Table 2 Major Adverse Drug Reactions During the J-LIT Extension 10 Study

	Initial study (6 years) (n=51,321)	Extension study (4 years) (n=19,905)	p value
	n (%)	n (%)	
Total number of ADRs	1,670 (3.25)	290 (1.46)	<0.001
Major ADRs			
Hepatic	500 (0.97)	55 (0.28)	<0.001
Musculoskeletal	439 (0.86)	89 (0.45)	<0.001
Gastrointestinal	291 (0.57)	21 (0.11)	<0.001
Generalized	200 (0.39)	32 (0.16)	<0.001
Skin	185 (0.36)	17 (0.09)	<0.001
Kidney	96 (0.19)	8 (0.04)	<0.001
Neurological	93 (0.18)	25 (0.13)	0.074
Laboratory abnormalities	67 (0.13)	11 (0.06)	0.006
Hematological	62 (0.12)	7 (0.04)	<0.001

ADRs, adverse drug reactions. Other abbreviation see in Table 1.

Serum Lipid Profile Over 10 Years

Compared with the baseline values, serum TC and LDL-C levels were markedly lower at 6 months after the start of simvastatin treatment, and similar levels were maintained during the 6-year and 10-year treatment periods (Fig 1). In contrast, the serum HDL-C level gradually became higher throughout the 10-year treatment period. The time course of the changes of lipid levels during the 10-year treatment period is shown in Fig 2. Serum TC, LDL-C, and TG levels showed a marked decrease after 6 months of treatment and then continued to decrease slightly during the remainder of the treatment period. The mean TC level decreased from 269 mg/dl at baseline to 217 mg/dl after 6 years and 215 mg/dl after 10 years of treatment. The average reduction was 19.3% after 6 years and 20.2% after 10 years. The mean LDL-C level decreased from 182 mg/dl at baseline to 129 mg/dl after 6 years and 126 mg/dl after 10 years of treatment, with the average reduction being 28.9% after 6 years and 30.6% after 10 years. The mean TG level decreased from 196 mg/dl at baseline to 155 mg/dl after 6 years and 147 mg/dl after 10 years, and the average reduction was 21.0% after 6 years and 25.2% after 10 years. In contrast, serum HDL-C continued to increase gradually throughout the treatment period. The mean HDL-C level increased from 52.6 mg/dl at baseline to 58.1 mg/dl after 6 years and 59.9 mg/dl after 10 years of simvastatin treatment, with the average increment being 10.5% after 6 years and 13.9% after 10 years. The ratios of continuing simvastatin treatment in the TC groups during treatment <180, 180–, 200–, 220–, 240– and 260– (mg/dl) were 76%, 79%, 78%, 72%, 65% and 57%, respectively.

ADRs

ADRs were reported in 290 patients, with an incidence of 1.46% during the 4-year extension period. The most frequent ADRs during the extension period are summarized in Table 2 and included musculoskeletal disorders (0.45%), hepatic disorders (0.28%), generalized disorders (0.16%), neurological disorders (0.13%), and gastrointestinal disorders (0.11%). During the initial J-LIT study, ADRs occurred in 3.25% of patients. No differences in the major ADRs were observed between both periods, except for a lower overall incidence during the extension period. During the 4-year extension study, as well as J-LIT itself, there were no episodes of rhabdomyolysis, which was defined as a creatine kinase (CK) level ≥10,000 IU/L, in association with muscular symptoms, by the ADR Assessment Subcommittee.

Table 3 Incidence of Coronary Events or Deaths During the 10-Year Follow-up Period

	Primary prevention (n=41,801) n (incidence)	Secondary prevention (n=4,599) n (incidence)
Coronary events	270 (0.90)	126 (3.99)
All deaths	1,336 (4.47)	289 (9.14)
Cardiac death	393 (1.32)	131 (4.15)
Cancer death	481 (1.61)	71 (2.25)
Other deaths	462 (1.55)	87 (2.75)

Incidence = no. of events or deaths/1,000 patient-years.

Incidence of Coronary Events and Death During Treatment

Coronary events occurred in 270 patients from the primary prevention cohort during the 10-year study period (an incidence of 0.90 events per 1,000 patient-years) and in 126 patients from the secondary prevention cohort (an incidence of 3.99 events per 1,000 patient-years) (Table 3).

During the entire course of the study, 1,336 patients in the primary prevention cohort died (an incidence of 4.47 events per 1,000 patient-years) and 289 patients in the secondary prevention cohort (9.14 events per 1,000 patient-years) (Table 3). The number of cardiac deaths was 393 (1.32 events per 1,000 patient-years) in the primary prevention cohort and 131 (4.15 events per 1,000 patient-years) in the secondary prevention cohort. The number of cancer deaths was 481 (1.61 events per 1,000 patient-years) and 71 (2.25 events per 1,000 patient-years), respectively, and the number of other deaths was 462 (1.55 events per 1,000 patient-years) and 87 (2.75 events per 1,000 patient-years), respectively.

Relative Risk of Events and Lipid Levels During 10 Years of Treatment

The relative risk of coronary events was analyzed in relation to the average serum lipid levels during the 10-year treatment period. Higher serum levels of TC, LDL-C, and TG were closely related to the relative risk of coronary events in the primary prevention cohort (Fig 3). Thus, the relative risk of coronary events was significantly higher in the patients with a TC level ≥240 mg/dl compared with those with a level <180 mg/dl. Also, patients with an LDL-C level ≥140 mg/dl had a far higher risk than those with a level <100 mg/dl, and patients with a TG level ≥300 mg/dl also had a higher risk of coronary events than patients with a level <150 mg/dl. In contrast, serum HDL-C was inversely related to the risk of coronary events and the relative risk was lower in patients with an HDL-C level ≥40 mg/dl compared with patients with a level <40 mg/dl.

In the secondary prevention cohort, the relative risk of coronary events was related to the serum TC or LDL-C level and was inversely correlated with the HDL-C level (Fig 4). Patients with a TC level ≥240 or LDL-C level ≥160 mg/dl had a higher risk than patients with a level <180 or <100 mg/dl, respectively, whereas patients with an HDL-C level ≥40 mg/dl had a lower risk than patients with a level <40 mg/dl. There was no correlation between the relative risk of coronary events and the serum TG level.

The relative risk of all-cause mortality was also analyzed in relation to the average serum lipid levels during the 10-year treatment period in the primary prevention cohort

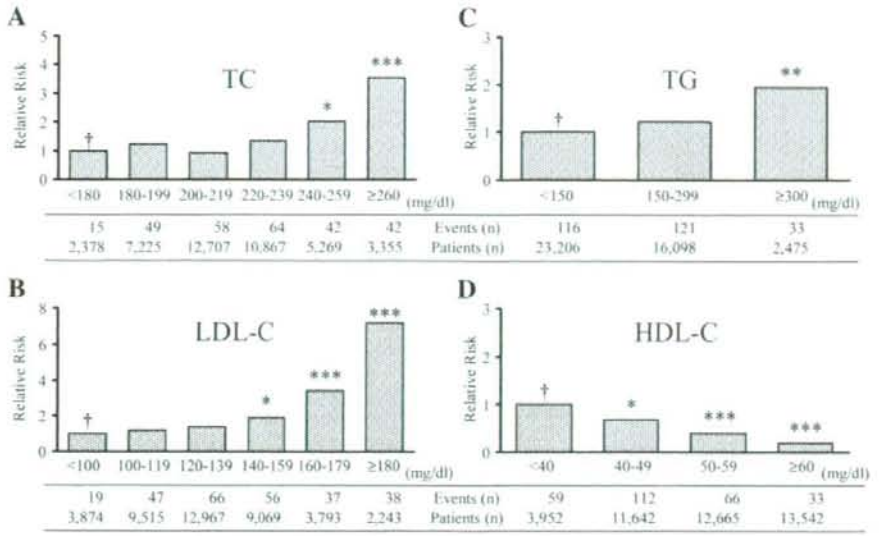

Fig 3. Relationship between the relative risk of coronary events and the serum levels of (A) TC, (B) LDL-C, (C) TG, and (D) HDL-C during 10 years of follow-up in the primary prevention cohort. Data were adjusted for age, sex, hypertension, diabetes mellitus, and smoking. *p<0.05, **p<0.01, ***p<0.001 vs † reference category. See Fig 1 for abbreviations.

