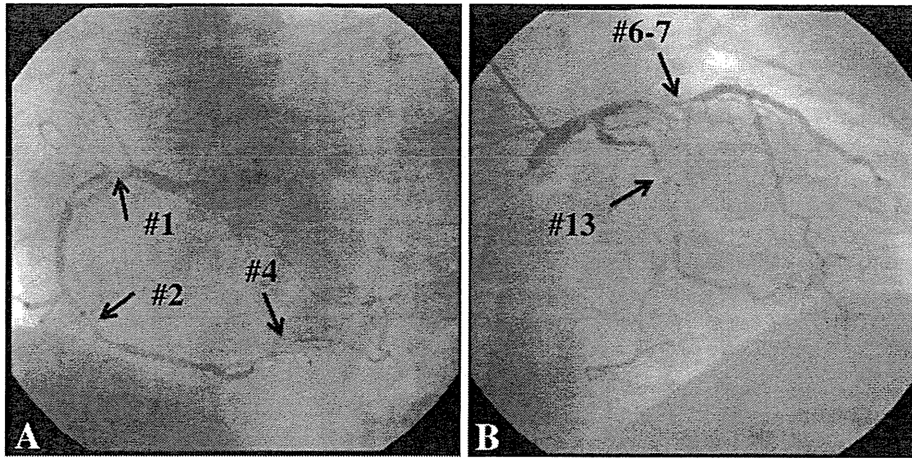

Figure 3. **Coronary angiography revealed severe multivessel disease with 99% stenosis at segment (#) 1, 90% stenosis at #2, 99% stenosis at #4 of the right coronary artery (A), 90% stenosis at #6-7 of the left anterior descending artery and 99% stenosis at #13 of the left circumflex artery (B).**

ity remains that a mutation of the LDLR gene other than these six mutations may have been present. However, on the other hand, compared with the LDLR gene mutation, fewer studies appear to have investigated the relationship between the LDLR genotype and its activity. A significant minority of patients (approximately 5%) who fulfill the criteria for FH with angiographically-proven coronary disease do not have a defective LDLR function or a detectable mutation in the LDLR gene (7).

Recently, several other genes that are candidates that may explain the FH phenotype have been reported, including APOB, PCSK9 and ARH (8). These nonLDLR mutations are relatively rare compared with the LDLR gene mutations whose prevalence of homozygote and heterozygote in FH are estimated to be $1/1\times10^6$ and 1/500, respectively. Additionally, the homozygote and heterozygote APOB are estimated to be $1/4\times10^6$ and 1/1,000, respectively, the heterozygote of PCSK9 is estimated to be <1/2,500 and the heterozygote of ARH is estimated to be $<1/5\times10^6$. In addition to searching for common LDLR mutations, we performed sequencing of the exons of the LDLR gene, the PCSK9 gene, the ARH gene and the APOB gene; however, no mutations that could explain the overt dyslipidemia observed in our patients were observed.

Xanthomas are a characteristic feature of FH and most usually measure a few centimeters in diameter (9). Multiple large tendinous xanthomas and advanced coronary artery atherosclerosis were noted in the present case. It has been reported that the presence of xanthomas increases the risk of cardiovascular disease in FH patients by as much as three times, suggesting that xanthomas and atherosclerosis may share a certain etiology (10). It has also been suggested that the severity of atherosclerosis and tissue lipid deposition, including the development of xanthomas and the width of the Achilles tendon, is correlated with the duration of hypercholesterolemia or the cholesterol-year score (11). It is not well known whether there is a correlation between the severity of the phenotype of FH, including multiple xanthomas and vascular diseases, and the genotype, including the sites of LDLR gene mutations. In our case, irrespective of the further extensive search for mutations in the LDLR, PCSK9, ARH and APOB genes, we were not able to identify any mutations in these genes that may potentially explain the patient's phenotype. Possible mechanisms, other than the genetic mutations examined in the current study, underlying prominent hypercholesterolemia, as observed in our patient, should be researched and identified in future studies.

