[11] Costet P, Hoffmann MM, Cariou B, Guyomarc'h Delasalle B, Konrad T, Winkler K. Plasma PCSK9 is increased by fenofibrate and atorvastatin in a non-additive fashion in diabetic patients. Atherosclerosis 2010;212:246–51.
[12] Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. J Med Chem 2000;43:527–50.
[13] Okazaki M, Sasamoto K, Muramatsu T, Hosaki S. Evaluation of precipitation and direct methods for HDL-cholesterol assay by HPLC. Clin Chem 1997;43:1885–90.
[14] Labeur C, Shepherd J, Rosseneu M. Immunological assays of apolipoproteins in plasma: methods and instrumentation. Clin Chem 1990;36:591–7.
[15] Arita Y, Kihara S, Ouchi N, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 1999;257:79–83.
[16] Hosoda K, Masuzaki H, Ogawa Y, et al. Development of radioimmunoassay for human leptin. Biochem Biophys Res Commun 1996;221:234–9.
[17] Dubuc G, Tremblay M, Pare G, et al. A new method for measurement of total plasma PCSK9: clinical applications. J Lipid Res 2010;51:140–9.
[18] Cariou B, Le Bras M, Langhi C, et al. Association between plasma PCSK9 and gamma-glutamyl transferase levels in diabetic patients. Atherosclerosis 2010;211:700–2.
[19] Lakoski SG, Lagace TA, Cohen JC, Horton JD, Hobbs HH. Genetic and metabolic determinants of plasma PCSK9 levels. J Clin Endocrinol Metab 2009;94:2537–43.
[20] Mbikay M, Sirois F, Mayne J, et al. PCSK9-deficient mice exhibit impaired glucose tolerance and pancreatic islet abnormalities. FEBS Lett 2010;584:701–6.
[21] Tenenbaum A, Motro M, Fisman EZ, Tanne D, Boyko V, Behar S. Bezafibrate for the secondary prevention of myocardial infarction in patients with metabolic syndrome. Arch Intern Med 2005;165:1154–60.
[22] Goldenberg I, Boyko V, Tennenbaum A, Tanne D, Behar S, Guetta V. Long-term benefit of high-density lipoprotein cholesterol-raising therapy with bezafibrate: 16-year mortality follow-up of the bezafibrate infarction prevention trial. Arch Intern Med 2009;169:508–14.
[23] Scott R, O'Brien R, Fulcher G, et al. Effects of fenofibrate treatment on cardiovascular disease risk in 9,795 individuals with type 2 diabetes and various components of the metabolic syndrome: the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study. Diabetes Care 2009;32:493–8.
[24] Lambert G, Ancellin N, Charlton F, et al. Plasma PCSK9 concentrations correlate with LDL and total cholesterol in diabetic patients and are decreased by fenofibrate treatment. Clin Chem 2008;54:1038–45.
[25] Chan DC, Hamilton SJ, Rye KA, et al. Fenofibrate concomitantly decreases serum proprotein convertase subtilisin/kexin type 9 and very-low-density lipoprotein particle concentrations in statin-treated type 2 diabetic patients. Diabetes Obes Metab 2010;12:752–6.
[26] Welder G, Zineh I, Pacanowski MA, Troutt JS, Cao G, Konrad RJ. High-dose atorvastatin causes a rapid sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol. J Lipid Res 2010;51:2714–21.
[27] Castro Cabezas M, de Bruin TW, de Valk HW, Shoulders CC, Jansen H, Willem Erkelens D. Impaired fatty acid metabolism in familial combined hyperlipidemia. A mechanism associating hepatic apolipoprotein B overproduction and insulin resistance. J Clin Invest 1993;92:160–8.
[28] De Man FH, Cabezas MC, Van Barlingen HH, Erkelens DW, de Bruin TW. Triglyceride-rich lipoproteins in non-insulin-dependent diabetes mellitus: post-prandial metabolism and relation to premature atherosclerosis. Eur J Clin Invest 1996;26:89–108.
[29] Ginsberg HN. Insulin resistance and cardiovascular disease. J Clin Invest 2000;106:453–8.
[30] Rashid S, Watanabe T, Sakaue T, Lewis GF. Mechanisms of HDL lowering in insulin resistant, hypertriglyceridemic states: the combined effect of HDL triglyceride enrichment and elevated hepatic lipase activity. Clin Biochem 2003;36:421–9.
[31] Nagasawa T, Inada Y, Nakano S, et al. Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARdelta agonist, on development of steatohepatitis in mice fed a methionine- and choline-deficient diet. Eur J Pharmacol 2006;536:182–91.
[32] Goa KL, Barradell LB, Plosker GL. Bezafibrate. An update of its pharmacology and use in the management of dyslipidaemia. Drugs 1996;52:725–53.
[33] Tenenbaum A, Motro M, Fisman EZ. Dual and pan-peroxisome proliferator-activated receptors (PPAR) co-agonism: the bezafibrate lessons. Cardiovasc Diabetol 2005;4:14.
[34] Eisenberg S, Gavish D, Oschry Y, Fainaru M, Deckelbaum RJ. Abnormalities in very low, low and high density lipoproteins in hypertriglyceridemia. Reversal toward normal with bezafibrate treatment. J Clin Invest 1984;74:470–82.
[35] Keating GM, Croom KF. Fenofibrate: a review of its use in primary dyslipidaemia, the metabolic syndrome and type 2 diabetes mellitus. Drugs 2007;67:121–53.
[36] Lee CH, Olson P, Evans RM. Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. Endocrinology 2003;144:2201–7.
[37] Ogawa S, Takeuchi K, Sugimura K, et al. Bezafibrate reduces blood glucose in type 2 diabetes mellitus. Metabolism 2000;49:331–4.
[38] Tenenbaum A, Motro M, Fisman EZ, et al. Peroxisome proliferator-activated receptor ligand bezafibrate for prevention of type 2 diabetes mellitus in patients with coronary artery disease. Circulation 2004;109:2197–202.
[39] Tenenbaum A, Motro M, Fisman EZ, et al. Effect of bezafibrate on incidence of type 2 diabetes mellitus in obese patients. Eur Heart J 2005;26:2032–8.
[40] Tenenbaum H, Behar S, Boyko V, et al. Long-term effect of bezafibrate on pancreatic beta-cell function and insulin resistance in patients with diabetes. Atherosclerosis 2007;194:265–71.
[41] Damci T, Tatliagac S, Osar Z, Ilkova H. Fenofibrate treatment is associated with better glycemic control and lower serum leptin and insulin levels in type 2 diabetic patients with hypertriglyceridemia. Eur J Intern Med 2003;14:357–60.
[42] Tan CE, Chew LS, Tai ES, et al. Benefits of micronised Fenofibrate in type 2 diabetes mellitus subjects with good glycemic control. Atherosclerosis 2001;154:469–74.
[43] Asano A, Kobayashi J, Murase Y, et al. Effects of fenofibrate therapy on plasma ubiquinol-10 and ubiquinone-10 levels in Japanese patients with hyperlipidemia and type 2 diabetes mellitus. Pharmacotherapy 2006;26:447–51.
[44] Flory JH, Ellenberg S, Szapary PO, Strom BL, Hennessy S. Antidiabetic action of bezafibrate in a large observational database. Diabetes Care 2009;32:547–51.
[45] Costet P, Cariou B, Lambert G, et al. Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. J Biol Chem 2006;281:6211–8.
[46] Koh KK, Quon MJ, Han SH, et al. Additive beneficial effects of fenofibrate combined with atorvastatin in the treatment of combined hyperlipidemia. J Am Coll Cardiol 2005;45:1649–53.
[47] Koh KK, Han SH, Quon MJ, Yeal Ahn J, Shin EK. Beneficial effects of fenofibrate to improve endothelial dysfunction and raise adiponectin levels in patients with primary hypertriglyceridemia. Diabetes Care 2005;28:1419–24.
[48] Hiuge A, Tenenbaum A, Maeda N, et al. Effects of peroxisome proliferator-activated receptor ligands, bezafibrate and fenofibrate, on adiponectin level. Arterioscler Thromb Vasc Biol 2007;27:635–41.
[49] Mori Y, Oana F, Matsuzawa A, Akahane S, Tajima N. Short-term effect of bezafibrate on the expression of adiponectin mRNA in the adipose tissues: a study in spontaneously type 2 diabetic rats with visceral obesity. Endocrine 2004;25:247–51.
[50] Kita R, Takamatsu S, Kimura T, Kokuryu H, Osaki Y, Tomono N. Bezafibrate may attenuate biliary damage associated with chronic liver diseases accompanied by high serum biliary enzyme levels. J Gastroenterol 2006;41:686–92.
[51] Noguchi Y, Tatsuno I, Suyama K, et al. Effect of fenofibrate on uric acid metabolism in Japanese hyperlipidemic patients. J Atheroscler Thromb 2004;11:335–40.
[52] Endo K, Miyashita Y, Sasaki H, et al. Probucol and atorvastatin decrease urinary 8-hydroxy-2′-deoxyguanosine in patients with diabetes and hypercholesterolemia. J Atheroscler Thromb 2006;13:68–75.

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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.

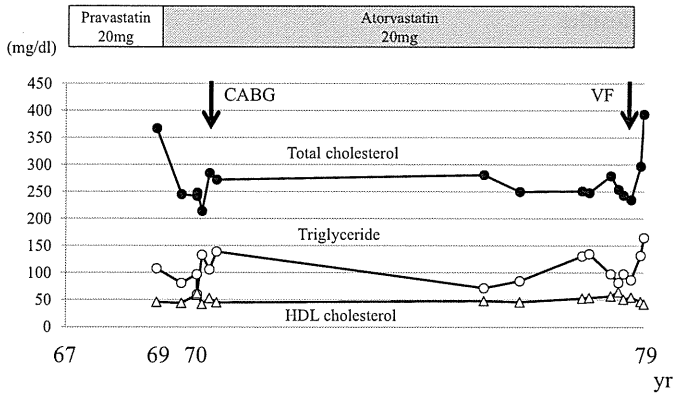

Fig. 1. Clinical course of the proband. Plasma concentration of the total cholesterol (solid circle), triglyceride (open circle), and HDL-C (open triangle) in the proband, and the major clinical events were illustrated. CABG; coronary artery bypass grafting, VF; ventricular fibrillation.

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.

