Apolipoprotein E Polymorphism Is Associated With Plasma Cholesterol Response in a 7-day Hospitalization Study for Metabolic and Dietary Control in NIDDM

KAORI MURAKAMI, PHD
MIZUO SHIMIZU, MD
NOBUHIRO YAMADA, MD
SHUN ISHIBASHI, MD
HITOSHI SHIMANO, MD
YOSHIO YAZAKI, MD
YASUO AKANUMA, MD

OBJECTIVE — To investigate the effects of apolipoprotein E phenotype, which is an important genetic factor determining plasma cholesterol level, on plasma lipoprotein metabolism during a 7-day diet therapy of diabetes.

RESEARCH DESIGN AND METHODS — Diet therapy was performed in 242 subjects with NIDDM. Plasma lipid levels and apolipoprotein A-I, B, and E levels on hospital days 2 and 9 were compared in three phenotype groups, ε2 (E2/2, and E2/3), ε3 (E3/3), and ε4 (E3/4 and E4/4).

RESULTS — No differences were observed in fasting blood glucose level, body mass index, age, duration of NIDDM, or medication among the three phenotype groups. Before starting the treatment, total plasma cholesterol did not vary by apolipoprotein E phenotype, although the mean plasma low-density lipoprotein cholesterol level in ε4 patients was higher than in either ε3 or ε2 patients (ε4, 145 ± 42; ε3, 131 ± 34; ε2, 128 ± 36 mg/dl; $P < 0.05$). Changes in fasting blood glucose/body mass index after the 7-day treatment were not different among the apolipoprotein E phenotype groups, whereas the decrease in plasma cholesterol level after the treatment was significantly greater in ε2 patients than in either ε3 or ε4 patients (ε2, 10.3 ± 14.2; ε3, 6.1 ± 11.0; ε4, 3.8 ± 9.6%; $P < 0.05$).

CONCLUSIONS — Total plasma cholesterol response to in-hospital diet therapy varies by apolipoprotein E phenotypes, with subjects with apolipoprotein E2 showing the greatest response, whereas those with apolipoprotein E4 show the least. We suggest that subjects with apolipoprotein E4 should be controlled more strictly than other subjects from a viewpoint of reducing plasma cholesterol levels.

FROM THE INSTITUTE FOR DIABETES CARE AND RESEARCH, ASAHI LIFE FOUNDATION, MARUNOUCHI; AND THE THIRD DEPARTMENT OF INTERNAL MEDICINE, UNIVERSITY OF TOKYO, HONGO, TOKYO, JAPAN.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO NOBUHIRO YAMADA, MD, THIRD DEPARTMENT OF INTERNAL MEDICINE, FACULTY OF MEDICINE, UNIVERSITY OF TOKYO, HONGO, TOKYO 113, JAPAN.

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apoE, APOLIPOPROTEIN E; apoA-I, APOLIPOPROTEIN A-I; apoB, APOLIPOPROTEIN B; NIDDM, NON-INSULIN-DEPENDENT DIABETES MELLITUS; WHO, WORLD HEALTH ORGANIZATION; FBG, FASTING BLOOD GLUCOSE; BMI, BODY MASS INDEX; LDL, LOW-DENSITY LIPOPROTEIN; VLDL, VERY-LOW-DENSITY LIPOPROTEIN; IDL, INTERMEDIATE-DENSITY LIPOPROTEIN; HDL_2, HIGH-DENSITY LIPOPROTEIN TYPE 2; P-S RATIO, RATIO OF POLYUNSATURATED FATTY ACIDS TO SATURATED FATTY ACIDS; V PLUS L, VERY-LOW-DENSITY LIPOPROTEIN PLUS LOW-DENSITY LIPOPROTEIN; OHA, ORAL HYPOGLYCEMIC AGENTS.