The authors state that they have no Conflict of Interest (COI).

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Original Article

Defining Patients at Extremely High Risk for Coronary Artery Disease in Heterozygous Familial Hypercholesterolemia

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***Aim*: Heterozygous patients with familial hypercholesterolemia (FH) are known to be associated with a high risk of coronary artery disease (CAD), which is a major determinant of their clinical outcome. The prognosis of heterozygous FH patients substantially varies, being dependent on the level of their CAD risk, and their therapeutic regimen should be individualized. We assessed critical levels of LDL-cholesterol (LDL-C) and Achilles tendon thickness (ATT) to identify heterozygous FH patients at "very high" risk for CAD.**
***Methods*: One hundred and nine heterozygous FH patients who had no history of CAD and had had their plasma lipid profile and ATT assessed before treatment were followed up until their first CAD event or 31 December 2010. Multivariable logistic regression models were used to analyze the correlation of LDL-C and/or ATT levels with the risk of developing CAD.**
***Results*: During the follow-up period, 21 of the 109 patients had a CAD event, diagnosed by coronary angiogram. Individuals in the highest tertile of LDL-C had a CAD risk 8.29-fold higher than those in the lowest tertile. Individuals in the highest tertile of the ATT group had a 7.82-fold higher CAD risk than those in the lowest tertile. Those who had either LDL-C ≥260 mg/dL or ATT ≥14.5 had a 23.94-fold higher CAD risk than those with LDL-C <260 mg/dL and ATT <14.5 mm.**
***Conclusions*: In heterozygous FH patients, LDL-C 260 mg/dL or higher and/or ATT 14.5 mm or thicker are useful markers for extracting patients at "very high" risk for CAD.**

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Key words; Familial hypercholesterolemia, LDL cholesterol, Coronary artery disease, Coronary risk, Achilles tendon thickness

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by hypercholesterolemia, skin and tendon xanthomas and a high risk of coronary artery disease (CAD) due to premature atherosclerosis[1)]. FH has the highest prevalence in genetic metabolic diseases, showing one per 300 to 500 heterozygous patients in the general population[1, 2)]. High low-density lipoprotein cholesterol (LDL-C) is the first symptom, appearing in heterozygous FH even from birth, and xanthomas in the Achilles tendon usually appear during or after the late 10s and are found in half of all patients by the age of 30[1)]. Coronary artery disease (CAD), which determines the prognosis of FH patients, appears during or after the third decade of life in men and the fifth decade in women[3-5)].

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CAD mortality in heterozygous FH is several times higher than that in the general population[1, 6-8]; therefore, it is very important to prevent CAD in heterozygous FH patients. The prognosis of heterozygous patients of FH varies substantially, such that some develop CAD at their 20s while others may not develop CAD until their seventh decade; therefore, the therapeutic regimen should be individualized according to the patients' risk of CAD.

Various risk factors for CAD have been identified in heterozygous patients with FH, such as age, sex, LDL-C, triglyceride (TG), HDL-C, Achilles tendon thickness (ATT), smoking, a family history of CAD, hypertension, diabetes mellitus, Lp(a), homocysteine and so on[3, 9-12]. Among these parameters, LDL-C and ATT are simple, specific and non-invasive to measure, and can easily be used by primary care physicians to evaluate the CAD risk. We therefore estimated the CAD risks in accordance with LDL-C and ATT in heterozygous FH patients in order to identify those at extremely high risk.