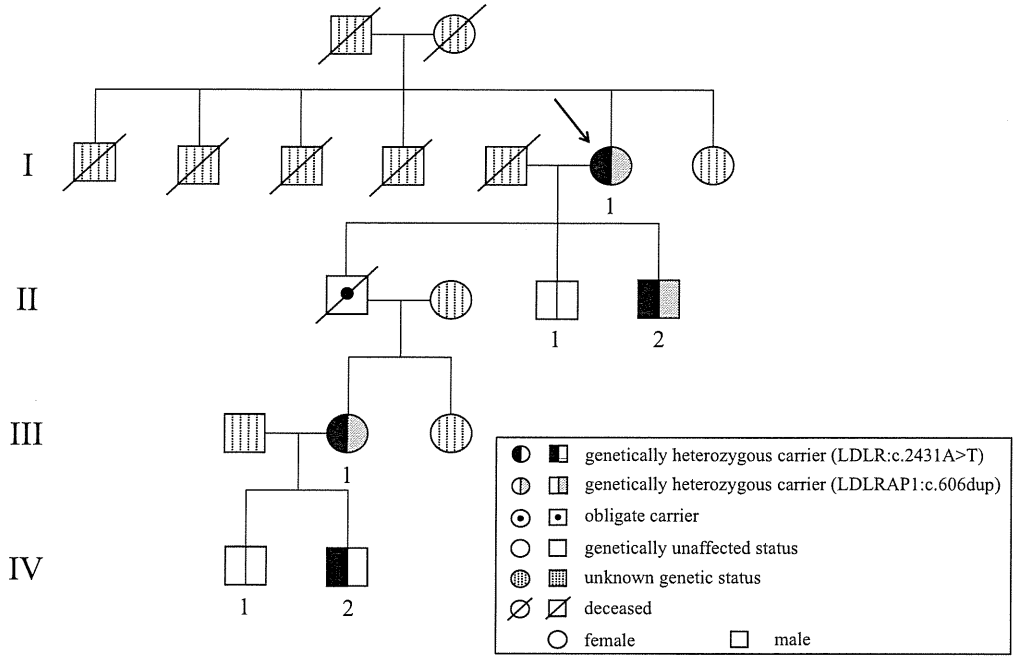

Fig. 2. Pedigree of the proband. Half-filled by black squares or circles indicate the heterozygous mutation carrier in LDLR (c.2431A > T). Half-filled by brown squares or circles indicate the heterozygous mutation carrier in LDLRAP1 (c.606dup). Square with a dot indicates the obligate carrier. Open squares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

References

[1] Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease, 8the ed., vol. 2. New York: McGraw-Hill; 2001. p. 2863–913.
[2] Soutar AK, Naoumova RP. Mechanisms of disease: genetic causes of familial hypercholesterolemia. Nat Clin Pract Cardiovasc Med 2007;4:214–25.
[3] Noguchi T, Katsuda S, Kawashiri MA, et al. The E32K variant of PCSK9 exacerbates the phenotype of familial hypercholesterolemia by increasing PCSK9 function and concentration in the circulation. Atherosclerosis 2010;210:166–72.
[4] Harada-Shiba M, Tajima S, Yokoyama S, et al. Siblings with normal LDL receptor activity and severe hypercholesterolemia. Arterioscler Thromb 1992;12:1071–8.
[5] Garcia CK, Wilund K, Arca M, et al. Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. Science 2001;292:1394–8.
[6] Pisciotta L, Oliva CP, Pes GM, et al. Autosomal recessive hypercholesterolemia (ARH) and homozygous familial hypercholesterolemia (FH): a phenotypic comparison. Atherosclerosis 2006;188:398–405.
[7] Harada K, Miyamoto Y, Morisaki H, et al. A novel Thr56Met mutation of the autosomal recessive hypercholesterolemia gene associated with hypercholesterolemia. J Atheroscler Thromb 2010;17:131–40.
[8] Daskalopoulou SS. When statin therapy stops: implications for the patient. Curr Opin Cardiol 2009;24:454–60.
[9] McGowan MP, Treating to New Target (TNT) Study Group. There is no evidence for an increase in acute coronary syndromes after short-term abrupt discontinuation of statins in stable cardiac patients. Circulation 2004;110:2333–5.
[10] Kiyohara T, Kiriyama R, Zamma S, et al. Enzyme immunoassay for cholesteryl ester transfer protein in human serum. Clin Chim Acta 1998;271:109–18.
[11] Barbagallo CM, Emmanuele G, Cefalù AB, et al. Autosomal recessive hypercholesterolemia in a Sicilian kindred harboring the 432insA mutation of the ARH gene. Atherosclerosis 2003;166:395–400.
[12] Inazu A, Jiang XC, Haraki T, et al. Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. J Clin Invest 1994;94:1872–82.
[13] Tada H, Kawashiri MA, Noguchi T, et al. A novel method for determining functional LDL receptor activity in familial hypercholesterolemia: application of the CD3/CD28 assay in lymphocytes. Clin Chim Acta 2009;400:42–7.
[14] Tada H, Kawashiri MA, Noguchi T, et al. Clinical impact of heterozygous carrier of autosomal recessive hypercholesterolemia on asymptomatic hyperlipidemic patients: evidence from familial gene analysis. Circulation 2008;118:S405.
[15] Inazu A, Mabuchi H. High-HDL syndrome. In: Ganten D, Ruckpaul K, editors. Encyclopedic reference of genomic and proteomics in molecular medicine, 1st ed., part 8. Springer; 2006. p. 792–5.

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The E32K variant of PCSK9 exacerbates the phenotype of familial hypercholesterolaemia by increasing PCSK9 function and concentration in the circulation

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ABSTRACT

Objective: Proprotein convertase subtilisin/kexin type 9 (PCSK9) regulates cholesterol trafficking by mediating degradation of cell-surface LDL receptors (LDLR). Gain-of-function *PCSK9* mutations are known to increase plasma LDL-C levels. We attempted to find gain-of-function *PCSK9* mutations in Japanese subjects and determine the frequency and impacts of these mutations, especially on circulating PCSK9 and LDL-C levels.

Methods: PCR-SSCP followed by direct sequence analysis was performed for all 12 exons and intronic junctions of the *PCSK9* in 55 subjects with clinically diagnosed familial hypercholesterolaemia (clinical-FH), who were confirmed to have no *LDLR* mutations. Among the mutations detected, *PCSK9* E32K was likely to be a gain-of-function mutation, and screening was performed by PCR-RFLP in clinical-FH and general Japanese controls. The levels of PCSK9 in plasma from subjects and in media of HepG2 cells transfected with PCSK9 constructs were measured by ELISA.

Results: We detected 7 *PCSK9* variants, including E32K. The frequency of *PCSK9* E32K in clinical-FH (6.42%) was significantly higher than that in controls (1.71%). Three cases representing homozygous FH phenotypes were double heterozygous for *PCSK9* E32K and *LDLR* C183S, C292X or K790X. Two cases were true homozygous for *PCSK9* E32K; to our knowledge, these are the first true homozygotes for gain-of-function *PCSK9* mutations reported to date. The *PCSK9* E32K mutant had over 30% increased levels of PCSK9 in plasma from the subjects and in media of transiently transfected HepG2 cells as compared with those in controls. Furthermore, LDL-C levels in the *PCSK9* E32K true homozygotes and heterozygotes were 2.10- and 1.47-fold higher than those in controls with comparable circulating PCSK9 levels, respectively, suggesting enhanced function of *PCSK9* E32K.

Conclusions: We found 2 true homozygotes for *PCSK9* E32K and 3 double heterozygotes for *PCSK9* E32K and *LDLR* mutations associated with autosomal dominant hypercholesterolaemia. This study provided evidence that *PCSK9* E32K significantly affects LDL-C levels *via* increased mass and function of PCSK9, and could exacerbate the clinical phenotypes of patients carrying *LDLR* mutations.

1. Introduction

Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) is the ninth member of the subtilisin-like serine convertase superfamily [1,2]. PCSK9 regulates plasma levels of LDL-cholesterol (LDL-C) by directing cell-surface LDL receptors (LDLR) to the lysosomes for degradation, resulting in reduced clearance and accumulation of LDL-C in the circulation [3]. *PCSK9* mutations that increase the degradation of LDLR are referred to as gain-of-function mutations and cause autosomal dominant hypercholesterolaemia (ADH) and premature coronary artery disease (CAD) [4]. Conversely, loss-of-function mutations in *PCSK9*, which decrease LDLR degradation, are associated with hypocholesterolaemia and less frequencies of CAD [5,6].

In the Japanese population, 60–70% of cases of ADH are caused by *LDLR* mutation, and no apolipoprotein B mutations have been identified to date [7,8]. Thus, gain-of-function *PCSK9* mutations could explain a part of the remaining 30% of ADH cases in the Japanese population.

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The only known function of PCSK9 as a convertase is in autocatalytic processing at the VFAQ152↓SIP site [2], and unlike the other proprotein convertases, PCSK9 does not appear to undergo a second cleavage event to release an active convertase [9,10]. The prodomain of PCSK9, which binds non-covalently to the mature convertase, acts as a chaperone to assist the folding of the mature convertase and also blocks access to the catalytic site of the mature convertase [1]. It is suggested from these mechanisms that mutations in the prodomain of *PCSK9* can markedly affect the function of PCSK9; in fact, many gain-of-function mutations and loss-of-function mutations in the prodomain region have been reported with almost the same frequency as those in the catalytic domain and C-terminal region [11–13].

Enzyme-linked immunoassays for measuring plasma PCSK9 have been established recently, and circulating levels of PCSK9 as well as sequence variations in *PCSK9* have been shown to affect plasma levels of LDL-C [4,14]. However, the relationship between mutations and circulating PCSK9 levels *in vivo* has not been fully elucidated [15,16]. Characterisation of the effects of naturally occurring gain-of-function mutations in *PCSK9* on circulating PCSK9 and LDL-C levels may provide important insight into the mechanism by which PCSK9 degrades LDLR.

In this study, we identified a gain-of-function *PCSK9* mutation in the prodomain region and clarified their lipid profiles and circulating PCSK9 levels in comparison to *LDLR* mutation heterozygotes. We also present data on gain-of-function *PCSK9* mutation true homozygotes and double heterozygotes with *LDLR* mutations, providing meaningful perspectives regarding the function of PCSK9.

2. Materials and methods

2.1. Subjects

In this study, we analysed 514 unrelated patients with clinically diagnosed familial hypercholesterolaemia (clinical-FH) attending the lipid clinic of Kanazawa University Hospital or the affiliated clinics in the Hokuriku district and 351 general Japanese men attending the medical clinic for their annual health examinations in the same district as controls. The diagnosis of FH was made according to the following criteria: (1) primary hypercholesterolaemia (total cholesterol above 230 mg/dL) with tendon xanthomas or (2) primary hypercholesterolaemia with and without tendon xanthomas in first-degree relatives of hypercholesterolaemic patients [17].

Screening was performed in the 514 subjects with clinical-FH, and 262 (51.0%) were confirmed to have mutations in *LDLR*. The two most common *LDLR* mutations were c.2431A>T mutation in exon 17 (K790X, 16.8%) and c.2312-3C>A mutation in the splice acceptor region of intron 15 (IVS15-3C/A, 4.5%). Of the remaining 252 subjects whose mutations were unidentified, 55 subjects were randomly assigned to a *PCSK9* mutation screening study to detect gain-of-function *PCSK9* mutations. Among the sequence variations detected, the relatively common c.94G>A variant (E32K) in the prodomain region of *PCSK9* was likely to be a gain-of-function mutation, and a larger scale screening was performed by restriction fragment length polymorphism (RFLP) in 514 clinical-FH and 351 general Japanese controls to determine the frequency of the *PCSK9* E32K variant. Relatives of *PCSK9* E32K carriers were further screened for *PCSK9* E32K and *LDLR* mutations.

2.2. Lipids and PCSK9 measurements

Serum total cholesterol (TC), triglyceride (TG) and HDL-cholesterol (HDL-C) concentrations were determined at accredited clinical laboratories using routine clinical methods. LDL-cholesterol (LDL-C) concentrations were calculated using the Friedewald equation as there were no subjects with serum triglycerides >400 mg/dL in the present study population. To distinguish the effects of gene mutations on circulating PCSK9 and LDL-C levels, plasma PCSK9 concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit targeting human PCSK9 (CycLex, Nagano, Japan) in 2 *PCSK9* E32K true homozygotes, 15 *PCSK9* E32K heterozygotes, 30 *LDLR* mutation heterozygotes (20 K790X and 10 IVS15-3C/A) and 20 control subjects. Lipid profile analysis and PCSK9 ELISA were performed using fasting blood samples collected when subjects were not taking any lipid-lowering drugs. Written informed consent was obtained from each of the subjects prior to participation in the study. The study protocol was approved by the Ethics Committee of the Graduate School of Medical Science, Kanazawa University.