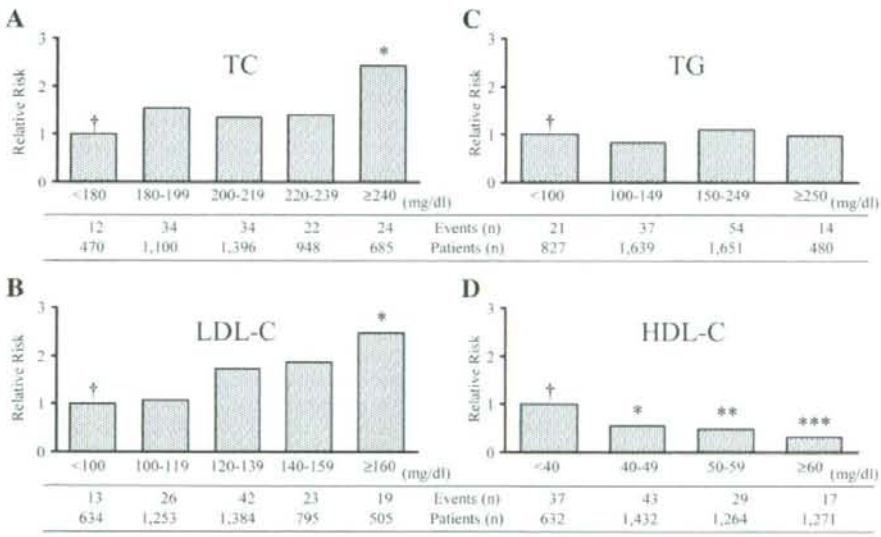

Fig 4. Relationship between the relative risk of coronary events and the serum levels of (A) TC, (B) LDL-C, (C) TG, and (D) HDL-C during 10 years of follow-up in the secondary prevention cohort. Data were adjusted for age, sex, hypertension, diabetes mellitus, and smoking. *p<0.05, **p<0.01, ***p<0.001 vs † reference category. See Fig 1 for abbreviations.

study (data not shown). The results were the same as those in the previous report[2]

Discussion

The J-LIT Extension 10 study was conducted to follow up patients who had been enrolled in the J-LIT study;[1–4] approximately 40% of the J-LIT patients agreed to continue the study for another 4 years. There were no significant differences in the baseline characteristics of the patients who were followed up for the additional 4-year period and of all patients in the original J-LIT study.

A low-dose of simvastatin (usually 5 mg/day) effectively maintained low serum TC, LDL-C, and TG levels in Japanese patients with hypercholesterolemia over 10 years. The mean reduction of serum TC and LDL-C levels after 10 years was 20.2% and 30.6%, respectively. In contrast, the serum HDL-C level gradually increased throughout the entire

study period, with the mean level rising from 52.6 mg/dl at baseline to 58.1 mg/dl after 6 years and 59.9 mg/dl after 10 years. These changes in HDL-C suggested that long-term, low-dose simvastatin therapy may have the additional benefit of increasing HDL-C levels.

As previously reported for the original J-LIT study[4] the magnitude of the reduction in TC (approximately 20%) or LDL-C (approximately 30%) achieved in Japanese patients treated with simvastatin at 5 mg/day corresponded to the effect of 20 mg/day in Western studies[9,10] There seems to be little difference in the pharmacokinetics and LDL-C reduction is enhanced by a low-fat diet[11,12] The reasons why Japanese patients respond so well to a low-dose of simvastatin are not clear. Dietary difference between Japanese and Western populations may at least partly account for differences in the sensitivity to simvastatin. Thus, low-dose statin treatment combined with a low-fat diet could benefit patients in Western countries.

The incidence of ADRs to simvastatin during the 4-year extension period was lower (1.5%) than that observed during the J-LIT period (3.3%)[4] which suggests that patients with a higher risk of developing ADRs experienced them during the initial 6-year treatment period. No cases of rhabdomyolysis (CK ≥10,000 IU/L combined with muscular symptoms) were reported throughout the 10-year study period. The low incidence of ADRs during the entire study period is considered to have been related to the low doses used (ie, simvastatin was given at 1/4 of the dose used in Western countries). Thus, the J-LIT Extension 10 study confirmed that a low dose of simvastatin is suitable for Japanese patients with hypercholesterolemia, not only to sufficiently reduce the serum lipid levels over the long term, but also to minimize ADRs.

There was no significant difference in the incidence of coronary events between the J-LIT period[2,3] and the extension period. Controlling serum lipids is thought to prevent an increase in the occurrence of coronary events. However, the overall mortality rate of the primary prevention cohort was higher during the 10-year period (4.47 deaths per 1,000 patient-years) than for the 6-year J-LIT period (3.69 deaths per 1,000 patient-years). The main factor contributing to the increase of mortality in the present study was probably aging, because the mean age had increased by 6 years for this extension study compared with that for the original J-LIT study.

We have already reported that in the J-LIT study the TC, LDL-C, and TG levels correlated with the incidence of coronary events, whereas HDL-C was inversely correlated, in Japanese patients with hypercholesterolemia receiving low-dose simvastatin treatment[2,3] The present extension 10 study confirmed those results with respect to a relationship between serum lipids and the incidence of coronary events. The serum LDL-C level was positively correlated with the incidence of coronary events, whereas serum HDL-C was inversely correlated in both the primary and secondary prevention cohorts. Especially in the primary prevention cohort, patients with a serum TC ≥240 or LDL-C ≥140 mg/dl developed coronary events more often than patients with TC <180 mg/dl or LDL-C <100 mg/dl. This is the first time such findings have been obtained from a very long-term study of Japanese patients.

Mantel-Teeuwisse et al reported that the rate of persistence with statin therapy was 61.5% at 1 year and decreased to 46.5% after 2 years in a general patient population from the Netherlands that included all age categories and both primary and secondary prevention cohorts[13] Similar surveys of elderly patients have also shown relatively low persistence rates[14,15] Elderly patients with and without recent CHD have low rates of continuing with statin therapy, suggesting that many patients who commence statins receive little or no benefit because of premature discontinuation.

In Japan, Nagashima et al[16] studied the percentage of patients who achieved the target LDL-C level (<100 mg/dl) specified by the Japan Atherosclerosis Society[17] and reported that only 29.9% of patients achieved it. The achievement rate among those on statin therapy was 41.3%, which was significantly higher than that (23.4%) among patients not receiving statins ($p<0.0001$). Those results suggest that many patients with dyslipidemia are not adequately treated in Western countries or in Japan. In the J-LIT and J-LIT Extension 10 studies, patients had higher continuation rates for statin therapy, probably because they were regularly monitored by their physicians. Because many patients who start statin therapy may receive little benefit from it as a result of premature discontinuation, we should aim to treat dyslipidemia with statins over the long-term in order to reduce cardiovascular events.

In conclusion, the present 10-year study confirmed that low-dose simvastatin therapy is suitable for Japanese patients with hypercholesterolemia, both for adequately controlling serum lipids and for minimizing ADRs. It was also suggested that the serum LDL-C level should be <140 mg/dl to decrease the risk of coronary events and should be maintained at a low level for as long as possible.

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Association of serum apolipoprotein B48 level with the presence of carotid plaque in type 2 diabetes mellitus

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ABSTRACT

Aims: The atherogenicity of chylomicron remnants has been discussed. We examined whether serum apoB48 level is associated with the presence of carotid plaque in type 2 diabetic patients.

Method: Forty type 2 diabetic patients (21 males and 19 females, 52.8 ± 11.8 years old; mean ± S.D.) were divided into two groups by the presence or absence of carotid plaque. The diurnal change of serum apoB48 level was measured by enzyme-linked immunosorbent assay.

Results: Fasting serum apoB48 level was higher in the subjects with carotid plaque than those without (6.5 ± 3.8 vs. 4.1 ± 1.9 μg/ml, $p = 0.01$). Age- and gender-adjusted analysis showed that the presence of carotid plaque was associated with fasting apoB48 (OR 1.43; 95% CI, 1.07–2.09, $p = 0.04$) and triglyceride (OR 1.14; 95% CI, 1.02–1.32, $p = 0.04$) levels. In normal LDL-cholesterol (<140 mg/dl) subjects, the presence of carotid plaque was associated with fasting apoB48 level (OR 2.16; 95% CI, 1.22–5.32, $p = 0.04$), but not associated with fasting triglyceride level (OR 1.11; 95% CI, 0.99–1.30, $p = 0.13$).

Conclusions: Serum apoB48 level was strongly associated with the presence of carotid plaque in type 2 diabetic patients.