Methods

Subjects

Of the patients referred to the lipid clinic at the National Cerebral and Cardiovascular Center (NCVC) from 1977 to 2007, 329 consecutive patients diagnosed as FH heterozygotes using previously described criteria[6] were subjected to this study. After diagnosis, the FH patients had medical checks according to the standard procedure for treating heterozygous FH in NCVC. The patients were subjected to a treadmill test for CAD screening just after their first visit to our clinic. Those who had a positive result on the treadmill test were subjected to a coronary angiogram (CAG), and diagnosed with CAD with 75% or more stenosis. Those who had a negative result on the treadmill or no significant stenosis by CAG were included in this study. Among the 329 FH patients, 229 were excluded: 53 had a past history of CAD, 160 had not had LDL-C measured before treatment, 76 had not had ATT thickness measured and 3 had TG more than 4.5 mmol/L, so 109 were followed up until their first CAD event or 31 December 2010. After the first visit to our clinic, dietary and drug treatment, including statins, was immediately started and continued.

During the course, those who had chest pain or a positive result on the treadmill test performed biennially were subjected to CAG, and diagnosed with CAD with 75% or more stenosis. Medical records of the patients were examined according to the analysis protocol approved by our institutional ethics committee (ID#M20-25-2).

Clinical and Laboratory Characteristics

Serum lipid and lipoprotein levels were measured at the time of initial diagnosis, before any lipid-lowering treatment. TC, TG and HDL-C levels were measured enzymatically with a commercial kit (Daiichi Pure Chemicals Co., Tokyo, Japan) using an automated analyzer (Hitachi model 704; Hitachi, Tokyo, Japan) in the clinical laboratory of the National Cerebral and Cardiovascular Center (NCVC). LDL-C was calculated by the Friedewald formula. ATT was measured by X-ray according to the method previously described[13]. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Hypertension was defined as the use of antihypertensive drugs or blood pressure ≥ 140 mmHg systolic or ≥ 90 mmHg diastolic or both at the first clinic visit (the criteria for hypertension of the Japanese Society of Hypertension Guidelines)[14]. Diabetes mellitus was defined according to the 2002 Guideline for the Treatment of Diabetes Mellitus of the Japanese Diabetes Society[15]. A family history of CAD was defined as positive by having within 2nd degree family members with CAD on the standardized questionnaire. Smoking was defined as positive by having a smoking habit at the first visit to NCVC on the patient report.

Statistical Analyses

Continuous variables are presented as the means ± SDs. 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.

Multivariable logistic regression analysis after adjusting for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD, and low HDL-C (<40 mg/dL) were used to analyze correlations of LDL-C levels or ATT levels and the development of CAD. LDL-C levels were categorized into tertiles: (1) LDL-C <206 mg/dL, (2) LDL ≥ 206 and <260 mg/dL, (3) LDL-C ≥ 260 mg/dL. ATT levels were also categorized into tertiles: (1) ATT <9.0 mm, (2) ATT ≥ 9.0 mm and <14.5 mm, (3) ATT ≥ 14.5 mm. All the confidence intervals were estimated at the 95% level and significance was set at $p<0.05$. All data were analyzed with the SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) statistical software package.

Table 1. Clinical characteristics of 109 patients with heterozygous FH classified with or without CAD

	Total $n=109$	CAD(−) $n=88$	CAD(+) $n=21$	p value
Age (years)	41.9±16.2	39.7±16.7	50.9±10.5	<0.01
Sex (male), n (%)	43 (39.4%)	30 (34.1%)	12 (57.1%)	0.052
Achilles tendon thickness (mm)	12.6±5.4	11.5±4.5	17.4±6.3	<0.0001
Skin xanthomas, n (%)	25 (22.9%)	16 (18.2%)	9 (42.9%)	0.052
Arcus cornea, n (%)	45 (41.3%)	27 (30.7%)	16 (76.2%)	0.001
Total cholesterol (mg/dL)	321±68	309±56	368±92	<0.001
Triglyceride (mg/dL)	139±82	134±85	156±65	0.272
HDL-C (mg/dL)	51±15	51±15	50±15	0.747
LDL-C (mg/dL)	242±70	232±59	287±92	0.001
Smoking (past or current), n (%)	42 (38.5%)	28 (31.8%)	14 (66.6%)	0.003
Hypertension, n (%)	19 (17.4%)	10 (11.4%)	9 (42.9%)	0.003
Diabetes mellitus, n (%)	9 (8.2%)	5 (5.7%)	4 (19.0%)	0.186
Family history of CAD, n (%)	47 (43.1%)	37 (42.0%)	10 (47.6%)	0.411