2.3. DNA analysis

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 32 point mutations previously identified in Japan, the multiplex ligation-dependent probe amplification (MLPA) method for large rearrangements using a P062B LDLR MLPA kit (MRC Holland, Amsterdam, Netherlands) and DNA sequencing method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) for the other mutations. MLPA and direct sequencing were performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Mutations in *PCSK9* were detected by polymerase chain reaction (PCR) single-strand conformational polymorphism (SSCP) followed by direct sequence analysis. SSCP analysis was performed as described previously [18]. *PCSK9* E32K mutation in exon 1 of the PCSK9 gene was determined using a PCR-RFLP method with primers 5′-TGAACTTCAGCTCCTGCACA-3′ and 5′-AACGCAAGGCTAGCACCA-3′. PCR products were digested at 37 °C overnight with 1 U of the restriction enzyme BslI (New England Biolabs, Ipswich, MA, USA). RFLP assay was designed such that the normal *PCSK9* sequence would be cut twice to generate fragments of 34, 49 and 171 bp and the E32K sequence would be cleaved once to generate fragments of 34 and 220 bp, to check that the restriction enzyme was digesting properly. PCR conditions for SSCP, RFLP and DNA sequencing were as follows. Each 25-mL reaction mixture contained 60 ng of DNA, 10 pmol of each primer, 0.25 mM of each dNTP and 1 U Taq polymerase (Biotech International, Perth, Australia) in PCR buffer containing 1.5 mM $MgCl_2$. Cycling conditions were 95 °C for 5 min followed by 5 cycles of step-down PCR consisting of 95 °C for 5 s, annealing temperature +5 °C for 30 s (decrease 1 °C each cycle) and 72 °C for 1 min, then 30 cycles of 95 °C for 5 s, annealing temperature for 30 s and 72 °C for 1 min, with a final extension for 5 min at 72 °C.

2.4. In vitro expression of PCSK9

The WT-PCSK9 plasmid (pCMV-PCSK9-FLAG) containing the sequence of the FLAG epitope tag fused to the 3′ end of the PCSK9 coding sequence, was a generous gift from Dr. Jay D. Horton, University of Texas Southwestern Medical Center, Dallas, TX, USA. To make the E32K-PCSK9 construct, PCR fragments containing the *PCSK9* E32K mutant sequence were generated from the genomic DNA of a *PCSK9* E32K homozygote with the primers used for RFLP.

Table 1
Distribution of *PCSK9* E32K mutation in a general Japanese population by LDL-cholesterol quintile.

	LDL-C quintile					Total (n=351)	ANOVA P value
	1 (n=69) LDL-C 47.8–88.1	2 (n=70) LDL-C 88.2–104.3	3 (n=72) LDL-C 104.4–122.5	4 (n=70) LDL-C 122.6–138.9	5 (n=70) LDL-C 139.0–222.4		
Number of PCSK9 E32K carrier	0	0	0	4	2	6	
%	0	0	0	5.71	2.86	1.71	
Age (years)	43.3±12.2	47.3±9.1	43.1±9.4	46.8±9.8	49.0±7.4	45.9±9.9	<0.001
TC (mg/dL)	146.3±18.0	170.5±13.9	184.6±14.0	203.1±12.3	225.2±19.4	186.0±31.2	<0.001
TG (mg/dL)	111.2±73.9	118.2±59.0	112.3±62.9	110.2±46.4	125.6±53.3	115.5±59.7	0.517
HDL-C (mg/dL)	51.9±11.6	50.3±14.0	49.9±11.7	51.4±11.4	46.0±11.2	49.9±12.1	<0.05
LDL-C (mg/dL)	72.2±10.9	96.5±5.4	112.2±5.4	129.7±4.4	154.1±14.0	113.0±29.2	<0.001

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.

Amplified fragments and pCMV-PCSK9-FLAG were digested at 37 °C overnight with the restriction enzymes NheI and SacII (New England Biolabs), purified with a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and ligated using T4 DNA ligase (Nippon Gene, Toyama, Japan). The whole coding sequence of the E32K-PCSK9 plasmid except for the G to A substitution at the position of E32K was confirmed to be identical to the WT-PCSK9 plasmid by DNA sequencing method. An empty plasmid, pcDNA3.1/myc his-c (Invitrogen, Carlsbad, CA, USA) was used as a control in the transfection experiments.

HepG2 cells obtained from the Health Science Research Resources Bank (HSRRB) were cultured in Dulbecco's modified Eagle's medium with low glucose and L-alanyl-glutamine (Gibco, Carlsbad, CA, USA) containing 1× penicillin-streptomycin solution (Wako Pure Chemical Industries, Osaka, Japan) and 10% foetal bovine serum (Gibco) in a humidified atmosphere (37 °C, 5% CO_2). HepG2 cells were then transiently transfected with the different PCSK9 constructs or empty plasmid using Lipofectamine 2000 Reagent (Invitrogen) in accordance with the manufacturer's instructions for the 24-well plates. PCSK9 levels in the media were determined using an ELISA kit after 24 h of incubation. The experiment was repeated 3 times with 4 wells in each group. Data were collected as the ratio to the WT-PCSK9 plasmid, and mean values were calculated.

2.5. Statistics

All data in the text and figures are expressed as means ± S.D. The frequency distribution of genotype was compared using standard χ^2 test. For multiple comparisons, the Tukey–Kramer HSD test was performed for variables with $P<0.05$ on F-test in one-way ANOVA. Linear correlations were analysed using Pearson's correlation coefficient analysis. 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. Identification of the LDLR and PCSK9 mutations

Screening of *LDLR* mutations in 514 clinical-FH subjects resulted in detection of 52 point mutations and 11 large rearrangements in 210 and 52 patients, respectively. Twenty-two of 55 randomly selected subjects who were free from *LDLR* mutations had the following *PCSK9* sequence variants: c.61_63dupCTG (L21dup, 8 heterozygotes), c.94G>A (E32K, 1 true homozygote and 2 heterozygotes), c.158C>T (A53V, 1 heterozygote), c.787G>A (G263S, 2 heterozygotes), c.1420A>G (I474V, 4 heterozygotes), c.2004C>A (S668R, 1 heterozygote) and c.2009A>G (E670G, 3 heterozygotes). Silent mutations that do not result in a change in the amino acid sequence of the protein product or occur within intronic regions were omitted. The *PCSK9* E32K true homozygote showed markedly higher plasma LDL-C level than the 2 heterozygotes (339 mg/dL *vs.* 222 and 248 mg/dL).

3.2. Screening of E32K mutation in exon 1 of the PCSK9 gene

Genotyping of the *PCSK9* E32K mutation in the general population indicated that *PCSK9* E32K occurred in 1.71% of subjects (n=6) and was detected in the highest and the second highest quintiles divided according to LDL-C level (Table 1). The frequency of *PCSK9* E32K variants in clinical-FH was significantly higher (6.24%, $P<0.01$) than that in the general population. Further analysis in the relatives of *PCSK9* E32K carriers showed that LDL-C concentrations in *PCSK9* E32K carriers were higher than those in the general population and lower than those in *LDLR* mutation heterozygotes (Fig. 1). The *PCSK9* E32K variant was associated with a 75% increase in LDL-C (197 mg/dL *vs.* 113 mg/dL, $P<0.01$) and a 31% increase in triglyceride (TG, 151 mg/dL *vs.* 115 mg/dL, $P<0.01$), and did not influence HDL-C level (Table S1).

3.3. PCSK9 E32K true homozygote and double heterozygote with LDLR mutation

There were 5 rare cases among the *PCSK9* E32K carriers in clinical-FH and their relatives as follows: subject I, a true homozygote for the *PCSK9* E32K with the highest LDL-C level in her family pedigree at 339 mg/dL (Fig. 2A); subject II, another *PCSK9* E32K true homozygote with a modest increase in LDL-C level at 246 mg/dL; subject III, a double heterozygote for c.611G>C (C183S) mutation of *LDLR* and *PCSK9* E32K (age: 1 year, LDL-C: 581 mg/dL and severe cutaneous xanthomatosis, Fig. 2C); subject IV, a double heterozygote for c.939C>A (C292X) mutation of *LDLR* and *PCSK9* E32K (age: 30 years, LDL-C: 425 mg/dL and severe tendon xanthomatosis); subject V, a double heterozygote for *LDLR* K790X and *PCSK9* E32K (age: 2 years, LDL-C: 248 mg/dL and severe cutaneous xanthomatosis, Fig. 2D). No mutations in *LDLR* were detected in *PCSK9* E32K carriers except for the 3 double heterozygotes described above.

3.4. Analysis of circulating PCSK9 levels in PCSK9 E32K carriers and LDLR mutation heterozygotes

Plasma PCSK9 levels in the *PCSK9* E32K heterozygotes were significantly higher than those in controls (349 ± 90 ng/mL *vs.* 266 ± 112 ng/mL, $P<0.05$), and those in *PCSK9* E32K true homozygotes (363 ng/mL) were similar to those in heterozygotes. Strong positive correlations were observed between PCSK9 and LDL-C levels in both the *PCSK9* E32K heterozygotes ($r=0.69$, $P<0.01$) and in control subjects ($r=0.64$, $P<0.01$, Fig. 3A). Further, the slope of the regression curve between plasma PCSK9 and LDL-C in *PCSK9* E32K heterozygotes ($y=0.383x+54.1$) was far steeper than that in controls ($y=0.186x+61.6$). Consequently, relative LDL-C levels were 1.47-fold higher in *PCSK9* E32K heterozygotes and 2.10-fold higher in true homozygotes than in controls with comparable cir-

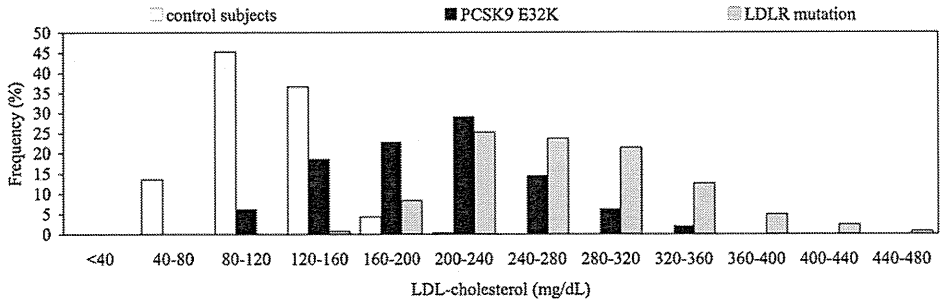

Fig. 1. Plasma LDL-C distribution in control subjects, *PCSK9* E32K carriers and *LDLR* mutation heterozygotes. Bars show the percentage of subjects in each group.