Diabetes mellitus is a major risk factor for atherosclerotic disorders such as ischemic heart disease and cerebrovascular disease. The prevalence of these atherosclerotic complications is increased two- to threefold in diabetic populations (1). Of risk factors for atherosclerotic diseases, hyperlipidemia is frequently found in diabetic subjects (2,3); the significant positive correlationship between plasma cholesterol level and the incidence of coronary heart disease is well known (4,5).

Lipoproteins containing apoE, such as VLDL, IDL, and HDL_2, have a high affinity to the LDL receptor (6). Three isoproteins of apoE (apoE2, apoE3, and apoE4) are encoded by three different alleles (ε2, ε3, and ε4, respectively; 7). The most common type of apoE is apoE3, encoded by ε3, whose prevalence is 75–85% of the total population. ApoE2 has markedly reduced the binding ability to LDL receptors, because Cys is substituted for Arg^{158} of apoE3, a residue in the binding domain critical for receptor binding. In apoE4, Arg is substituted for Cys^{112} of apoE3. Epidemiological studies showed higher incidence of ischemic heart disease in subjects with apoE4 than in those with apoE2 (7,8). Recently, we reported the increased prevalence of the allele for ε4 in subjects with multi-infarct dementia (9). These observations have been explained by the significant association of apoE polymorphism with the levels of plasma cholesterol and LDL cholesterol (10). Subjects with apoE2 have lower plasma cholesterol levels than those with apoE3, and subjects with apoE4 have the highest. To prevent the progression of atherosclerosis, we need to correct plasma lipid abnormalities based on the genetic backgrounds of individual subjects. ApoE polymorphism is a good genetic marker, which regulates plasma cholesterol level and the LDL receptor. In this study, we examined the effects of apoE polymorphism on plasma choles-

terol level during diet therapy in diabetes mellitus.

RESEARCH DESIGN AND METHODS

— ApoE phenotype was determined in 333 patients who were hospitalized in the Institute for Diabetes Care and Research of Asahi Life Foundation for the metabolic control of diabetes mellitus. This study was performed on the 242 patients with complete data. The 91 subjects not studied had similar apoE phenotypes to those studied. Of the 242 patients studied, 174 were male, 68 were female, 88 were hypertriglyceridemic (≥150 mg/dl), and 55 were hypercholesterolemic (≥240 mg/dl). On admission, 96 patients were treated with diet only, 86 patients with sulfonylureas, and 60 patients with insulin. Average caloric intake, cholesterol intake, and the P/S ratio in diet before hospitalization were 31 kcal · kg^{-1} body weight · day^{-1}, 290 mg/day, and 0.93, respectively. No significant difference was observed in the average daily caloric intake (ε2, 31.5 ± 7.8; ε3, 30.8 ± 8.7; ε4, 30.9 ± 5.6 Kcal · kg body $weight^{-1}$ · day^{-1}), cholesterol intake (ε2,289 ± 75; ε3, 289 ± 84; ε4, 294 ± 71 mg/day), and P/S ratio (ε2, 0.94 ± 0.19; ε3, 0.92 ± 0.22; ε4, 0.94 ± 0.20) among apoE phenotype subgroups. During hospital days 2 to 9, all patients were treated with a diet of 25 kcal · kg body $weight^{-1}$ · day^{-1} without any changes of medication. Of the total calories, 20, 55, and 25% were protein, carbohydrate, and fat, respectively. The P/S ratio was 0.93, and cholesterol intake was 275 mg/day. All patients were diagnosed with NIDDM according to WHO criteria (11). All patients had normal liver, kidney, and thyroid function. No subjects were receiving drugs for either hyperlipidemia or hypertension (diuretics or β-blocking agents), and no female subjects were receiving oral contraceptive or postmenopausal hormone-replacement therapy.