Table 2. Clinical characteristics at first visit in heterozygous patients of FH classified by LDL-C Levels (Mean±SD)

LDL-C (mg/dL) categories	LDL-C <206 $n=36$	206≦LDL-C<260 $n=36$	LDL-C ≥260 $n=37$	p value
Age (years)	43.7±15.6	42.0±17.5	40.0±15.8	0.645
Sex (male), n (%)	14 (38.9%)	13 (36.1%)	15 (40.5%)	0.928
Body mass index (kg/m^2)	22.2±3.3	22.5±3.2	22.8±6.8	0.880
Total cholesterol (mg/dL)	258±28	308±20	394±55	<0.001
Triglyceride (mg/dL)	149±102	134±67	134±74	0.672
HDL-C (mg/dL)	54±16	51±15	47±14	0.100
Smoking (past or current), n (%)	15 (41.7%)	10 (27.8%)	15 (40.5%)	0.385
Hypertension, n (%)	5 (13.9%)	6 (16.7%)	3 (8.1%)	0.660
Diabetes mellitus, n (%)	2 (5.6%)	3 (8.3%)	2 (5.4%)	0.831
Family history of CAD, n (%)	17 (47.2%)	14 (38.9%)	16 (43.2%)	0.775
Achilles tendon thickness (mm)	10.7±4.2	12.5±5.5	14.6±5.8	0.282
CAD, n (%)	5 (13.9%)	3 (8.3%)	13 (35.1%)	0.02

Results

Characteristics of the Patients Subjected to Analysis of the Correlations of LDL-C and CAD

Among 109 patients, 21 (19.3%) developed CAD during the subsequent period. There was a significantly higher prevalence of hypertension, skin xanthomas, arcus cornea and smoking in the CAD (+) group. Mean age, ATT, TC and LDL-C were significantly higher in the CAD (+) group than in the CAD (−) group (**Table 1**).

LDL-C Levels and Development of CAD

The clinical characteristics of patients categorized into tertiles according to their LDL-C levels are shown in **Table 2**. They clearly show that parameters other than TC levels were not significantly different in each tertile. Higher LDL-C was associated with higher TC and the incidence of CAD.

To examine the influence of conventional coronary risk factors, logistic regression analyses for CAD were performed. The multivariable adjusted odds ratios (ORs) for CAD are shown in **Table 3**. Individuals in the highest tertile (LDL-C ≥260 mg/dL) had a 8.29-fold increased risk of CAD incidence compared with those in the lowest tertile (LDL-C <206 mg/dL) (adjusted odds ratio (OR) 8.29, 95 % CI 1.33-51.47, $p=0.023$). No significant increase in the odds of future CAD in the second tertile (206≦LDL-C<260 mg/dL) (adjusted OR 0.42, 95%CI 0.05-3.26, $p=0.409$).

Table 3. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to LDL-C

LCL-C categories	n	Odds Ratio	95% CI	*p* value
LDL-C <206 mg/dL	36	1.0 (referent)	-	-
206≦LDL-C<260 mg/dL	36	0.42	0.05-3.26	0.409
LDL-C ≥260 mg/dL	37	8.29	1.33-51.47	0.023

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD, and low HDL-C (<40 mg/dL).