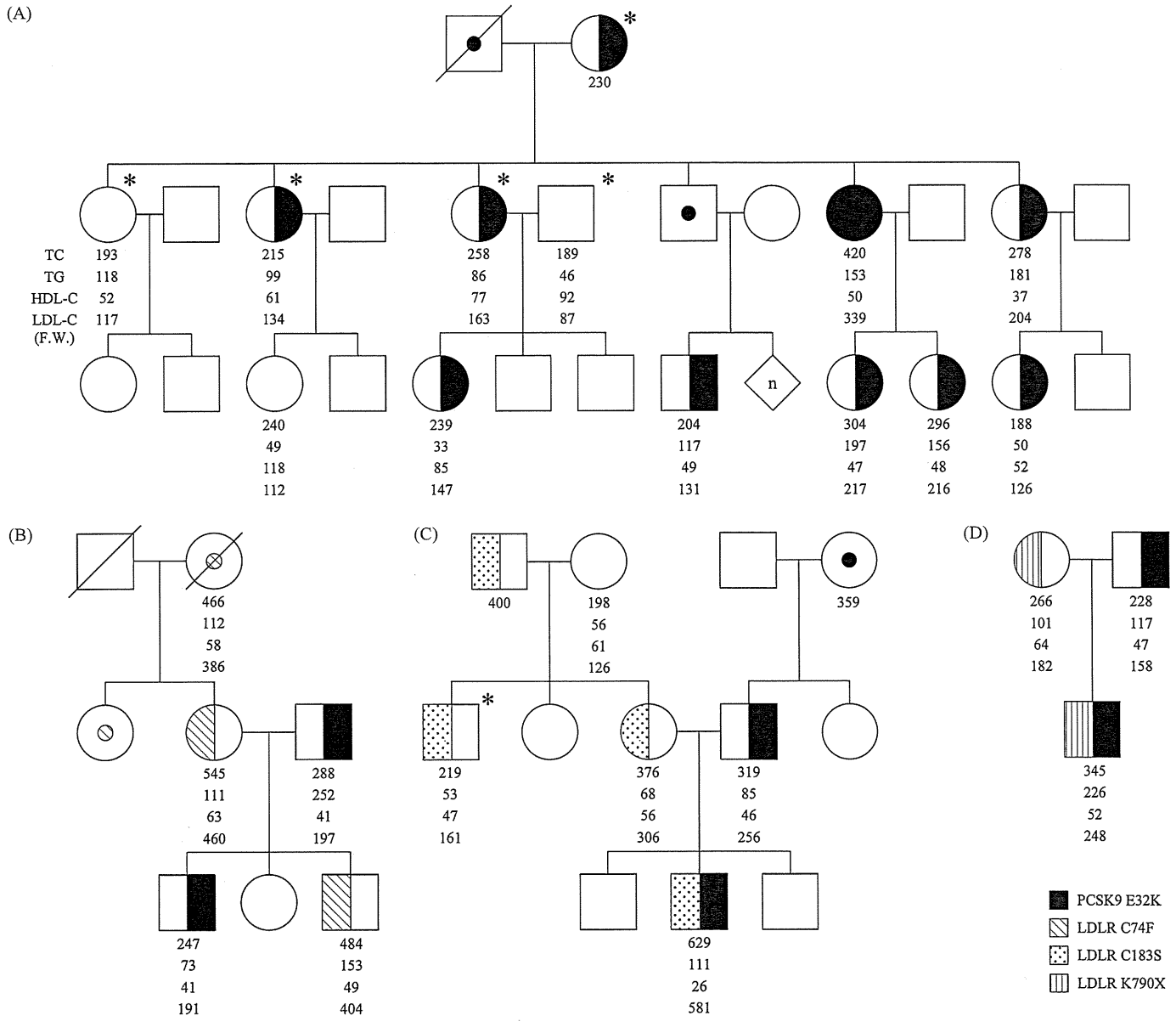

Fig. 2. Pedigrees of gain-of-function *PCSK9* mutation families caused by the *PCSK9* E32K. (A) Pedigree of subject with true homozygous *PCSK9* E32K mutation. (B) Pedigree affected by a single mutation of *PCSK9* E32K or *LDLR* C74F in each subject. (C) Family with a double heterozygote of *PCSK9* E32K and *LDLR* C183S mutations. (D) Pedigree of a double heterozygote with *PCSK9* E32K and *LDLR* K790X mutations. Relatives of another *PCSK9* E32K true homozygote and a double heterozygote with *PCSK9* E32K and *LDLR* C292X mutations did not participate in this study. Half-shaded symbols indicate carriers of a single mutation of *PCSK9* or *LDLR* and filled symbol indicates true homozygote. Plasma TC, TG, HDL-C and LDL-C values (mg/dL) are shown below each symbol. Asterisks indicate the subjects treated with lipid-lowering drugs at the time of the study. Hatched lines represent deceased family members. Diamond symbols labelled "n" indicate number and sex of individuals unknown.

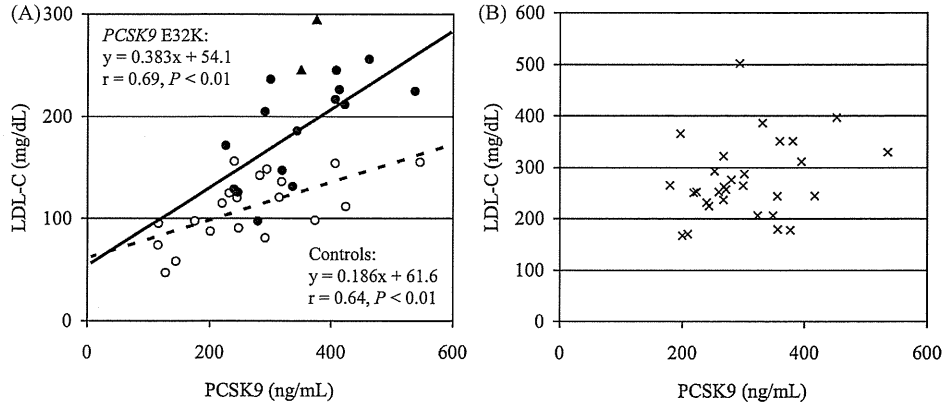

Fig. 3. Relationships between circulating levels of PCSK9 and LDL-C in (A) 20 control subjects (open circles), 15 *PCSK9* E32K heterozygotes (closed circles), 2 *PCSK9* E32K true homozygotes (closed triangles) and (B) 30 *LDLR* mutation heterozygotes (20 K790X and 10 IVS15-3C/A, crossed symbols).

culating PCSK9 levels (Table S2). In heterozygous *LDLR* mutation carriers, there were slight increases in plasma PCSK9 levels at 304 ± 83 ng/mL and the correlation between plasma PCSK9 and LDL-C levels was not significant ($r = 0.25$, $P = 0.40$, Fig. 3B). The results were similar when the relation was analysed in *LDLR* K790X carriers ($r = 0.12$, $P = 0.64$) and in *LDLR* IVS15-3C/A carriers ($r = 0.33$, $P = 0.34$), respectively. Plasma PCSK9 and TG levels were positively correlated in control subjects ($r = 0.48$, $P < 0.05$) and *LDLR* mutation heterozygotes ($r = 0.40$, $P < 0.05$), whereas the correlation did not reach statistical significance in *PCSK9* E32K carriers ($r = 0.44$, $P = 0.10$).

3.5. Effects of PCSK9 E32K mutation on secretion of PCSK9

To examine whether the *PCSK9* E32K mutation affects the secretion of PCSK9, the amounts of PCSK9 were measured in media of HepG2 cells transiently transfected with WT-PCSK9, E32K-PCSK9 or empty plasmid. Consistent with *in vivo* results, HepG2 cells transfected with E32K-PCSK9 secreted significantly larger amounts of PCSK9 into the media than those with WT-PCSK9 after 24 h of incubation (139 ± 13% *vs.* 100 ± 3%, $P < 0.01$, Fig. 4). PCSK9 levels in the media of HepG2 cells transfected with empty plasmid were almost undetectable (0.7 ± 0.3%) under our experimental conditions.

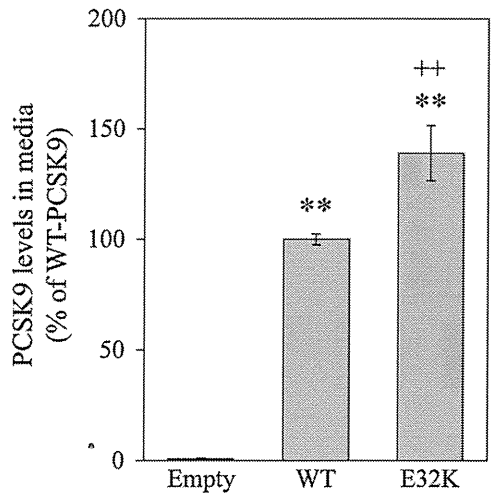

Fig. 4. Secretion of PCSK9 in media from HepG2 cells transiently transfected with WT-PCSK9, E32K-PCSK9 or empty plasmid. After 24 h of incubation, the media were collected and subjected to ELISA. The values are expressed relative to the amount of PCSK9 in the media of WT-PCSK9-transfected HepG2 cells and are given as means ± S.D. from three independent experiments. $^{**}P < 0.01$ *vs.* empty plasmid, $^{++}P < 0.01$ *vs.* WT-PCSK9.

4. Discussion

In this study, 55 clinical-FH subjects without *LDLR* mutations were screened for *PCSK9* mutations and 7 variants were detected in 22 subjects. One of the variant carriers was true homozygous for *PCSK9* E32K and showed markedly higher LDL-C levels than *PCSK9* E32K heterozygotes. On further screening for *PCSK9* E32K variant in the general population, 6 carriers of *PCSK9* E32K were detected among individuals with higher LDL-C levels (Table 1), which was similar to the results of Miyake et al. who detected *PCSK9* E32K variant in the higher LDL-C group and anti-hypercholesterolaemia treatment group [19]. The lipid profiles of *PCSK9* E32K carriers and the significantly higher frequency of *PCSK9* E32K in clinical-FH than that in controls of the same district suggested this should be classified as a gain-of-function mutation. The remaining 6 *PCSK9* variants detected here were also described in their study, and the capabilities of these variants to cause FH were considered to be very low.

Consistent with previous findings [14], plasma PCSK9 levels were positively correlated with LDL-C levels in both control subjects and *PCSK9* E32K heterozygotes (Fig. 3A). The slope of the regression curve in *PCSK9* E32K heterozygotes was almost double that in control subjects (Fig. 3A). Further, *PCSK9* E32K true homozygotes showed 2.10-fold higher LDL-C levels than controls with comparable PCSK9 levels and the value was almost the square of that in heterozygotes (1.47), suggesting the impact of circulating *PCSK9* E32K on LDL-C level (Table S2). Although the media of HepG2 cells transfected with E32K-PCSK9 plasmid as well as the plasma in *PCSK9* E32K carriers showed more than 30% higher PCSK9 concentrations compared with controls in our ELISA study (Fig. 4, Table S2), circulating PCSK9 levels in *PCSK9* E32K true homozygotes were similar to those in heterozygotes. In a recent study, a much more potent gain-of-function *PCSK9* mutation c.1120G>T (D374Y) was associated with lower plasma PCSK9 levels [16]. We presume that the increased function of PCSK9, which in turn enhances its own clearance, would in part lower the plasma PCSK9 concentration in *PCSK9* E32K true homozygotes like *PCSK9* D374Y carriers. The effect of *PCSK9* E32K was relatively mild, and thus age, gender and other metabolic markers, such as insulin and glucose, would considerably influence the plasma levels of PCSK9 [15,20,21].

In the heterozygous FH subjects with *LDLR* mutations, plasma PCSK9 levels were similar to those in controls but plasma levels of LDL-C were twice those in controls after adjusting for circulating PCSK9 levels. A positive correlation between plasma PCSK9 and LDL-C levels was not observed in *LDLR* mutation carriers (Fig. 3B), and therefore we speculated that high LDL-C levels relative to PCSK9 levels in *LDLR* mutation carriers would reflect the far more

potent impact of *LDLR* mutations on LDL-C concentration than that of circulating PCSK9.