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1. Introduction

Dyslipidemia is related to the pathogenesis of atherosclerosis. The relationship between cardiovascular disease (CVD) and hypercholesterolemia, increased low-density lipoprotein cholesterol (LDL-C) level, or decreased high-density lipoprotein cholesterol (HDL-C), has been well documented. In contrast, the association between CVD and abnormality in triglyceride (TG) metabolism is still debated. Several prospective studies have shown positive association between serum TG level and CVD [1–3]; however, other studies have demonstrated that TG level was no longer associated with atherosclerosis after adjustment for HDL-C [4,5]. The heterogeneity of TG-rich lipoproteins (i.e., chylomicron, VLDL, and those remnant lipoproteins) would make it difficult to evaluate the atherogenicity of hypertriglyceridemia. Recently, it was reported that elevated nonfasting TG level was associated with increased risk of cardiovascular events [6]. It suggests an association between increased remnant lipoproteins and increased risk of CHD. However, it has not been clinically

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established which TG-rich lipoproteins play a crucial role in atherosclerosis.

Chylomicron and its remnant have a characteristic apolipoprotein, apolipoprotein B48 (apoB48), each having one apoB48 molecule per particle. Hence, apoB48 could be a good marker for TG-rich lipoproteins derived from intestine. Several clinical studies indicated that apoB48 level was associated with atherosclerosis [7–10], but the association was not confirmed in other studies [11,12]. The inconsistent results may be due in part to differences in assay method for apoB48. To date, apoB48 level has been commonly evaluated by SDS-PAGE. However, the apoB48 value based on SDS-PAGE is essentially semi-quantitative [13]. Recently, an enzyme-linked immunosorbent assay (ELISA) method for apoB48 was newly established [14,15]. The ELISA method is simple and quantitative, and it has been used in some clinical studies [10,14].

Type 2 diabetes mellitus (DM) is one of the important risk factors for CVD. The mortality and morbidity of diabetic patients are markedly high compared to non-diabetic subjects [16]. In diabetic patients, dyslipidemia is recognized as an important CVD risk factor. A characteristic pattern of dyslipidemia in DM is high TG, low HDL-C, elevated small dense LDL-C, and postprandial lipemia. In type 2 DM, the production and clearance of TG-rich lipoproteins are disturbed [17]. Previous studies in type 2 DM demonstrated that apoB48 level was elevated [18], and apoB48-containing lipoproteins were increased in production and decreased in clearance [19]. Therefore, serum apoB48 level is expected to be increased throughout the day in type 2 DM; however, at present, little is known about the diurnal change. Furthermore, it remains uncertain whether serum apoB48 level is associated with atherosclerosis in type 2 DM. In the present study, we examined the diurnal change of serum apoB48 level and its association with atherosclerosis in type 2 DM.

2. Subjects and methods

2.1. Subjects

Basal data of 147 type 2 diabetic patients admitted to Nippon Medical School Hospital (Tokyo, Japan) for glycemic control were checked. Among these patients, 107 were excluded for the following reasons: treatment with steroid or lipid lowering drugs, complication with diabetic proliferative retinopathy or macroalbuminuria (>300 mg/day), primary hyperlipidemia, liver diseases, chronic renal diseases (>1.2 mg/dl of serum creatinine), infection, malignancy, endocrine diseases, recent major surgery or illness. After receipt of informed consent, 40 patients [21 males, 19 females; age 30–72 years (52.8 ± 11.8, mean ± S.D.)] were enrolled as the subjects of the present study. The subjects were divided into two groups by the presence or absence of carotid plaque (plaque (+) or (−), respectively). Standard lifestyle modifications (exercise and dietary changes) had been adopted for each subject before admission. Six subjects were treated with insulin (14.3% and 15.8% in plaque (+) and (−), respectively, $p = 0.89$) and 20 were treated with oral agents for diabetes (42.9% and 57.9%, $p = 0.34$), including sulphonylurea, glimepiride, metformin, thiazolidinedione, and alpha-glucosidase inhibitor. Fifteen subjects did not take any medication for diabetes (42.9% and 26.3%, $p = 0.30$). There was no bias in treatment for diabetes between the two groups. This study was approved by the Nippon Medical School Hospital Ethics Committee.

2.2. Blood specimen preparation

The study-related assessments were performed within 10 days after admission. All subjects were assessed after an overnight fast of 14 h. The total calories of daily diet (kcal/day) were calculated as 27.5 (kcal) × ideal body weight (IBW). IBW (kg) was calculated as 22 × [height (m)]^2 according to the recommendation of the Japan Society for the Study of Obesity. The nutritional composition of daily diet was 20% fat, 25% protein, and 55% carbohydrate. Each subject was provided three meals per day, at 8:00, 12:00, and 18:00. To assess the diurnal changes in serum apoB48, serum lipids, and plasma glucose (PG), blood specimens were obtained at 30 min before and 2 h after each meal (7:30, 10:00, 11:30, 14:00, 17:30, and 20:00), night (23:00), and early morning (3:00). Blood was drawn from the cubital vein and collected in test tubes. The blood samples collected from 7:30 to 14:00 were immediately centrifuged, and the samples from 17:00 to 3:00 were kept at 4 °C and centrifuged the next morning. To analyze PG, a portion of each blood sample was collected into a tube with NaF and anticoagulant, and centrifuged immediately after blood collection. Serum and plasma were immediately collected after the centrifugations, and stored at −80 °C until assayed.

2.3. Clinical and biochemical assessment

Subjects underwent a physical examination (height, weight, blood pressure, and waist circumference). Blood pressure was measured with a standard mercury sphygmomanometer in a sitting position in the morning. Smoking habits, a familial history of CVD, and duration of DM were assessed by interview. Smoking was defined as current or past smoking. Serum total cholesterol (TC), HDL-C, and TG were measured by enzymatic methods (LABOSPECT 008, Hitachi, Tokyo, Japan). Glycated albumin (GA) was also measured enzymatically (JCA-BM12, Japan Electron Optics Laboratory, Tokyo, Japan). LDL-C level was calculated by the Friedewald formula [20]. Plasma glucose (PG) was measured by a glucose oxidase method (ADAMS Glucose GA-1170, Arkray, Kyoto, Japan), and hemoglobin A_{1c} (HbA_{1c}) was measured by high performance liquid chromatography (ADAMS A1c HA-8160, Arkray). The plasma levels of lipoprotein lipase mass and adiponectin were measured by ELISA (Daiichi Pure Chemicals and Otsuka pharmaceutical, Tokyo, Japan, respectively). Serum apoB48 level was measured by ELISA using an anti-human apoB48 monoclonal antibody (B-48-151) as previously described (Fujirebio, Tokyo, Japan) [14,15]. The apoB48 assay was highly reproducible, with coefficients of variation of 1.9–3.1% and 2.2–4.4% for intra- and inter-assay, respectively.

Carotid artery status was examined by high-resolution B-mode ultrasonography (SDU-2000, Shimadzu, Kyoto, Japan; iU22 and EnVisor, Philips Medical Systems, Andover, MA, USA; LOGIQ 7, GE Healthcare, Tokyo, Japan). The ultrasound devices

were used with electrical linear transducers (3–12 MHz). Subjects were placed in a supine position with the neck hyper-extended. Carotid plaque was assessed in common carotid artery, the carotid artery bifurcation, and internal carotid artery bilaterally. Plaque was defined as a localized intima-media thickness (IMT) ≥1.0 mm with marked protuberance. All measurements were performed by well-trained doctors.

2.5. Statistical analysis

Values are presented as means ± S.D. Statistical analysis was performed by χ^2 test or Fisher's exact test, as appropriate, and Student's *t*-test or Welch's *t*-test, as appropriate. Logistic regression analysis was used to calculate odds ratios of each variable for carotid plaque. Because TG values were highly dispersed, the value TG/10 was used for the calculation of odds ratio. Data were analyzed with JMP 6 software (SAS Institute, Cary, NC). All statistical tests were two-sided, and $p < 0.05$ were considered significant.

3. Results

3.1. Clinical and laboratory data

The clinical and laboratory data of all subjects are presented in Table 1. There were no differences in gender distribution, age, duration of DM, smoking habit, body mass index, or blood pressure between the plaque (+) and (−) groups. The two groups were similar in fasting TC, LDL-C, PG, and HbA_{1c} levels. The fasting serum apoB48 and TG levels were significantly higher in plaque (+) than in plaque (−).

3.2. Diurnal changes in apoB48, lipids, and glucose

Diurnal changes in serum apoB48 and TG levels are shown in Fig. 1A and B. Serum apoB48 level was sharply increased after breakfast and reached peaks after lunch and dinner, then returned toward the fasting levels at 3:00 in both plaque (+) and plaque (−). ApoB48 and TG levels were significantly higher in plaque (+) than in plaque (−) throughout the day, with the exception of a few time points. PG level was elevated after each meal in a similar pattern for both groups (Supplementary Fig. 1A). The diurnal fluctuation of TC and HDL-C levels was small and similar in both groups (Supplementary Fig. 1B and C).