Table 4. Clinical characteristics at first visit in heterozygous patients of FH classified by ATT levels (mean ± SD)

ATT (mm) categories	ATT <9 *n*=36	9≦ATT<14.5 *n*=37	ATT ≥14.5 *n*=36	*p* value
Age (years)	39.7±18.3	39.4±16.4	45.2±13.5	0.177
Sex (male), n (%)	11 (30.6%)	13 (35.1%)	18 (50.0%)	0.207
BMI (kg/m²)	22.3±2.8	21.7±2.8	23.1±2.7	0.883
Total cholesterol (mg/dL)	293±42	319±66	350±79	0.002
Triglycerides (mg/dL)	140±106	134±69	142±67	0.505
HDL-C (mg/dL)	57±14	47±14	48±15	0.916
LDL-C (mg/dL)	208±44	245±67	274±78	0.003
Smoking habit, n (%)	9 (25.0%)	13 (35.1%)	14 (38.9%)	0.001
Hypertension, n (%)	4 (11.1%)	2 (5.4%)	8 (22.2%)	0.094
Diabetes mellitus, n (%)	1 (2.8%)	1 (2.7%)	5 (13.9%)	0.125
Family history of CAD, n (%)	16 (44.4%)	17 (46.0%)	14 (38.9%)	0.815
CAD, n (%)	2 (5.6%)	4 (10.8%)	15 (41.7%)	<0.001

ATT Levels and Development of CAD

The clinical characteristics of patients categorized into tertiles according to their ATT levels are shown in **Table 4**. Higher ATT levels were associated with higher TC and LDL-C levels, smoking and the incidence of CAD.

The multivariable adjusted OR for CAD is shown in **Table 5**. Individuals in the highest tertile group of ATT ≥14.5 mm had a 7.82-fold increased risk of CAD compared with those in the ATT <9.0 mm group (95%CI 1.28-47.7, p=0.001). No significant increase in the odds of future CAD in the group with 9≦ATT<14.5 mm (adjusted OR 1.42, 95%CI 0.18-11.14, p=0.740).

LDL-C and/or ATT Levels and Development of CAD

To estimate the future risk for CAD using the combination of LDL-C and ATT thickness, the patients were divided into 3 groups, (1) LDL-C <260 mg/dL and ATT <14.5 mm, (2) LDL-C <260 and ATT ≥14.5, or LDL-C ≥260 and ATT <14.5, (3) LDL-C ≥260 and ATT ≥14.5. OR for CAD was calculated for these groups and shown in **Table 6**. Those who had both LDL-C ≥260 and ATT ≥14.5 had a 20.62-fold increased risk of CAD compared with those with LDL-C <260 and ATT <14.5 (95%CI 2.91-145.89). Those with either LDL-C ≥260 or ATT ≥14.5 had a 23.62-fold increased risk of CAD compared with those with LDL-C <260 and ATT < 14.5 (95%CI 3.11-184.16).

Discussion

As the prognosis of heterozygous FH patients varies substantially, the therapeutic regimen should be determined according to the CAD risk of individual patients. High levels of LDL-C and ATT are clinical signs already found at a young age and can be measured easily and non-invasively by family physicians in primary care, so they can be good markers for estimating the future CAD risk of FH. In the present study, we demonstrated the critical levels of LDL-C and ATT for estimation of the CAD risk in Japanese heterozygous patients with FH.

Several studies on the Japanese population have indicated that the serum cholesterol level is correlated significantly with the risk of CAD[16, 17]. The increased CAD incidence seems exponential with the serum cholesterol level in the general population, and it can be

Table 5. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to ATT levels

ATT (mm) categories	n	Odds Ratio	95% CI	*p* value
ATT <9 mm	36	1.0 (referent)	–	–
9≦ATT<14.5 mm	37	1.42	0.18-11.14	0.740
ATT ≥ 14.5 mm	36	7.82	1.28-47.7	0.001

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD and low HDL-C (<40 mg/dL).