Although there is consensus regarding the mechanism of LDLR degradation by PCSK9, there is debate over its role in very low-density lipoprotein (VLDL) receptor (VLDLR) regulation. Poirier et al. demonstrated that PCSK9 induces degradation of VLDLR independent of the presence of LDLR [22], whereas Zhang et al. reported that the selectivity of PCSK9 for the LDLR is due to Leu318 in epidermal growth factor-like repeat (EGF)-A domain, which is not present in VLDLR [3]. With regard to VLDL production, fasting led to a marked decrease in LDLR levels in mice overexpressing PCSK9, which was associated with an increase in VLDL production rate [23]. In gain-of-function *PCSK9* S127R, the lipoprotein kinetic study using stable isotopes indicated a 3-fold increase in apolipoprotein B100 and VLDL production rate [24]. In the present study, there was a positive correlation between plasma PCSK9 and TG levels in controls, which was consistent with recent reports [15,20,21], whereas this correlation did not reach statistical significance in *PCSK9* E32K carriers despite marked increase in plasma PCSK9 and TG levels (Table S2). An *in vivo* lipoprotein kinetic study of the *PCSK9* E32K true homozygote using stable isotopes would provide more precise information on the lipoprotein kinetics in gain-of-function *PCSK9* mutations.

Previous studies and our results indicated that the gain-of-function mutation *PCSK9* E32K is relatively common and causes milder hypercholesterolaemia than *LDLR* mutations and other potent gain-of-function *PCSK9* mutations such as D374Y [19,25]. Interestingly, *PCSK9* E32K heterozygotes were clearly distinguished from *LDLR* C74F heterozygotes with regard to plasma LDL-C levels within the family (Fig. 2B). Similar to other gain-of-function mutations of *PCSK9* [25,26], *PCSK9* E32K could worsen the lipid profiles in subjects true homozygous or double heterozygous with *LDLR* mutation as in one patient in the present study (Fig. 2A, C and D), although the levels of plasma LDL-C varied widely from 246 to 581 mg/dL. There were no further mutations in *LDLR* in any of these subjects and they were apparently different from those homozygous for *LDLR* mutation in that their LDL-C levels could be reduced to values (<280 mg/dL) comparable to those in *LDLR* mutation heterozygotes by drug treatment.

In the family affected with *PCSK9* E32K and *LDLR* K790X, the presence of lipid-lowering gene mutation was suggested by the relatively low cholesterol levels considering their gene mutations, but at the same time, it is also possible that the combined gene mutations would interact with each other and affect the lipid profiles of double heterozygous subjects (Fig. 2D). The mutations encoded in 50-residue cytoplasmic domain of LDLR (amino acids 790–839), which is important in directing the receptors to coated pits and facilitating rapid endocytosis of bound LDL [27], are considered to be internalisation-defective alleles and we confirmed that T-lymphocytes of *LDLR* K790X heterozygotes showed defective uptake of DiI-LDL [28]. Recently, Bottomley et al. demonstrated that the EGF sub-fragments of the LDLR were able to counteract the inhibitory effect of PCSK9 in LDL uptake assays [29]. Likewise, in *LDLR* K790X heterozygotes, the intact EGF-AB domain of the internalisation-defective *LDLR* K790X may act as a decoy to protect the remaining half of normal LDLR against PCSK9-mediated degradation. In this regard, the weaker association between plasma levels of PCSK9 and LDL-C in subjects with *LDLR* K790X compared to subjects with *LDLR* IVS15-3C/A ($r=0.12$ *vs.* $r=0.34$) may support the inhibitory effect of *LDLR* K790X on the function of PCSK9, although neither relation reached statistical significance.

In summary, we found 2 true homozygotes for *PCSK9* E32K and 3 double heterozygotes for *PCSK9* E32K and *LDLR* mutations associated with ADH. To our knowledge, this is the first report of gain-of-function *PCSK9* mutation true homozygosity, providing evidence that *PCSK9* E32K significantly affects LDL-C levels *via* increased function and mass of PCSK9, and could exacerbate the clinical phenotypes of patients carrying *LDLR* mutations.

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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.2009.11.018.

References

[1] Seidah NG, Benjannet S, Wickham L, et al. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. Proc Natl Acad Sci USA 2003;100:928–33.
[2] Naureckiene S, Ma L, Sreekumar K, et al. Functional characterization of Narc 1, a novel proteinase related to proteinase K. Arch Biochem Biophys 2003;420:55–67.
[3] Zhang DW, Lagace TA, Garuti R, et al. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. J Biol Chem 2007;282:18602–12.
[4] Abifadel M, Varret M, Rabes JP, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 2003;34:154–6.
[5] Rashid S, Curtis DE, Garuti R, et al. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc Natl Acad Sci USA 2005;102:5374–9.
[6] Cohen JC, Boerwinkle E, Mosley Jr TH, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 2006;354:1264–72.
[7] Yu W, Nohara A, Higashikata T, Lu H, Inazu A, Mabuchi H. Molecular genetic analysis of familial hypercholesterolemia: spectrum and regional difference of LDL receptor gene mutations in Japanese population. Atherosclerosis 2002;165:335–42.
[8] Miyake Y, Yamamura T, Sakai N, Miyata T, Kokubo Y, Yamamoto A. Update of Japanese common LDLR gene mutations and their phenotypes: mild type mutation L547V might predominate in the Japanese population. Atherosclerosis 2009;203:153–60.
[9] Benjannet S, Rhainds D, Essalmani R, et al. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. J Biol Chem 2004;279:48865–75.
[10] Cunningham D, Danley DE, Geoghegan KF, et al. Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. Nat Struct Mol Biol 2007;14:413–9.
[11] Lambert G, Charlton F, Rye KA, Piper DE. Molecular basis of PCSK9 function. Atherosclerosis 2009;203:1–7.
[12] Leigh SE, Leren TP, Humphries SE. Commentary PCSK9 variants: a new database. Atherosclerosis 2009;203:32–3.
[13] Costet P, Krempf M, Cariou B. PCSK9 and LDL cholesterol: unravelling the target to design the bullet. Trends Biochem Sci 2008;33:426–34.
[14] Lambert G, Ancellin N, Charlton F, et al. Plasma PCSK9 concentrations correlate with LDL and total cholesterol in diabetic patients and are decreased by fenofibrate treatment. Clin Chem 2008;54:1038–45.
[15] Lakoski SG, Lagace TA, Cohen JC, Horton JD, Hobbs HH. Genetic and metabolic determinants of plasma PCSK9 levels. J Clin Endocrinol Metab 2009;94:2537–43.
[16] Humphries SE, Neely RD, Whittall RA, et al. Healthy individuals carrying the PCSK9 p.R46L variant and familial hypercholesterolemia patients carrying PCSK9 p.D374Y exhibit lower plasma concentrations of PCSK9. Clin Chem 2009 [Epub ahead of print].
[17] Mabuchi H, Koizumi J, Shimizu M, Takeda R. Development of coronary heart disease in familial hypercholesterolemia. Circulation 1989;79:225–32.
[18] Spinardi L, Mazars R, Theillet C. Protocols for an improved detection of point mutations by SSCP. Nucleic Acids Res 1991;19:4009.
[19] Miyake Y, Kimura R, Kokubo Y, et al. Genetic variants in PCSK9 in the Japanese population: rare genetic variants in PCSK9 might collectively contribute to plasma LDL cholesterol levels in the general population. Atherosclerosis 2008;196:29–36.
[20] Dubuc G, Tremblay M, Pare G, et al. A new method for measurement of total plasma PCSK9—clinical applications. J Lipid Res 2009 [Epub ahead of print].
[21] Baass A, Dubuc G, Tremblay M, et al. Plasma PCSK9 is associated with age, sex, and multiple metabolic markers in a population-based sample of children and adolescents. Clin Chem 2009;55:1637–45.

[22] Poirier S, Mayer G, Benjannet S, et al. The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. J Biol Chem 2008;283:2363–72.
[23] Lambert G, Jarnoux AL, Pineau T, et al. Fasting induces hyperlipidemia in mice overexpressing proprotein convertase subtilisin kexin type 9: lack of modulation of very-low-density lipoprotein hepatic output by the low-density lipoprotein receptor. Endocrinology 2006;147:4985–95.
[24] Ouguerram K, Chetiveaux M, Zair Y, et al. Apolipoprotein B100 metabolism in autosomal-dominant hypercholesterolemia related to mutations in PCSK9. Arterioscler Thromb Vasc Biol 2004;24:1448–53.
[25] Leren TP. Mutations in the PCSK9 gene in Norwegian subjects with autosomal dominant hypercholesterolemia. Clin Genet 2004;65:419–22.
[26] Pisciotta L, Priore Oliva C, Cefalu AB, et al. Additive effect of mutations in LDLR and PCSK9 genes on the phenotype of familial hypercholesterolemia. Atherosclerosis 2006;186:433–40.
[27] Davis CG, van Driel IR, Russell DW, Brown MS, Goldstein JL. The low density lipoprotein receptor. Identification of amino acids in cytoplasmic domain required for rapid endocytosis. J Biol Chem 1987;262:4075–82.
[28] Tada H, Kawashiri MA, Noguchi T, et al. A novel method for determining functional LDL receptor activity in familial hypercholesterolemia: application of the CD3/CD28 assay in lymphocytes. Clin Chim Acta 2009;400:42–7.
[29] Bottomley MJ, Cirillo A, Orsatti L, et al. Structural and biochemical characterization of the wild type PCSK9-EGF(AB) complex and natural familial hypercholesterolemia mutants. J Biol Chem 2009;284:1313–23.

CARDIO VASCULAR DIABETOLOGY

ORIGINAL INVESTIGATION Open Access

Age, gender, insulin and blood glucose control status alter the risk of ischemic heart disease and stroke among elderly diabetic patients

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Abstract

Background: We analyzed the effects of insulin therapy, age and gender on the risk of ischemic heart disease (IHD) and cerebrovascular accident (CVA) according to glycemic control.

Methods and Results: We performed a prospective cohort study (Japan Cholesterol and Diabetes Mellitus Study) of type 2 diabetes patients (n = 4014) for 2 years. The primary endpoint was the onset of fatal/non-fatal IHD and/or CVA, which occurred at rates of 7.9 and 7.2 per 1000 person-years, respectively. We divided diabetic patients into four groups based on age (≤ 70 and > 70) and hemoglobin A1C levels (≤ 7.0 and > 7.0%). Multiple regression analysis revealed that IHD was associated with high systolic blood pressure and low HDL-C in patients under 70 years of age with fair glycemic control and was associated with low diastolic blood pressure in the older/fair group. Interestingly, insulin use was associated with IHD in the older/poor group (OR = 2.27, 95% CI = 1.11-5.89; p = 0.026) and was associated with CVA in the older/fair group (OR = 2.09, 95% CI = 1.06-4.25; p = 0.028). CVA was associated with lower HDL-C and longer duration of diabetes in younger/poor glycemic control group. Results by stepwise analysis were similar. Next, patients were divided into four groups based on gender and diabetic control (hemoglobinA1C < or > 7.0%). Multiple regression analysis revealed that IHD was associated with high systolic blood pressure in male/fair glycemic control group, age in male/poor control group, and short duration of diabetic history in females in both glycemic control groups. Interestingly, insulin use was associated with IHD in the male/poor group(OR = 4.11, 95% CI = 1.22-8.12; p = 0.018) and with CVA in the female/poor group(OR = 3.26, 95% CI = 1.12-6.24; p = 0.02). CVA was associated with short duration of diabetes in both female groups.

Conclusions: IHD and CVA risks are affected by specific factors in diabetics, such as treatment, gender and age. Specifically, insulin use has a potential role in preventing IHD but may also be a risk factor for CVA among the diabetic elderly, thus revealing a need to develop improved treatment strategies for diabetes in elderly patients. The Japan Cholesterol and Diabetes Mellitus Study was formulated to evaluate them(Umin Clinical Trials Registry, clinical trial reg. no. UMIN00000516; http://www.umin.ac.jp/ctr/index.htm).