3.3. Association of apoB48 with plaque

The results of logistic regression analysis showed that fasting serum apoB48 and TG levels were significantly associated with the presence of carotid plaque, regardless of adjustment for age and gender (Table 2). Other variables were not associated with the presence of carotid plaque.

3.4. Analyses of subjects with normal LDL-C level (<140 mg/dl)

To exclude the involvement of LDL-C level on the association between apoB48 and carotid plaque, we examined this factor in normal LDL-C (<140 mg/dl) subjects ($n = 25$). The normal LDL-C level of < 140 mg/dl was defined according to the recommendation of Japan Atherosclerosis Society [21]. Among the normal LDL-C subjects, 12 subjects were with carotid plaque [plaque (+)] and 13 were without the plaque [plaque (−)].

Table 1 – Clinical and laboratory data for all subjects

	Total ($n = 40$)	Plaque (+) ($n = 21$)	Plaque (−) ($n = 19$)	p value (+) vs. (−)
Male/female	21/19	13/8	8/11	0.21
Age (year)	52.8 ± 11.8	53.4 ± 9.9	52.1 ± 13.9	0.72
Duration of DM (year)	5.9 ± 6.3	5.9 ± 7.0	5.9 ± 5.7	0.99
Familial history of CVD (%)	10.0	14.3	5.3	0.37
Smoking (%)	57.5	66.7	47.4	0.22
BMI (kg/m^2)	24.1 ± 3.9	23.9 ± 3.1	24.4 ± 4.7	0.68
Waist circumference (cm)	83.8 ± 12.8	82.5 ± 13.3	85.5 ± 12.5	0.48
Blood pressure (mmHg)				
Systolic	121 ± 12	122 ± 11	120 ± 13	0.59
Diastolic	73 ± 10	73 ± 9	73 ± 11	0.80
TC (mg/dl)	210 ± 43	217 ± 38	201 ± 47	0.23
HDL-C (mg/dl)	52 ± 17	49 ± 14	55 ± 19	0.21
LDL-C (mg/dl)	130 ± 32	136 ± 30	123 ± 32	0.19
TG (mg/dl)	140 ± 73	163 ± 84	115 ± 48	0.03
Glucose (mg/dl)	177 ± 59	181 ± 67	172 ± 50	0.64
HbA_{1c} (%)	9.8 ± 2.1	10.1 ± 2.2	9.5 ± 1.9	0.40
GA (%)	28.0 ± 19.1	27.8 ± 8.5	28.1 ± 10.0	0.92
Adiponectin (μg/ml)	7.1 ± 4.4	6.3 ± 3.8	8.0 ± 4.9	0.25
LPL (ng/ml)	35.9 ± 14.4	34.4 ± 13.2	37.7 ± 15.7	0.49
ApoB48 (μg/ml)	5.4 ± 3.3	6.5 ± 3.8	4.1 ± 1.9	0.01

Values are means ± S.D. Plaque (+), subjects with carotid plaque; plaque (−), subjects without carotid plaque; DM, diabetes mellitus; CVD, cardiovascular disease; BMI, body mass index; TC, total cholesterol; HDL-C, HDL cholesterol; TG, triglyceride; LDL-C, LDL cholesterol; HbA_{1c}, hemoglobin A_{1c}; GA, glycated albumin; LPL, lipoprotein lipase; apoB48, apolipoproteinB48.

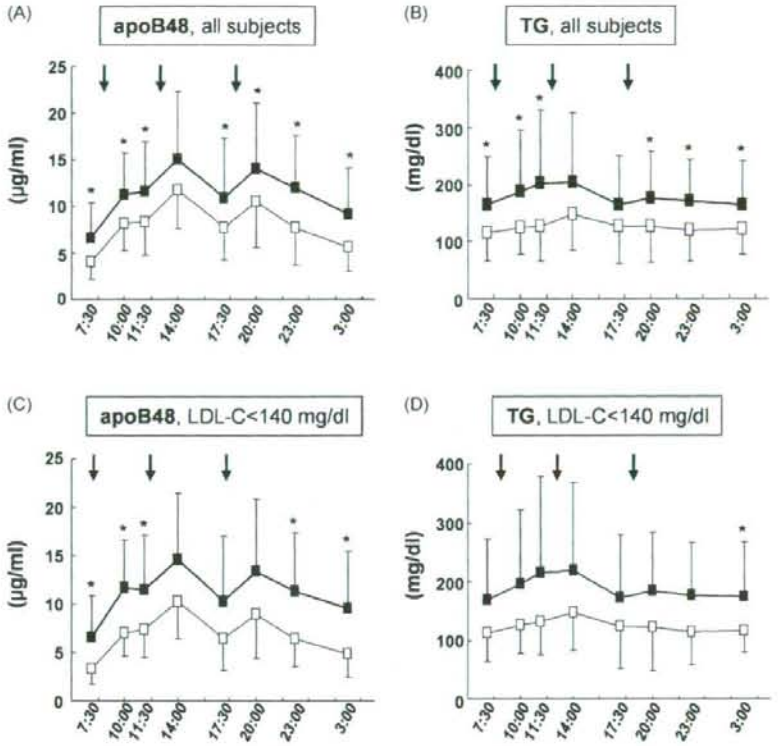

Fig. 1 – Diurnal changes of serum apolipoprotein B48 (apoB48, A and C) and triglyceride (TG, B and D) levels in the subjects with (■) and without (□) carotid plaque. A and B, all subjects (19 and 21 subjects with and without carotid plaque, respectively). C and D, normal LDL-cholesterol (LDL-C <140 mg/dl) subjects (13 and 12 subjects with and without carotid plaque, respectively). Values are means ± SD. *p < 0.05 vs. subjects without plaque. Arrows indicate breakfast, lunch, and dinner at 8:00, 12:00, and 18:00, respectively.

In the normal LDL-C subjects, fasting apoB48 level was significantly higher in plaque (+) than in plaque (–); however, the fasting TG level and other parameters were not different between the two groups (Table 3). The diurnal pattern of apoB48 level in the normal LDL-C subjects (Fig. 1C) was similar to that in all subjects (Fig. 1A). ApoB48 level was significantly higher in plaque (+) than in plaque (–) throughout the day, with the exception of a few time points (Fig. 1C). In contrast, TG level was not significantly different between the two groups, except at 3:00 (Fig. 1D). The diurnal changes of PG, TC, and HDL-C are similar in both plaque (+) and (–) (data not shown). Table 4 shows the results of logistic regression analysis in the normal LDL-C subjects. Regardless of gender and age adjustment, the fasting apoB48 level was significantly

Table 2 – Unadjusted, and age- and gender-adjusted odds ratios for the presence of carotid plaque in all subjects (n = 40)

Variable	Unadjusted		Adjusted	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Age	1.01 (0.96–1.07)	0.71		
Gender (male)	1.50 (0.80–2.87)	0.21		
Smoking	1.49 (0.79–2.88)	0.22	1.33 (0.63–2.82)	0.45
HDL-C	0.97 (0.93–1.01)	0.21	0.98 (0.92–1.02)	0.34
LDL-C	1.01 (0.99–1.04)	0.19	1.02 (0.10–1.05)	0.12
TG	1.13 (1.02–1.28)	0.04	1.14 (1.02–1.32)	0.04
HbA_{1c}	1.15 (0.84–1.60)	0.39	1.16 (0.84–1.66)	0.39
ApoB48	1.42 (1.08–2.03)	0.03	1.43 (1.07–2.09)	0.04

CI, confidence interval; HbA_{1c}, hemoglobin A_{1c}; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; TG, triglycerides; ApoB48, apolipoprotein B48.