Table 6. Multivariate-adjusted odds ratio for CAD by logistic regression analyses according to both ATT and LDL-C levels

LDL-C and ATT categories	n	Odds Ratio	95% CI	*p* value
LDL-C <260, ATT <14.5 mm	54	1.0 (referent)	–	–
LDL-C <260, ATT ≥ 14.5 mm or LDL-C ≥ 260, ATT <14.5 mm	37	23.94	3.11-184.16	0.002
LDL-C ≥ 260, ATT ≥ 14.5 mm	18	20.62	2.91-145.89	0.002

Multivariable logistic regression models for CAD are adjusted for age, sex, hypertension, diabetes mellitus, smoking, family history of CAD and low HDL-C (<40 mg/dL).

considered low until it hits a certain "threshold". The findings of the relationship with LDL-C levels and the onset of CAD in FH patients seem to show a "right shift" of this profile as LDL-C 2-fold higher and CAD incidence more than 10-fold. Previous studies have reported that higher LDL-C is related with the higher risk factors for the development of CAD even in heterozygous FH patients[18, 19], whereas other factors, such as age, gender, hypertension, smoking, or other lipid abnormalities, such as low HDL-C and high TG reportedly contribute to the increased risk[3, 12, 18-22]. Bujo *et al.* reported that male gender, age over 50, smoking, hypertension, diabetes mellitus, TG >150 mg/dL and HDL-C <40 mg/dL were risk factors for CAD in FH by multicenter, cross-sectional analysis[3].

As we reported in a previous paper, drug treatment including statins may influence the outcome of CAD[5]. The name and dose of drugs prescribed to the patients during the course are listed in **Table 7**. Because all FH patients had had intensive drug therapy to prevent the development of atherosclerosis, no comparison could be made with those without drug therapy. It was also impossible to analyze the difference in drugs statistically because there were so many patterns of prescription and most patients changed the type and dose of drugs several times during the course.

LDL-C levels under drug treatment may also affect the outcome. The mean LDL-C under drug treatment did not increase the odds ratio for CAD (odds ratio: 0.983, 95%CI: 0.97-1.00); however the relationship between mean LDL-C in the pre-treatment period and CAD risk remained due to pre-exposure to high LDL-C before treatment, although the absolute risk of CAD might be decreased at any LDL-C level by intensive drug treatment during the course.

Table 7. Lipid-lowering drugs in heterozygous FH patients during the course

	Dose/day
cholestyramine	4-12 g
colestimide	0.5-3 g
probucol	250-1,000 mg
pravastatin	10-30 mg
simvastatin	5-20 mg
fluvastatin	20-60 mg
atorvastatin	5-40 mg
pitavastatin	1-4 mg
rosuvastatin	2.5-20 mg
fenofibrate	100-200 mg
bezafibrate	100-400 mg
ezetimibe	5-10 mg

Civerira *et al.* reported that heterozygous FH with tendon xanthomas has a 3.1-fold increased risk of premature CAD compared with those without it[23]. The Achilles tendon was reported to be thicker in FH patients with CAD than in those without CAD in both sexes[12]. Persistent high LDL-C causes cholesterol depositions in the tendons and results in tendon xanthomas[1]. Achilles tendon xanthomas have been used

as one of the criteria for clinical diagnosis of FH because of their high sensitivity and specificity[1, 24]. A strongly positive correlation was observed between ATT and cholesterol-year scores in FH patients[25, 26], suggesting that ATT reflects both the severity and duration of hypercholesterolemia. ATT is an important factor that can be measured quantitatively as the deposition of cholesterol in the tissue. The present study showed that ATT is a good marker for evaluating the risk for CAD, indicating that there is a strong correlation between the deposition of cholesterol in extravascular tissue and the stenosis of coronary arteries. ATT should be used not only as a diagnostic parameter for FH but also, and more importantly, as a prognostic factor that indicates the need for a more aggressive approach for patients at high risk.

In conclusion, LDL-C ≥260 mg/dL and ATT ≧14.5 mm or thicker are useful criteria for identifying patients at "very high" risk of CAD in Japanese heterozygous FH. Patients with either of these risk factors require more intensive cholesterol-lowering therapy and a more careful medical check-up for CAD.

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