Keywords: Elderly, Diabetes mellitus, Insulin, Cerebral ischemia, Ischemic heart disease

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Background

Elderly patients with type 2 diabetes mellitus (T2DM) have much higher risks of ischemic heart disease (IHD) and cerebrovascular accident (CVA) compared to younger diabetic patients. Because of these risks, diabetes may shorten an individual's life span by approximately 10 years [1]. A considerable number of studies have assessed IHD and CVA risk factors in culturally diverse groups of diabetic patients less than 70 years of age. With regard to glycemic control, a recent meta-analysis of several large clinical studies revealed that intensive and strict glycemic control was more effective than standard control for preventing IHD [2]. This review analyzed five trials, including the United Kingdom Prospective Diabetes Study (UKPDS), Action to Control Cardiovascular Risk in Diabetes (ACCORD), and Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE). The meta-analysis study concluded that intensive glucose control [decreasing hemoglobin A1C (HbA1C) levels by 0.9%] was superior to standard control for preventing IHD. However, intensive glucose control did not seem to have any effect on stroke rates or overall survival [2-5]. Furthermore, most studies focused exclusively on patients under the age of 70 and did not examine elderly diabetic patients. Additionally, the authors did not evaluate whether specific diabetes treatments, such as insulin, had any effect on the risk of IHD and CVA.

The Japanese population has lower rates of IHD and higher rates of CVA than the U.S. and European populations [6]. However, the rate of IHD is much higher among Japanese diagnosed with diabetes [6,7]. Although it has been shown that elderly diabetic individuals have a higher risk of IHD than younger, non-diabetic patients, there is insufficient evidence regarding the associations between age, diabetic control, CVA, and IHD [8]. The present study, the Japan-CDM (Japan Cholesterol and Diabetes Mellitus Investigation), was a nationwide observational cohort study that enrolled 4,014 Japanese individuals with diabetes [7]. We recently reported the possibility of a change in the relationship between atherosclerotic risk factors and IHD or CVA based on age [9]. In other words, we identified a significant relationship between lower HDL or higher LDL cholesterol levels and the occurrence of IHD in subjects older than 65 years old. Lower HDL cholesterol was also significantly related to CVD in subjects over 65 years of age and especially in those older than 75. Lower HDL cholesterol is an important risk factor for IHD and CVD, especially in diabetic elderly individuals. Based on these data, the goal of this study is to evaluate the relationships between age, diabetic control, CVA and IHD in Japanese T2DM patients.

Methods

Patients

We recruited diabetic individuals examined at 40 institutions throughout Japan between September 2004 and March 2005. Patients who had experienced previous myocardial infarctions or cerebrovascular accidents requiring hospitalization were excluded from the study. Other exclusion criteria included the following: a history of or complications related to serious heart disease (such as acute heart failure); serious hepatic or renal disease, such as non-compensated liver cirrhosis and chronic renal failure requiring hemodialysis; malignancy; intention to undergo surgery; any illness with a poor prognosis; and the recruiting physician's judgment that a patient was inappropriate for inclusion in the study.

Study design

This multicenter prospective longitudinal cohort study included 4,014 diabetic individuals examined on a consecutive outpatient basis (1,936 women and 2,078 men; mean age = 67.4 ± 9.5 years, range = 35-83 years, median = 70.4 years). The one-year and two-year follow-up rates were 98.2% and 92.3%, respectively (Figure 1). Primary endpoints were the onset of IHD (comprising fatal or nonfatal myocardial infarction; development of unstable angina; or the need for coronary revascularization procedures, either coronary artery bypass grafting or percutaneous coronary intervention because of angina or an acute coronary syndrome) or CVA (stroke with neurological deficit, except transient ischemic attack). Secondary endpoints were sudden cardiac death due to causes other than myocardial infarction, transient ischemic attack, subarachnoid hemorrhage and all-cause mortality. We collected blood samples from the patients to evaluate their plasma lipid levels, blood glucose levels and HbA1C levels during the same month each year. Informed consent was obtained from all subjects participating in the study. The study was approved by the Institutional Review Boards of the participating hospitals and the relevant safety monitoring boards [10]. The guidelines of the Japan Atherosclerosis Society, which state that LDL values should be less than 120 mg/dL and HDL values should be higher than 40 mg/dL in diabetic individuals, and the diagnostic criteria for T2DM of the American Diabetes Association were used [11,12]. All reported events were confirmed by the organizing committee.

Group allocation by age and glycemic control

We investigated the relationships between age, diabetic control, IHD, and CVA. Because the median age and hemoglobin A1C values of the patients in this study were 70.4 years and 7.0%, respectively, we divided the

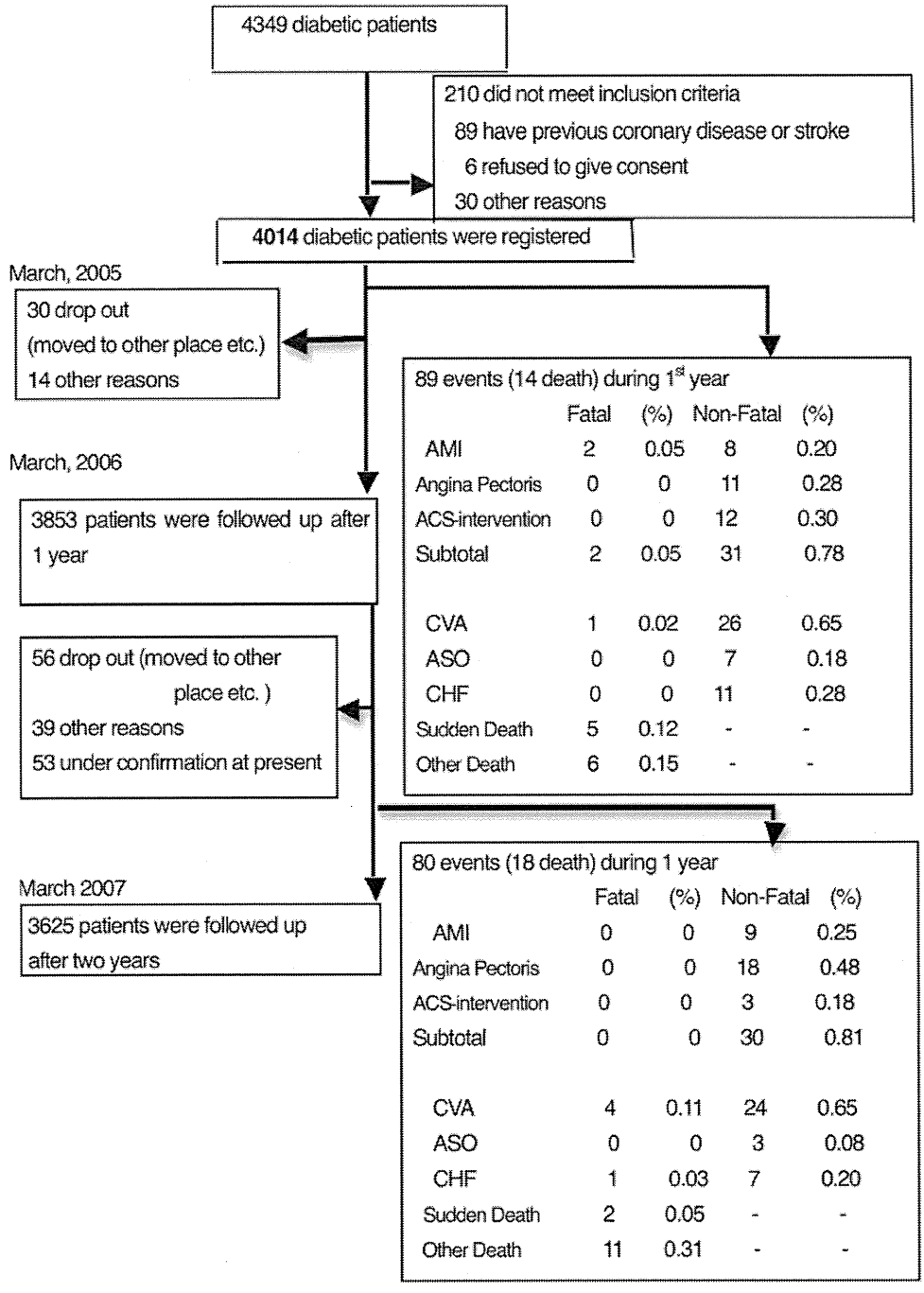

Figure 1 Trial design and cardiovascular events that occurred after the first and second years among the diabetic individuals included in the study. AMI: acute myocardial infarction, ACS: acute coronary syndrome, CVA: cerebrovascular accident (stroke), ASO: arteriosclerosis obliterans, CHF: congestive heart failure.

patients into four groups based on age (≤ 70 and > 70) and hemoglobin A1C levels (≤ 7.0 and > 7.0%). The four groups were as follows: 1) NF: under 70 years of age with fair glycemic control (n = 870); 2) NP: under 70 years of age with poor glycemic control (n = 1,072); 3) OF: over 70 years of age with fair glycemic control (n = 923); and 4) OP: over 70 years of age with poor glycemic control (n = 1,149).

Group allocation by gender and glycemic control

We investigated the relationships between gender, diabetic control, IHD, and CVA. We divided the patients into four

groups based on gender and hemoglobin A1C levels (≤ 7.0 and > 7.0%). The four groups were as follows: 1) MF: males with fair glycemic control (n = 1063); 2) MP: males with poor glycemic control (n = 950); 3) FF: females with fair glycemic control (n = 859); and 4) FP: females with poor glycemic control (n = 1,142).

Statistics

Results are presented as means ± SD (standard deviation) of the data analyzed. All statistical analyses were performed using JMP (version 7, SAS Institute Inc., Cary, NC). The incidence of IHD and CVA were analyzed according to age, which was stratified as 70 years or younger vs. older than 70 years. We used the chi-square test and Fisher's exact test for categorical comparisons of the data. Differences in the means of continuous measurements were tested using the Mann-Whitney U test. We selected possible significant predictors by conducting a univariate logistic regression analysis (P < 0.10) and performed multiple logistic regression analysis to identify important risk factors for IHD and CVA. Additionally, stepwise regression analysis was used to confirm the relationships between IHD, CVA, and clinical variables. P-values less than 0.05 were considered to be statistically significant.

Results

A total of 152 cardiovascular events (IHD and CVA) and 17 deaths due to other etiologies occurred during the 2-year study period. IHD and CVA occurred at rates of 7.9 and 7.2 per 1000 person-years, respectively. Table 1 shows the baseline characteristics of the four patient groups. In the NF group, fasting blood glucose levels, diastolic blood pressure readings and the female/male ratio were higher among patients using insulin than among patients not using insulin. Conversely, LDL-C was lower and HDL-C tended to be higher among patients using insulin than those not using insulin in the NF group. In the NP group, triglyceride and LDL-C levels were lower and HDL-C levels were higher among patients using insulin. In both the NF and NP groups, the history of diabetes was longer for patients using insulin. In both the OF and OP groups, the duration of diabetes was not different for insulin users compared to non-users. In the OP group, more females than males used insulin. We adjusted the data for these baseline differences in the following analyses.

Multiple regression analysis examining the relationship between the risk of IHD and clinical variables for each group divided by age and glucose control

Table 2 shows the relationship between IHD and each clinical measurement, such as LDL-C level and insulin treatment for each group divided by age and glucose control levels (HbA1C). We performed multiple regression analysis for variables that showed a possible relationship with IHD risk by univariate analysis (P < 0.1).

Patients 70 years of age or younger: Univariate analysis revealed that higher systolic blood pressure, higher LDL-C and lower HDL-C were associated with IHD risk among patients in the NF group. However, only systolic blood pressure (OR = 1.81, 95% CI = 1.19-2.93; p = 0.009) and HDL-C level (OR = 0.46, 95% CI = 0.26-0.85; p = 0.006) were confirmed to be significantly associated with IHD risk by the multiple regression analysis.