Table 3 – Clinical and laboratory data in normal LDL-C (<140 mg/dl) subjects

	Total ($n = 25$)	Plaque (+) ($n = 12$)	Plaque (−) ($n = 13$)	p value (+) vs. (−)
Male/female	14/11	7/5	7/6	0.82
Age (year)	51.1 ± 12.5	50.0 ± 3.7	52.0 ± 3.5	0.71
Duration of DM (year)	4.8 ± 5.3	3.7 ± 1.5	5.8 ± 1.5	0.32
Familial history of CVD (%)	8.0	8.3	7.6	0.43
Smoking (%)	60.0	66.7	53.8	0.76
BMI (kg/m^2)	24.0 ± 3.4	23.5 ± 1.0	24.5 ± 3.7	0.50
Waist circumference (cm)	83.2 ± 13.3	79.5 ± 3.8	86.9 ± 3.8	0.18
Blood pressure (mmHg)				
Systolic	120 ± 13	122 ± 4	118 ± 4	0.37
Diastolic	71 ± 11	71 ± 11	71 ± 12	0.89
TC (mg/dl)	189 ± 34	199 ± 31	179 ± 35	0.15
HDL-C (mg/dl)	49 ± 14	49 ± 14	49 ± 14	0.89
LDL-C (mg/dl)	111 ± 22	116 ± 6	107 ± 6	0.32
TG (mg/dl)	140 ± 83	169 ± 23	113 ± 22	0.09
Glucose (mg/dl)	183 ± 65	196 ± 72	172 ± 59	0.37
HbA_{1c} (%)	9.7 ± 2.1	10.0 ± 0.6	9.3 ± 0.6	0.45
GA (%)	28.0 ± 9.9	28.1 ± 2.9	27.9 ± 2.8	0.96
Adiponectin (μg/ml)	6.9 ± 4.3	6.5 ± 1.3	7.3 ± 1.2	0.61
LPL (ng/ml)	35.9 ± 15.0	37.1 ± 4.6	34.8 ± 4.4	0.73
ApoB48 (μg/ml)	4.8 ± 3.6	6.5 ± 4.4	3.2 ± 1.5	0.03

Values are means ± S.D. Plaque (+), subjects with carotid plaque; plaque (−), subjects without carotid plaque; DM, diabetes mellitus; CVD, cardiovascular disease; BMI, body mass index; TC, total cholesterol; HDL-C, HDL cholesterol; TG, triglyceride; LDL-C, LDL cholesterol; HbA_{1c}, hemoglobin A_{1c}; GA, glycated albumin; LPL, lipoprotein lipase; apoB48, apolipoproteinB48.

associated with the presence of carotid plaque. The fasting TG level and other variables were not associated with the presence of carotid plaque.

4. Discussion

The present study demonstrated that fasting serum apoB48 level was associated with the presence of carotid plaque in type 2 DM and that diurnal apoB48 level was significantly higher in the plaque (+) group than in the plaque (−) group, throughout most of the day. The present result that apoB48 is associated with atherosclerosis is consistent with several previous studies [7–10]. To date, possible underlying mechanisms for the association have been suggested in several in vitro experiments. Flood et al. [22] indicated that apoB48 possesses a binding site to arterial wall proteoglycans, and the interaction will induce the retention of apoB48-containing lipoproteins. It is also reported that chylomicron remnants were taken up by macrophages via LDL receptor-related protein [23] and apoB48 receptor [24,25]. These results suggest that apoB48-containing lipoproteins could penetrate into vascular subendothelial space, and where it could be involved in macrophage foam cell formation. In fact, apoB48-containing lipoproteins penetrated into the artery wall and were retained within the subendothelial space of the carotid artery in Watanabe heritable hyperlipidemic rabbits [26]. Furthermore, ApoB48 was also identified in human atherosclerotic plaque [27].

It is well established that high LDL-C level is a strong determinant of atherosclerosis; however, high LDL-C level was not significantly associated with the presence of carotid plaque in the present study. This result might be due to the range and the mean value of LDL-C in the subjects. In the present study, the range was not particularly wide and the mean value was not high (130 ± 32 mg/dl), which might be the

Table 4 – Unadjusted, and age- and gender-adjusted odds ratios for the presence of carotid plaque in normal LDL-cholesterol (<140 mg/dl) subjects ($n = 25$)

Variable	Unadjusted		Adjusted	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Age	0.99 (0.92–1.05)	0.70		
Gender (male)	1.10 (0.49–2.46)	0.82		
Smoking	1.31(0.59–3.04)	0.51	1.38 (0.50–4.21)	0.54
HDL-C	1.00 (0.94–1.06)	0.88	1.00 (0.93–1.08)	0.90
LDL-C	1.02 (0.98–1.07)	0.31	1.03 (0.99–1.08)	0.21
TG	1.11 (0.99–1.29)	0.11	1.11 (0.99–1.30)	0.13
HbA_{1c}	1.17 (0.80–1.73)	0.43	1.17 (0.80–1.78)	0.43
ApoB48	1.91 (1.17–4.03)	0.04	2.16 (1.22–5.32)	0.04

CI, confidence interval; HbA_{1c}, hemoglobin A_{1c}; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; TG, triglycerides; ApoB48, apolipoprotein B48.

reason that significance was not observed in the association of LDL-C with carotid plaque. The present study indicated that apoB48 level was strongly associated with carotid plaque. Even when LDL-C was within the normal range, an elevated apoB48 level would be a risk factor for atherosclerosis in type 2 DM.

In general, age, hypertension, history of smoking, and low HDL-C predict carotid atherosclerosis. However, these factors were not associated with the presence of carotid plaque in this study. The lack of association may be due to the characteristics and small number of the subjects. In our study, the proportion of age ≤45 years was 20%. This small proportion of young subjects may not have a statistical power, because carotid plaque area was reported to be strongly related to age, increased between 45 and 70 years of age markedly, and measurable plaque were more detected in >45-year subjects [28]. With respect to blood pressure, most of our subjects had normal blood pressure (the proportions of systolic blood pressure <135 mmHg and diastolic blood pressure <85 mmHg were 85% and 88%, respectively). Low HDL-C (<40 mg/dl) subjects were 20% in our study. These biases of the subjects may be the reason why the conventional atherosclerotic risk factors did not associated with the presence of carotid plaque in the present study. The lack of association between smoking habit and carotid plaque might be because we defined smoking as both current and past smoking, and did not account daily cigarette consumption.

ApoB48-containg lipoproteins were reported to be increased in production and decreased in clearance in type 2 DM [19], but little is known about its diurnal profile. The present study showed that the diurnal level of apoB48 fluctuated more than that of TG in type 2 DM, and was higher in plaque (+) than in plaque (−) throughout the day. The diurnal profile of apoB48 level would reflect the metabolism of chylomicron and its remnants, and high level of fasting apoB48 would indicate the accumulation of chylomicron remnants. These results suggest that apoB48 is more useful as a maker for atherogenic lipoprotein abnormality than TG. Campos et al. [29] reported the diurnal change in apoB48 level in normolipidemic subjects, indicating that the apoB48 level in both light and dense VLDL fraction were increased sharply after breakfast, increased slightly after lunch, and not increased immediately after dinner. In contrast, in the present study, serum apoB48 level was sharply increased after each meal. One reason for the inconsistent results may be the difference in the assay method for apoB48. Campos et al. evaluated apoB48 level in each lipoprotein fraction by SDS-PAGE, whereas we evaluated that in serum by ELISA. In addition, the difference in the subjects (normolipidemia vs. type 2 DM) might affect the discordance in the diurnal apoB48 profiles.

In conclusion, serum apoB48 level was strongly associated with the presence of carotid plaque in type 2 DM and was higher in the subjects with carotid plaque than in the subjects without the plaque throughout the day. The measurement of serum apoB48 may be useful to evaluate the risk of atherosclerosis. The present results warrant further studies, in large scale and prospective design, to confirm the significance of the measurement of apoB48.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.diabres.2008.04.028.

5. Conflict of interest

The authors declare that they have no conflict of interest.

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SHORT COMMUNICATION

INSIG2 gene rs7566605 polymorphism is associated with severe obesity in Japanese

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Abstract The single nucleotide polymorphism (SNP) rs7566605 in the upstream region of the insulin-induced gene 2 (*INSIG2*) is associated with the obesity phenotype in many Caucasian populations. In Japanese, this association with the obesity phenotype is not clear. To investigate the relationship between rs7566605 and obesity in Japanese, we genotyped rs7566605 from severely obese subjects [n = 908, body mass index (BMI) $\geq$ 30 kg/m^2] and normal-weight control subjects (n = 1495, BMI < 25 kg/m^2). A case–control association analysis revealed that rs7566605 was significantly associated with obesity in Japanese. The P value in the minor allele recessive mode was 0.00020, and the odds ratio (OR) adjusted for gender and age was 1.61 [95% confidential interval (CI) = 1.24–2.09]. Obesity-associated phenotypes, which included the level of BMI, plasma glucose, hemoglobin A1c, total cholesterol,

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triglycerides, high-density lipoprotein (HDL) cholesterol, and blood pressure, were not associated with the rs7566605 genotype. Thus, rs7566605 in the upstream region of the *INSIG2* gene was found to be associated with obesity, i.e., severe obesity, in Japanese.

Keywords Insulin-induced gene 2 · Obesity · Japanese population · Association · SNP

Introduction

Obesity has become one of the major issues in public health, medicine, and the economy (Kopelman 2000). Obesity is considered to be important due to its relationship with various complications, such as diabetes mellitus, dyslipidemia, and hypertension. A combination of these dysfunctions is now defined as the metabolic syndrome that significantly increases the risk of cardiovascular disease (Wilson and Grundy 2003). Genetic and environmental factors contribute to the development of obesity (Maes et al. 1997; Barsh et al. 2000; Rankinen et al. 2006). Due to the recent progress in single nucleotide polymorphism (SNP) genotyping techniques, it is possible to conduct genome-wide screens to identify common genetic variants associated with obesity. We conducted a large-scale case–control association study and found that secretogranin III (*SCG3*) (Tanabe et al. 2007) and myotubularin-related protein 9 (*MTMR9*) (Yanagiya et al. 2007) confer susceptibility to the obesity phenotype in the Japanese population. Genome-wide association studies have shown that variations in the upstream region of the insulin-induced gene 2 (*INSIG2*) (Herbert et al. 2006) and in the fat-mass and obesity-associated gene (*FTO*) (Frayling et al. 2007; Scuteri et al. 2007; Hinney et al. 2007) are associated with the obesity phenotype. We recently reported the association between variations in the *FTO* gene and severe obesity in Japanese (Hotta et al. 2008). An association between rs7566605 in the upstream region of the *INSIG2* gene and obesity was also found in several Caucasian and Hispanic American populations (Herbert et al. 2006; Hall et al. 2006; Lyon et al. 2007; Liu et al. 2008). However, results from some reports with respect to these associations could not be reproduced (Lyon et al. 2007; Smith et al. 2007; Boes et al. 2008); further, these associations are not observed in the Indian (Kumar et al. 2007), Chinese (Yang et al. 2008) and Japanese populations (Tabara et al. 2008). Thus, the association between rs7566605 in the *INSIG2* gene and obesity in Japanese remains controversial.

To investigate the relationship between the *INSIG2* gene and obesity in Japanese, we performed a case–control association study involving patients with severe adult obesity (BMI $\geq$ 30 kg/m^2) and normal weight controls (BMI $<$ 25 kg/m^2). We found that rs7566605 was significantly associated with severe adult obesity.

Materials and methods

Study subjects

Severely obese subjects were recruited from among the outpatients of medical institutes. Patients with secondary obesity and obesity-related hereditary disorders were excluded from this study, as were patients with medication-induced obesity. Control subjects were recruited from among subjects who had undergone a medical examination for the screening of common diseases. Each subject provided written informed consent, and the protocol was approved by the ethics committee of each institution and that of RIKEN. The sample size for the severely obese subjects (BMI $\geq$ 30 kg/m^2) was 908 (male:female ratio, 418:590; age, 49.1 $\pm$ 14.2 years; BMI, 34.5 $\pm$ 5.4 kg/m^2), whereas that for the normal weight controls (BMI $<$ 25 kg/m^2) was 1,495 (male:female ratio, 672:823; age, 48.1 $\pm$ 16.5 years; BMI, 21.6 $\pm$ 2.1 kg/m^2). Subjects' clinical features are illustrated in Table 1.

DNA preparation and SNP genotyping

Genomic DNA was prepared from the blood samples of each subject with a Genomix kit (Talent Srl, Trieste, Italy). SNP rs7566605 reported in a previous genome-wide association study (Herbert et al. 2006) was genotyped with TaqMan probe (C_29404113_20; Applied Biosystems; Foster City, CA, USA).

Statistical analysis

Genotype or allele frequencise were compared between cases and controls in three different modes. In the first

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Table 1 Clinical characterization of obese and control subjects

	Obese	Control	*P* value[a]
Sample size	908	1495	–
Gender (M/F)	418/490	672/823	–
Age (year)	49.1 ± 14.2	48.1 ± 16.5	0.050
BMI (kg/m^2)	34.50 ± 5.39	21.65 ± 2.07	<0.000001
Glucose (mg/dl)	129.1 ± 49.7	97.7 ± 23.8	<0.000001
HbA1c (%)	6.5 ± 1.8	5.1 ± 0.6	<0.000001
Total cholesterol (mg/dl)	210.1 ± 38.0	201.2 ± 36.4	<0.000001
Triglycerides (mg/dl)	155.6 ± 111.0	104.0 ± 73.1	<0.000001
HDL cholesterol (mg/dl)	53.1 ± 18.8	65.1 ± 15.6	<0.000001
Systolic blood pressure (mmHg)	136.4 ± 18.2	123.4 ± 17.8	<0.000001
Diastolic blood pressure (mmHg)	83.8 ± 12.0	76.0 ± 11.1	<0.000001

Data are mean ± standard deviation

HbA1c hemoglobin A1c, *HDL* high-density lipoprotein

[a] *P* values were analyzed using Mann–Whitney *U* test

mode, i.e, the allele frequency mode, allele frequencies were compared with a 2 × 2 contingency table. In the second mode, i.e., the minor allele recessive mode, frequencies of the homozygous genotype for the minor allele were compared with a 2 × 2 contingency table. In the third mode, i.e., the minor allele dominant mode, frequencies of the homozygous genotype for the major allele were compared with a 2 × 2 contingency table. A test of independence was performed using Pearson's χ^2 method. Odds ratio (OR) and 95% confidence interval (CI) were calculated by Woolf's method. The rs7566605 genotype was transformed to a multidichotomous variable, i.e., homozygosity with C alleles versus the other genotypes. The OR adjusted for age and gender was calculated by multiple logistic regression analysis, with genotype, age, and gender as independent variables. Hardy–Weinberg equilibrium was assessed using the χ^2 test (Nielsen et al. 1998). A simple comparison of clinical data among the different genotypes was performed by one-way analysis of variance (ANOVA). Statistical analyses were performed with StatView 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Case–control association study

We successfully genotyped rs7566605 by the TaqMan assay and performed tests of independence between the phenotype and genotype of obesity in severely obese subjects (BMI ≥ 30 kg/m^2) and normal weight controls (BMI < 25 kg/m^2). The minor allele frequency (MAF) of rs7566605 in the control group was 0.31. This was consistent with data obtained from the haplotype map of the human genome (HapMap). As shown in Table 2, rs7566605 demonstrated significant association with the obesity phenotype [recessive mode, $P = 0.00020$, and the OR (95% CI) was 1.62 (1.26–2.10)]. The rs7566605 genotype was transformed to a multidichotomous variable, i.e., CC homozygote versus the other genotypes. Multiple logistic regression analysis was performed, with genotype, age, and gender as independent variables. The *P* values for age, gender, and genotype were 0.21, 0.51, and 0.00030, respectively. OR (95% CI) was 1.61 (1.24–2.09). Our data indicated that rs7566605 in the *INSIG2* gene was associated with severe obesity in Japanese.

A deviation from the Hardy–Weinberg equilibrium was detected in cases ($P = 0.0015$), because this SNP is associated with obesity and cases were selected by phenotype. Cases were selected on the basis of BMI, and the prevalence of subjects with a BMI ≥30 kg/m^2 is only 2–3% in Japan (Yoshiike et al. 2002). Cases may be biased and not representative of the general population. Thus, it is not unexpected that cases were not in accordance with Hardy–Weinberg equilibrium.

Analysis of various quantitative phenotypes with rs7566605

To investigate whether the genotypes of SNP rs7566605 are associated with the phenotypes of metabolic disorders, we compared the following among the different genotypes in cases, controls, and combined groups: ANOVA results; BMI; levels of fasting plasma glucose; hemoglobin A1c (HbA1c); total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol; and blood pressure. Quantitative phenotypes with respect to BMI and levels of fasting plasma glucose; HbA1c; total cholesterol, triglycerides, and HDL cholesterol; and blood pressure were not found to be significantly associated with the rs7566605 genotypes in either cases or controls (Table 3). Systolic and diastolic blood pressures were significantly lower in the GG homozygote in the control group. Blood pressure was higher in GG homozygote in the obese group. Thus, the rs7566605 genotype was not associated with blood pressure.