Patients over 70 years of age: Lower diastolic blood pressure was associated with IHD risk among patients in the OF group (OR = 0.65, 95% CI = 0.48-0.96; p = 0.016). Insulin use was significantly associated with IHD risk in the OP group (OR = 2.27, 95% CI = 1.11-5.89; p = 0.026) (Table 2). The duration of diabetes did not affect IHD risk in either group.

Multiple regression analysis examining the relationship between clinical variables and the risk of CVA for each group divided by age and glucose control

Table 3 shows the relationship between CVA and each clinical measurement as well as the insulin treatment for each group.

Patients 70 years of age or younger: CVA tended to occur more frequently among patients with higher HbA1C levels than in those with lower HbA1C levels (OR = 4.11, 95% CI = 1.01-13.4; p = 0.067) in the NF group. Lower HDL-C (OR = 0.43, 95% CI = 0.23-0.78; p = 0.006) and a longer duration of diabetes (OR = 1.06, 95% CI = 1.01-1.11; p = 0.018) were associated with CVA risk among patients in the NP group.

Patients over 70 years of age: Insulin use was associated with CVA risk in the OF group (OR = 2.09, 95% CI = 1.06-4.25; p = 0.028) (Table 2). Univariate analysis revealed a trend toward an association between lower HDL-C levels and CVA in the OF and OP groups, but this association was not statistically significant according to the results of the multiple regression analysis.

Stepwise regression analysis

Stepwise multiple regression analyses were also performed separately for IHD and CVA risks. The results were very similar to those of the multiple regression analyses described above, except that LDL-C was associated with IHD in the NF group (Table 3).

Regarding IHD risk, higher systolic blood pressure (p = 0.003), lower HDL-C (p = 0.017), and higher LDL-C (p = 0.035) were all associated with IHD risk in the NF group. Insulin use (p = 0.005) and age (p = 0.015) were significantly associated with IHD in the OP group.

Regarding CVA risk, higher levels of hemoglobin A1C were associated with CVA risk in the NF group (p = 0.03), and lower HDL-C (p = 0.004) and the duration of diabetes

Table 1 Base line profile of patients

= < 70y.o.	HbA1C = < 7.0 (Gp.MF)			HbA1C 7.0 < (Gp.MP)		
	Insulin (-)	Insulin (+)	P	Insulin (-)	Insulin (+)	P
	Mean(SD)	Mean(SD)		Mean(SD)	Mean(SD)	
Duration of Diabetes (yrs)	6.67 (7.50)	12.18 (9.31)	0.012*	9.53(8.05)	12.48 (8.15)	0.011*
FBS (mg/dl)	145.2 (44.4)	158.2 (55)	0.022*	183.7(63.4)	173.7(71)	0.134
SBP (mmHg)	134.4 (15.5)	133.4 (17.6)	0.409	133.4(17.7)	132.7 (18.3)	0.893
DBP (mmHg)	77 (12.3)	73.2 (14.8)	0.011*	76 (10.9)	73.4(11)	0.04*
TG (mg/dl)	148.3(119.9)	135.5 (65.3)	0.844	154.1(111.6)	141.9 (184)	0.03*
HDL-C(mg/dl)	55(14.2)	57.2 (15.1)	0.062	52.8(13.4)	59.7(21.5)	0.001**
LDL-C(mg/dl)	116.3(31.9)	110.5 (27.9)	0.034*	123.4(35.9)	120.2(33.3)	0.52
	%	%	P	%	%	P
Gender (Male/total)	61.8	49.6	0.011*	53.3	47.5	0.116
Anti-hyperlipidemic drugs	53.9	49.3	0.131	52.7	58.6	0.477
statin	41.4	39.0	0.122	48.5	45.7	0.444
Oral diabetic drugs	44.4	10	< 0.001**	82.0	6.7	< 0.001**
sulfonylurea	43.0	9.0	< 0.001**	79.4	8.6	< 0.001**
Anti-hypertensive drugs	66.1	47.9	0.042*	54.8	51.0	0.921
70 +y.o.	**HbA1C = < 7.0 (Gp.OF)**			**HbA1C 7.0 < (Gp.OP)**		
	Insulin (-)	Insulin (+)	P	Insulin (-)	Insulin (+)	P
	Mean(SD)	Mean(SD)		Mean(SD)	Mean(SD)	
Duration of Diabetes (yrs)	10.83 (8.75)	12.2 (9.67)	0.12	10.67 (9.17)	11.0 (9.50)	0.43
FBS (mg/dl)	146.2 (47.1)	159.1 (59)	0.052	174.5 (70.5)	181.7 (75.5)	0.224
SBP (mmHg)	136.2 (16.4)	135.7 (17)	0.896	136.4 (15.9)	136.2 (17)	0.822
DBP (mmHg)	71.7 (11.2)	70.4 (11.6)	0.256	73.1 (11.3)	73.1 (11.1)	0.832
TG (mg/dl)	127.5 (73.4)	124.3 (75.3)	0.168	130.8 (75)	124.6 (69.3)	0.53
HDL-C (mg/dl)	55.1 (16)	55 (17.3)	0.953	54.7 (16.1)	54.7 (16.5)	0.888
LDL-C (mg/dl)	114.1 (29)	112.3 (30.3)	0.704	118.2 (30.7)	117.8 (33.6)	0.541
	%	%	P	%	%	P
Gender (Male/total)	54	45.9	0.061	53.2	37.9	0.035*
Anti-hyperlipidemic drugs	50.2	46.0	0.140	46.7	50	0.307
statin	43.9	40.2	0.003**	43.3	42.5	0.167
Oral diabetic drugs	62.6	18.5	< 0.001**	84.6	19.1	< 0.001**
sulfonylurea	60.1	12.6	< 0.001**	79.4	8.0	< 0.001**
Anti-hypertensive drugs	68.2	59.9	0.904	60.9	54.6	0.370

Characteristics of the patients in each of the four groups, including insulin use. These groups were stratified by glycemic control and age.
*p < 0.05, **p < 0.01.

(p = 0.01) were associated with CVA risk in the NP group. Insulin use (p = 0.038) was associated with CVA risk among patients in the OF group. Lower HDL-C levels tended to be associated with CVA in the OF group (p = 0.056) and the OP group (p = 0.08), although these associations were not statistically significant.

Influence of insulin therapy on IHD and CVA risk

The results indicated that insulin therapy was associated with IHD risk in the OP group and with CVA risk in the OF group (Figure 2). Interestingly, patients 70 years of age or younger who used insulin tended to have slightly decreased incidences of IHD and CVA, whereas patients over 70 years of age using insulin tended to have an increased incidence of CVA (Figure 2a and 2b).

Interestingly, insulin use was associated with IHD in the MP group (OR = 4.11, 95% CI = 1.22-8.12; p = 0.018) and with CVA in the FP group (OR = 3.26, 95% CI = 1.12-6.24; p = 0.02).

Multiple regression analysis examining the relationship between clinical variables and the risk of IHD for each group divided by gender and glucose control

Multiple regression analysis was performed to evaluate the relationship between IHD and each clinical measurement, such as LDL-C level and insulin treatment for

Table 2 The relationship between IHD, CVA and clinical variables

IHD = < 70 y.o.	**Mean(SD)**	**Hgb A1C = < 7.0 (Gp.MF)**		**7.0 < HgbA1C (Gp.MP)**	
Number of events		**17**		**15**	
		Univariate	**Multivariate**	**Univariate**	**Multivariate**
Male/Female	0.96	0.75 (0.40-1.37)		1.03 (0.62-1.74)	
Age (y.o.)	60.9(7.9)y.o.	1.06 (0.98-1.20)		1.03 (0.96-1.13)	
Duration of diabetes (years)	9.15(8.22)	1.04^{+} (0.99-1.09)		1.03 (0.96-1.08)	
Hemoglobin A1C(%)	7.38(1.31)	1.16 (0.40-4.37)		1.01 (0.60-1.50)	
Triglyceride (mg/dl)	146.9(129.5)	1.01 (0.99 - 1.03)		1.01 (0.94-1.04)	
LDL-Chol (mg/dl)	119.2(34.1)	**1.13* (1.01-1.28)**	**1.12^{+}(1.00-1.28)**	1.04 (0.87-1.21)	
HDL-Chol (mg/dl)	55.4(16.2)	**0.54* (0.29-0.91)**	**0.46*(0.26-0.85)**	0.78 (0.53-1.08)	
Systolic BP(mmHg)	133.5(17.5)	**1.58* (1.11-2.24)**	**1.81**(1.19-2.93)**	1.07 (0.80-1.49)	
Diastolic BP **(mmHg)**	75.5(11.7)	0.94 (0.63-1.58)		0.96 (0.63-1.62)	
Insulin user (%)	32.80%	0.96 (0.37-1.93)		1.06 (0.61-1.81)	
IHD 70 < y.o.	Mean(SD)	**Hgb A1C = < 7.0 (Gp.OF)**		**7.0 < HgbA1C (Gp.OP)**	
Number of events		25		25	
		Univariate	Multivariate	Univariate	Multivariate
Male/Female	1	1.12 (0.71-1,81)		1.41 (0.83-2.97)	
Age (y.o.)	75.4(4.3)	0.99 (0.88-1.09)		0.83 (0.66-0.99)	
Duration of diabetes (years)	10.18(9.08)	1.02 (0.98-1.06)		1.02 (0.93-1.08)	
Hemoglobin A1C (%)	7.26 (1.15)	1.74 (0.65-5.55)		1.37 (0.77-2.16)	
Triglyceride (mg/dl)	129.2 (62.2)	1.00 (1.00-1.09)		1.01 (0.99-1.04)	
LDL-Chol (mg/dl)	116.1 (30.6)	0.99 (0.78-1.11)		1.05 (0.87-1.24)	
HDL-Chol (mg/dl)	54.7 (16.2)	0.99 (0.73-1.26)		0.79 (0.50-1.18)	
Systolic BP (mmHg)	135.9 (17.1)	0.86 (0.69-1.12)		0.88 (0.61-1.25)	
Diastolic BP (mmHg)	72.2 (10.9)	**0.65* (0.46-0.95)**	**0.65*(0.46-0.95)**	0.98 (0.59-1.78)	
Insulin user (%)	33.90%	0.97 (0.51-1.64)		**3.48*(1.42-15.24)**	**3.48*(1.42-15.24)**
CVA = < 70 y.o.	Mean(SD)	**Hgb A1C = < 7.0 (Gp.MF)**		**7.0 < HgbA1C (Gp.MP)**	
Number of events		17		15	
		Univariate	Multivariate	Univariate	Multivariate
Male/Female	1.16	1.16 (0.65-2.26)		1.06(0.58-1.98)	
Age (y.o.)	60.9 (7.9)	1.01 (0.94-1.11)		1.02(0.94-1.13)	
Duration of diabetes (years)	9.15 (8.22)	**0.81*(0.60-0.99)**		**1.08*(1.01-1.14)**	**1.06*(1.01-1.11)**
HemoglobinA1C(%)	7.38 (1.31)	4.34*(1.07-25.8)	4.11^{+}(1.01-13.40)	0.77(0.35-1.35)	
Triglyceride (mg/dl)	146.9(129.5)	1.00 (0.98-1.02)		1.01(0.97-1.07)	
LDL-Chol (mg/dl)	119.2 (34.1)	0.99 (0.96-1.14)		0.98(0.79-1.17)	
HDL-Chol (mg/dl)	55.4 (16.2)	0.78 (0.49-1.18)		**0.60**(0.35-0.63)**	**0.43**(0.23-0.78)**
Systolic BP (mmHg)	133.5 (17.5)	0.98 (0.71-1.41)		0.99(0.75-1.44)	
Diastolic BP (mmHg)	75.5 (11.7)	1.26 (0.76-2.17)		1.01(0.62-1.83)	
Insulin user (%)	32.80%	0.89 (0.35-1.77)		0.74(0.35-1.18)	
CVA 70 < y.o.	Mean(SD)	**Hgb A1C = < 7.0 (Gp.OF)**		**7.0 < HgbA1C (Gp.OP)**	
Number of events		16		22	
		Univariate	Multivariate	Univariate	Multivariate
Male/Female	0.96	1.33 (0.79-2.40)		0.68 (0.36-1.17)	
Age (y.o.)	75.4 (4.3)	1.04 (0.93-1.14)		1.05 (0.93-1.17)	
Duration of diabetes (years)	10.18 (9.08)	1.01 (0.96-1.05)		1.04 (0.98-1.09)	
HemoglobinA1C(%)	7.26 (1.15)	1.27 (0.47-4.29)		0.74 (035-1.32)	
Triglyceride (mg/dl)	129.2 (62.2)	1.01 (0.91-1.07)		1.05 (0.97-1.19)	
LDL-Chol (mg/dl)	116.1 (30.6)	0.96 (0.74-1.10)		1.04 (0.88-1.21)	