Table 2 Association of rs7566605 in the *INSIG2* gene with severe obesity

Sample (sample size)	No. of subjects (%)			No. of chromosomes (%)		HWE test[a]	
	CC	CG	GG	C	G	χ^2	*P* value
Case (n = 908)	127 (14)	365 (40)	416 (46)	619 (34)	1,197 (66)	10.1	0.0015
Control (n = 1495)	136 (9)	664 (44)	695 (46)	936 (31)	2,054 (69)	1.6	0.21

Allele frequency mode[b]			Minor allele recessive mode[b]			Minor allele dominant mode[b]		
χ^2	*P* value	OR[c] (95% CI)	χ^2	*P* value	OR[c] (95% CI)	χ^2	*P* value	OR[c] (95% CI)
4.0	0.046	1.13 (1.00–1.28)	13.9	0.00020	1.62 (1.26–2.10)	0.1	0.75	1.03 (0.87–1.21)

[a] Hardy–Weinberg equilibrium test

[b] Association test was performed in three different modes as described in the "Materials and methods", and the results in the three modes are shown

[c] Odds ratio (OR) with 95% confidence interval (CI)

Table 3 Comparison of various quantitative phenotypes among different genotypes at rs7566605 in obese and control subjects

	Obese[a]			Control[a]		
	CC (n = 127)	CG (n = 365)	GG (n = 416)	CC (n = 136)	CG (n = 664)	GG (n = 695)
Age (year)	48.5 ± 15.2	48.0 ± 14.0	50.0 ± 14.2	47.7 ± 17.4	48.1 ± 16.7	48.4 ± 16.4
P value[b]		0.13			0.89	
BMI (kg/m^2)	35.22 ± 6.91	34.28 ± 5.09	34.52 ± 5.21	21.70 ± 2.06	21.68 ± 2.07	21.60 ± 2.10
P value		0.25			0.73	
Glucose (mg/dl)	127.8 ± 44.1	129.3 ± 50.3	129.1 ± 50.8	102.0 ± 40.6	98.0 ± 21.7	97.0 ± 21.9
P value		0.96			0.23	
HbA1c (%)	6.4 ± 1.7	6.5 ± 1.8	6.5 ± 1.8	5.1 ± 0.8	5.1 ± 0.6	5.1 ± 0.6
P value		0.93			0.81	
Total cholesterol (mg/dl)	209.1 ± 39.3	209.3 ± 37.5	210.4 ± 38.1	204.1 ± 35.0	199.4 ± 36.9	202.2 ± 36.3
P value		0.90			0.23	
Triglycerides (mg/dl)	147.1 ± 85.4	160.1 ± 127.3	153.9 ± 102.7	99.6 ± 58.2	103.0 ± 65.5	105.6 ± 82.3
P value		0.51			0.62	
HDL cholesterol (mg/dl)	51.6 ± 14.1	52.7 ± 15.9	53.8 ± 22.2	67.3 ± 14.8	64.9 ± 15.8	65.0 ± 15.6
P value		0.52			0.45	
SBP[c] (mmHg)	134.8 ± 15.3	136.8 ± 18.7	136.6 ± 18.4	122.9 ± 15.8	125.4 ± 17.9	121.9 ± 18.0
P value		0.57			0.0019	
DBP[d] (mmHg)	82.9 ± 11.1	84.6 ± 11.7	83.4 ± 12.4	76.5 ± 11.3	77.0 ± 11.1	75.1 ± 11.1
P value		0.26			0.008	

Data are mean ± standard deviation

BMI body mass index, *HbA1* hemoglobin A1c, *HDL* high-density lipoprotein

[a] Data of each quantitative phenotype were compared among different genotypes at the rs7566605 in obese and control subjects

[b] *P* values were analyzed using analysis of variance in each group of obese and control subjects

[c] Systolic blood pressure

[d] Diastolic blood pressure

Discussion

Recent genome-wide association studies have shown that rs7566605 in the upstream region of the *INSIG2* gene is associated with obesity (Herbert et al. 2006). Associations between rs7566605 and the obesity phenotype have been observed in many Caucasian subjects (Herbert et al. 2006; Hall et al. 2006; Lyon et al. 2007; Liu et al. 2008). However, these associations were controversial with regard to Asian subjects (Yang et al. 2008; Tabara et al. 2008). The association between rs7566605 and BMI may be hard to be replicated in the Asian general population due to the relatively smaller average BMI value and smaller proportion of obesity with BMI >30 kg/m^2 in Asians compared with

those in Caucasians. Indeed, criteria of obesity is BMI >30 kg/m^2 in Caucasians and BMI >25 kg/m^2 in Japanese. We could show the contribution of rs7566605 to obesity in Japanese using severely obese patients with a BMI ≥30 kg/m^2 as cases. SNP rs7566605 could also contribute to the development of severe obesity in Japanese.

Allele frequency was 0.31–0.34 in Japanese, just as observed in European subjects, and the CC genotype was also associated with severe obesity in Japanese, as previously reported (Herbert et al. 2006; Hall et al. 2006; Lyon et al. 2007; Liu et al. 2008). However, CC genotype was not significantly associated with BMI in obese and control groups, although CC homozygotes had higher BMI. Thus, it is possible that our study did not have sufficient power to detect the association between rs7566605 and BMI. The CC genotype would be thrifty variation and have an advantage for survival before modern times. Subjects with CC genotype would be susceptible to obesity in recent years. As a result, the number of CC homozygotes would increase in severely obese group, leading to a deviation from Hardy–Weinberg equilibrium.

Since rs7566605 exists approximately 10 kb upstream from *INSIG2*, SNPs may affect the transcriptional activity of *INSIG2*. INSIG2 is expressed ubiquitously. It was downregulated by insulin in the liver and involved in fatty acid synthesis (Yabe et al. 2003; Takaishi et al. 2004). INSIG2 also mediates feedback control of cholesterol synthesis (Goldstein et al. 2006). Although serum total cholesterol, HDL cholesterol, and triglycerides were not significantly different among genotypes, it is possible that INSIG2 is related to obesity as it affects lipid metabolism.

In summary, our study indicated that rs7566605 in the upstream region of the *INSIG2* gene may influence the risk of severe obesity in Japanese.

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Effects of EPA on coronary artery disease in hypercholesterolemic patients with multiple risk factors: Sub-analysis of primary prevention cases from the Japan EPA Lipid Intervention Study (JELIS)

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Abstract

Background: Japan EPA Lipid Intervention Study (JELIS) was a large-scale clinical trial examining the effects of eicosapentaenoic acid (EPA) on coronary artery disease (CAD) in hypercholesterolemic patients. Herein, we focused on risk factors other than low-density lipoprotein cholesterol (LDL-C) to investigate the effects of EPA on CAD among JELIS primary prevention cases.

Methods: Hypercholesterolemic patients on statin therapy but without evidence of CAD ($n = 14,981$) were randomly assigned to an EPA group ($n = 7503$) or a control group ($n = 7478$). The relationships between incident CAD, the number of CAD risk factors (hypercholesterolemia; obesity; high triglyceride (TG) or low high-density lipoprotein cholesterol (HDL-C); diabetes; and hypertension) and EPA treatment were investigated.

Results: For the control and EPA groups combined, a higher number of risk factors was directly associated with an increased incidence of CAD. Incidence was lower for the EPA group than for the control group regardless of the numbers of risk factors. Compared to patients with normal serum TG and HDL-C levels, those with abnormal levels (TG ≥ 150 mg/dL; HDL-C <40 mg/dL) had significantly higher CAD hazard ratio (HR: 1.71; 95% CI: 1.11–2.64; $P = 0.014$). In this higher risk group, EPA treatment suppressed the risk of CAD by 53% (HR: 0.47; 95% CI: 0.23–0.98; $P = 0.043$).

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Conclusions: Multiple risk factors besides cholesterol are associated with markedly increased incidence of CAD. High TG with low HDL-C represents a particularly potent risk factor. EPA was effective in reducing the incidence of CAD events for patients with this dyslipidemic pattern, suggesting that EPA may be especially beneficial in patients who with abnormal TG and HDL-C levels (NCT00231738).
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Keywords: JELIS; Eicosapentaenoic acid; Primary prevention; Coronary artery disease; Risk factors; HDL-C; Triglycerides

1. Introduction

Eicosapentaenoic acid (EPA) is one of the n-3 polyunsaturated fatty acids (PUFA) found large quantities in fish oil. Ever since Dyerberg and Bang reported that EPA levels were high in the blood and diets of Greenland Inuit (who have low prevalence of atherosclerotic diseases [1]), the preventive effects of n-3 PUFA, including EPA, has been examined in many epidemiological and clinical studies [2–6]. Most studies have found that intake of fish and fish oil are related to reduced risk for total mortality, sudden death and coronary artery disease (CAD). Furthermore, randomized controlled intervention trials have suggested the suppressive effects of fish and fish oil consumption on CAD [7].