Table 2 The relationship between IHD, CVA and clinical variables *(Continued)*

HDL-Chol (mg/dl)	54.7 (16.2)	**0.61*(0.38-0.93)**		**0.71*(0.47-1.02)**	**0.71^{+}(0.49-1.02)**
Systolic BP (mmHg)	135.9 (17.1)	0.80 (0.64-1.04)		1.25 (0.91-1.72)	
Diastolic BP (mmHg)	72.2 (10.9)	0.83 (0.55-1.34)		1.09 (0.68-1,85)	
Insulin user (%)	33.90%	**1.93* (1.05-3.62)**	**1.93* (1.05-3.62)**	1.11 (0.53-2.24)	

$^{+}$P < 0.1, *P < 0.05, **P < 0.01

Clinical characteristics of diabetic individuals and the results of the univariate and multiple regression analyses examining the association between various clinical variables and IHD and CVA risk with stratification by age group. Patients were divided into two age categories: ≤ 70 years of age vs. > 70 years of age. Odds ratios and the corresponding 95% confidence intervals, which are in parentheses following the odds ratios, are shown. Abbreviations: Male/Female: ratio of male patients to female patients; Hemoglobin A1C (%): glycated hemoglobin A1C; LDL-C (mg/dl): Low-density lipoprotein cholesterol; HDL-C (mg/dl): High-density lipoprotein cholesterol; Systolic BP (mmHg): systolic blood pressure; Diastolic BP (mmHg): diastolic blood pressure.

Bold characters indicate that a factor was found to be statistically significant by the univariate or multivariate regression analyses (p < 0.05).

each group divided by gender and glucose control levels (HbA1C) (Table 4).

Male patients: IHD was associated with high systolic blood pressure in the male/fair glycemic control group, age in the male/poor control group. Interestingly, insulin use was associated with IHD in the male/poor group (OR = 4.11, 95% CI = 1.22-8.12; p = 0.018).

Female patients: IHD was associated with a short duration of diabetic history in the female/fair and female/poor groups.

Multiple regression analysis examining the relationship between clinical variables and the risk of CVA for each group divided by gender and glucose control

Multiple regression analysis was performed to evaluate the relationship between CVA and each clinical measurement and insulin treatment for each group divided by gender and glucose control levels (HbA1C) (Table 4).

Male patients: CVA was not significantly associated with any variables.

Female patients: CVA was associated with a short duration of diabetic history in the female/fair and female/poor groups. Insulin use was associated with CVA in the female/poor group (OR = 3.26, 95% CI = 1.12-6.24; p = 0.02).

Table 3 The stepwise multiple regression analyses to the onset of IHD or CVA

Ischemic Heart Diseases		
= < 70 y.o.	HbA1C = < 7.0 (Gp.MF)	Systolic BP 0.016* HDL-C 0.035* LDL-C 0.049* Gender 0.036*
	7.0 < HbA1C (Gp.MP)	None
70 y.o. <	HbA1C = < 7.0 (Gp.OF)	Diastolic BP 0.033*
	7.0 < HbA1C (Gp. OP)	Insulin 0.006** Age 0.016*
Cerebrovascular Attacks (Stroke)		
= < 70 y.o.	HbA1C = < 7.0 (Gp.MF)	(HbA1C 0.059)
	7.0 < HbA1C (Gp.MP)	HDL-C 0.028*
70 y.o. <	HbA1C = < 7.0 (Gp.OF)	Insulin 0.028* (HDL-C 0.086)
	7.0 < HbA1C (Gp. OP)	None

Discussion

In the present study, IHD was associated with a higher systolic blood pressure and a lower HDL-C in patients in the 70 years of age or younger patients with fair glycemic control and a lower diastolic blood pressure in older patients with fair glycemic control. Insulin use was associated with IHD in the **OP** group, whereas it was associated with CVA in the **OF** group. CVA was associated with lower HDL-C and a longer duration of diabetes in patients in the NP group. The results obtained by stepwise analysis were similar, except that LDL-C was associated with IHD in patients in the NP group. In the elderly, insulin use and glycemic control may contribute differently to IHD and CVA risks.

The frequency of diabetes increases with age, and there are many elderly diabetic individuals. However, the risk factors for IHD and CVA have not been clearly defined in elderly diabetics. Furthermore, there is insufficient clinical evidence regarding the effects of insulin therapy on IHD and CVA risks in elderly patients [13,14]. Therefore, this study examined the effect of insulin therapy on IHD and CVA risks among elderly diabetics. This study also examined the possibility that IHD and CVA risk factors varied by age.

In the present study, differences in the IHD and CVA risks by gender were not evident. IHD was associated with high systolic blood pressure in the MF group, age in the MP control, short duration of diabetic history in the FF and FP groups. Insulin use was associated with IHD in the MP group and with CVA in the FP group (OR = 3.26, 95% CI = 1.12-6.24; p = 0.02). CVA was associated with short duration of diabetes in both female groups.

IHD risk factors

American guidelines for diabetic control suggest that diabetic individuals under 70 years of age have a risk of developing IHD similar to that for non-diabetic individuals

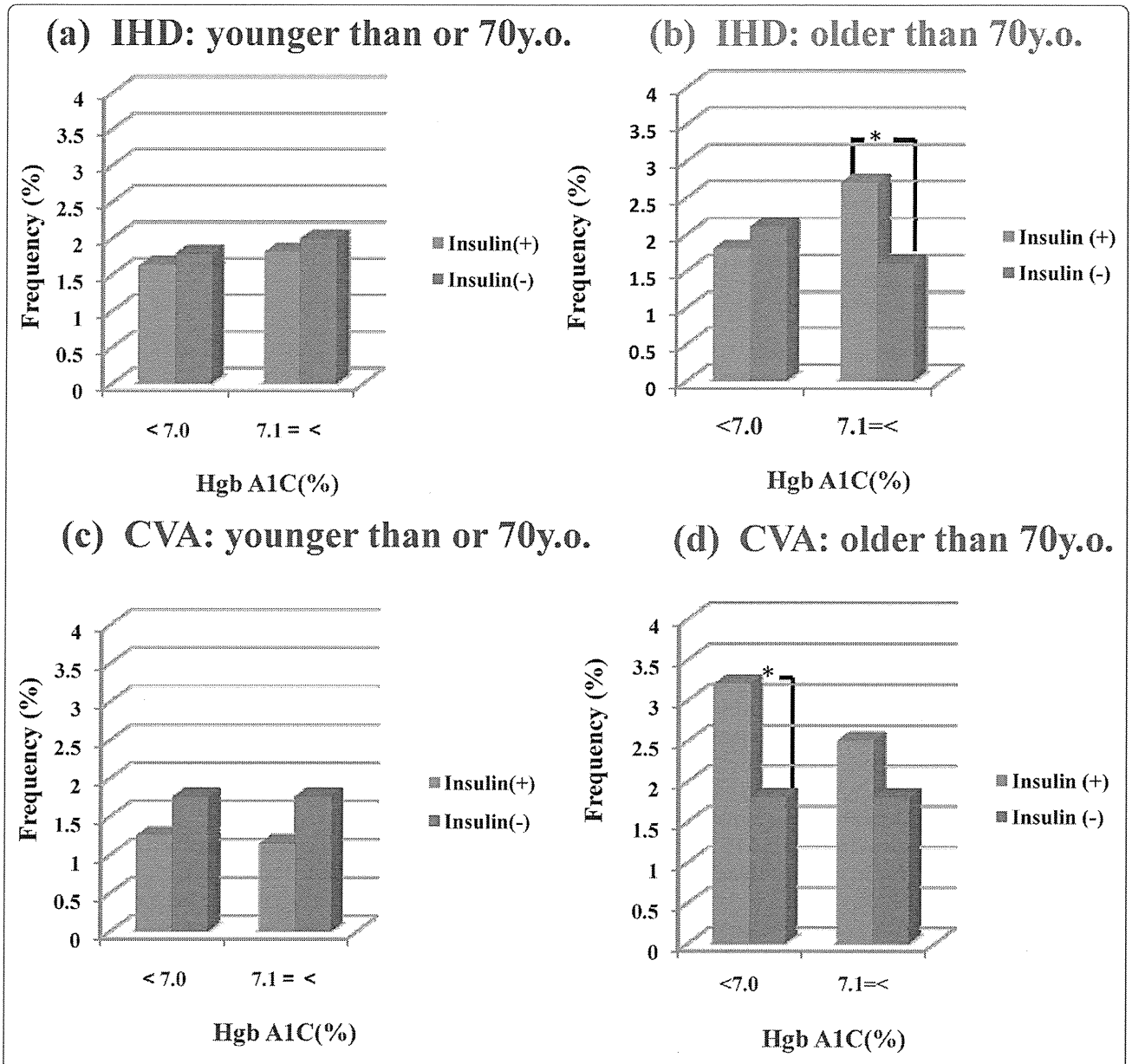

Figure 2 Effects of age and insulin therapy on the risk of IHD and CVA among diabetic patients stratified by glycemic control (measured by hemoglobin A1C, stratified as ≤ 7.0% vs. > 7.0%). Incidence of ischemic heart disease (IHD), left column, and cerebrovascular accident (CVA), right column. Patients with lower hemoglobin A1C values, upper column, and higher hemoglobin A1C values, lower column. (a) Incidence of ischemic heart disease (IHD) (≤ 70 years of age). (b) Incidence of ischemic heart disease (IHD) (> 70 years of age). (c) Incidence of stroke (CVA) (≤ 70 years of age). (d) Incidence of stroke (CVA) (> 70 years of age). These data were adjusted for age and gender. *p < 0.05 Blue column: subjects using insulin. Red column: subjects not using insulin.

having a prior myocardial infarction [8], and the results from the present study support this concept (Figure 2). We found that the incidence of IHD among diabetic Japanese was relatively high and comparable with that of IHD in Western countries, such as the U.K. [3,15]. The incidence of IHD in our study was approximately three times higher than the incidence rates previously reported in Japanese trials, such as Mega study investigating patients under 70 years of age, not all of whom had diabetes [16,17].

In the present study, among diabetic individuals in the NF group, higher systolic blood pressure and lower HDL-C were associated with a risk of IHD. Systolic blood pressure, not diastolic pressure, was confirmed as a classical IHD risk factor from previous studies [18-20]. The age of diabetic patients in those reports was less