Using a highly purified (≥98%) EPA, not a mixture of several fatty acids, i.e., fish oil, we conducted a randomized controlled trial, the Japan EPA Lipid Intervention Study (JELIS; ClinicalTrials.gov number, NCT00231738) [8], and reported that pure EPA suppressed CAD even in Japanese hypercholesterolemic patients who routinely consume a large amount of EPA and DHA from fish [5]. In the JELIS, EPA had no significant effect on total cholesterol (TC) or low-density lipoprotein cholesterol (LDL-C) levels indicating that EPA can lower CAD risk by mechanisms other than LDL-C lowering.

Besides LDL-C, other risk factors for CAD include obesity, dislipidemia, impaired glucose metabolism and hypertension. When present together in the same patients, these risk markers constitute a syndrome called the visceral fat syndrome, syndrome X, insulin-resistant syndrome and the metabolic syndrome [9–14]. Compared to patients with only one of these risk factors, incidence of CAD in patients with multiple factors is higher [15,16]. In addition, we assumed that EPA would suppress CAD even in patients at high risk. The present study focused on CAD risk associated with increasing numbers of non-LDL-C risk factors in hypercholesterolemic patients and the effects of EPA on the risk for CAD in these patients.

2. Materials and methods

2.1. Study design and patients

The study design of the JELIS, including inclusion and exclusion criteria, has been reported in detail [17]. Briefly, hypercholesterolemic patients with serum TC levels ≥250 mg/dL (men: 40–75 years; women: postmenopausal-75 years) were followed for up to 5 years (mean: 4.6 years) using the prospective, randomized, open-label, blinded endpoint evaluation (PROBE) method. A total of 18,645 patients were registered and randomly assigned to either the EPA with statin (EPA group) or to statin alone (control group). Eighty percent ($n = 14,981$) of the patients had no history of CAD and are the subject of this report.

2.2. Procedures

Dietary guidance was provided for all patients before the start of and during the study. All patients received 10 mg of pravastatin or 5 mg of simvastatin administered once a day. In the EPA group, two 300-mg capsules containing EPA ethylester (EPA-E) with >98% purity were administered 3 times/day, for a total daily dose of 1800 mg.

2.3. Primary endpoint

The primary endpoint was major coronary events (MCE), comprising: sudden cardiac death; fatal myocardial infarction; nonfatal myocardial infarction; unstable angina pectoris including hospitalization for documented ischemic episodes; and angioplasty/stenting or coronary artery bypass grafting. MCE was reported by primary physicians and was examined by the case report committee without knowledge of groups' assignment.

2.4. Risk factors

The following five risk factors were of primary interest for this report and were defined as indicated at the time of registration:

A. *Hypercholesterolemia*: untreated serum TC ≥250 mg/dL (all subjects in JELIS had serum TC ≥250 mg/dL).
B. *Obesity*: body mass index (BMI) ≥25 kg/m^2.
C. *Dyslipidemia*: serum triglyceride (TG) ≥150 mg/dL and/or high-density lipoprotein cholesterol (HDL-C) <40 mg/dL.
D. *Diabetes*: physician-diagnosis or fasting plasma glucose ≥126 mg/dL and/or hemoglobin A_{1C} ≥6.5%.
E. *Hypertension*: physician-diagnosis or systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg.

Furthermore, all subjects were divided into the following four subgroups: based on the following serum TG and HDL-C levels at the time of registration:

1. TG <150 mg/dL and HDL-C ≥40 mg/dL (low TG/high HDL-C group).
2. TG ≥150 mg/dL and HDL-C ≥40 mg/dL (high TG/high HDL-C group).
3. TG <150 mg/dL and HDL-C <40 mg/dL (low TG/low HDL-C group).
4. TG ≥150 mg/dL and HDL-C <40 mg/dL (high TG/low HDL-C group).

2.5. Statistical analysis

All analyses were intention-to-treat with the level of significance set at $P < 0.05$ (two-sided). A Wilcoxon two-sample test was used to compare continuous variables. A chi-square test was used to compare class variables. Kaplan–Meier methods, the log-rank test, and the Cox proportional hazard model were used for survival analysis. The Cox proportional hazard model was adjusted for age, gender, smoking, diabetes and hypertension. However, we chose age, gender and smoking as adjusted factors to analyze the relationships between multiple risk factors and the incidence of MCE. We computed the power to detect the difference in CAD incidence between EPA and control groups for patients with high TG and low HDL-C. With a total of 957 patients in the high TG/low HDL-C group, we could detect a difference in MCE incidence of 1% vs. 0.5% with a power of 57%. The analysis plan for this sub-study was pre-specified according to the study hypothesis before the analysis was initiated. All analyses were conducted using SAS software (version 8.12; SAS Institute, Cary, NC).

3. Results

Subject characteristics have been previously reported [8]. The number of patients with hypertension and/or diabetes differed slightly from that originally published because the original designations were based only on physician-diagnosed diabetes or hypertension only. As a result, number of patients with diabetes was 1238 in control group, and 1258 in EPA group. In the same way, number of patients with hypertension was 4004 in control group, and 4015 in EPA group.

Risk for MCE increased in both the EPA and the control groups with increasing numbers of risk factors. The incidence of MCE was lower, but not statistically significant, for the EPA group than for the control group with each number of risk factors (Fig. 1).

Compared to the low TG/high HDL-C reference group, HR for MCE was increased only in the high TG/low HDL-C group (HR: 1.71; 95% CI: 1.11–2.64; $P = 0.014$; Fig. 2). Other risk factors were compared to the high TG/low HDL-C group, those in the low TG/high HDL-C group had no significant differences in TC or LDL-C but the proportions of male patients, smokers and drinkers; BMI; the prevalence of diabetes and high diastolic blood pressure. Furthermore,

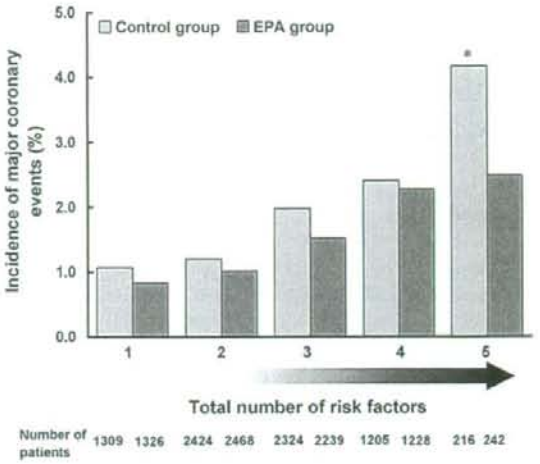
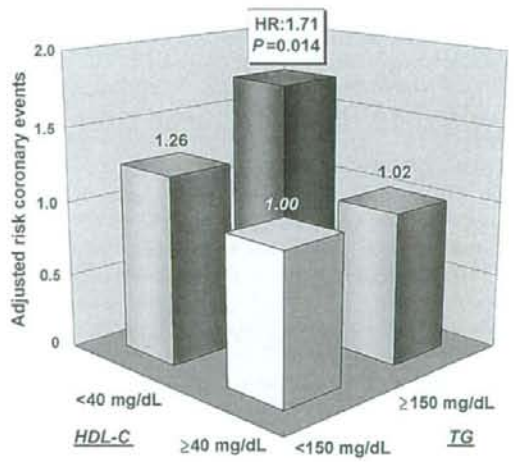


Fig. 1. Multiple risk factors and the incidence of MCE. Number of risk factors at the time of registration was counted: Risk A, hypercholesterolemia (all patients); Risk B, body mass index (BMI) ≥25; Risk C, triglyceride ≥150 mg/dL or HDL-cholesterol <40 mg/dL; Risk D, diabetes; Risk E, hypertension. The Cox proportional hazard model was adjusted for age, gender, smoking. $^{*}P < 0.05$ vs. risk number 1 in the control group.



TG and HDL-C (mg/dL)	Events	(%)	P	HR	95%CI
TG<150 , HDL-C≥40	86 / 6614	1.3		1.00	
TG≥150 , HDL-C≥40	97 / 6096	1.6	0.923	1.02	0.75 - 1.37
TG<150 , HDL-C<40	4 / 196	2.0	0.663	1.26	0.46 - 3.46
TG≥150 , HDL-C<40	32 / 957	3.3	0.014	1.71	1.11 - 2.64

Fig. 2. Incidence of MCE and triglyceride and HDL-cholesterol levels at the time of registration for the combined EPA and control group. Hazard ratio and P value adjusted for age, gender, smoking, diabetes, and hypertension. HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; HR, hazard ratio; CI, confidence interval.