We measured body weight, plasma glucose level, plasma cholesterol level, plasma triglyceride level, plasma HDL cholesterol level, and plasma apoA-I, apoB, and apoE levels in the morning on hospital days 2 and 9 after 14 hr fasting. Apolipoproteins were determined by a single radial immunodiffusion method (12) and lipids by enzymatic methods (13). Plasma V plus L cholesterol level and LDL cholesterol were calculated as follows: V plus L cholesterol = (cholesterol) − (HDL cholesterol) and LDL cholesterol = (cholesterol) − (HDL cholesterol) − (0.2 × triglyceride). The calculated LDL-cholesterol levels do not always reflect the real levels when the plasma triglyceride level is >300 mg/dl. We found that 18 subjects had plasma triglyceride levels >300 mg/dl; therefore, we calculated both V plus L cholesterol and LDL cholesterol. Statistical significance was determined by a Student's *t* test. All data were expressed as means ± SD.

Radiological examination of chest and abdomen was performed to evaluate aortic calcification. X rays of chest (posterior-anterior view) and abdomen (lateral view) were taken with the patient in an erect posture. One of us analyzed the radiological findings in random order without knowledge of the clinical status. When any visible calcification was found on the roentogram, we defined aortic calcification positive.

ApoE phenotype was determined as follows. Blood was collected into a tube containing 0.01% EDTA and centrifuged at 4°C to isolate plasma. We delipidated 7 μl of plasma with ethanol-ether (1:1 vol/vol) twice at −20°C, and the precipitated protein was dissolved in 10 mM Tris-HCl containing 8 M urea and 10 mM dithiothreitol, then subjected to 5% polyacrylamide gel isoelectric focusing (14). After running isoelectric focusing at 30 W for 1.5 h, the proteins were electrically transferred to nitrocellulose paper at 67 V for 3 h. After the nitrocellulose paper was incubated with anti-human apoE goat serum (Daiichi Pure Chemicals, Tokyo, Japan) and then with [^{125}I]protein A (2000 cpm/ng, 0.5 μg/ml), autoradiography was performed (15).

Table 1—*Prevalence of the common alleles for the gene locus of apoE*

Population studied	*N*	ε2	ε3	ε4
This study	333	0.042	0.863	0.095
Japanese (7)	880	0.035	0.851	0.112
Caucasians (7)	5805	0.080	0.769	0.150

RESULTS

— Of 333 patients, phenotypes of E2/2, E2/3, E3/3, E2/4, E3/4, and E4/4 were found in 1, 23, 251, 3, 50, and 5 patients, respectively (Table 1). The prevalence of each allele, ε2, ε3, and ε4, for apoE was 4.2, 86.3, and 9.5%, respectively. The clinical background of subjects studied (n = 242) is shown in Table 2. No significant differences were noted in age, duration of illness, BMI, FBG level, or treatment for diabetes mellitus among three phenotype groups. The ε2 group (16- to 68-yr of age; 16 males and 7 females) consisted of subjects with E2/2 and E2/3; the ε3 group (21- to 74-yr of age; 130 males and 44 females) had subjects with E3/3; and the ε4 group (18- to 68-yr of age; 28 males and 17 females) had subjects with E3/4 and E4/4. Patients with ε2 appeared to have a shorter duration of illness. One subject with E2/2 was included in the ε2 group; he did not exhibit hyperlipidemia. The ε2 group included more hypertriglyceridemic subjects than either the ε3 or ε4 group (52.2, 36.2, and 14.8% of subjects in ε2, ε3, and ε4 groups, respectively), and hypercholesterolemic subjects were found in 26.1, 21.3, and 26.7% of subjects in each subgroup, respectively.

As shown in Tables 3–5, on hospital day 2 before subjects started diet therapy, no significant differences were observed in mean plasma cholesterol, triglyceride, HDL cholesterol, or V plus L cholesterol levels among apoE subgroups, although the mean plasma LDL-

Table 2—*Clinical characteristics of subjects*

	N	Age (yr)	Duration of diabetes (yr)	BMI (kg/m^2)	FBG (mg/dl)	HbA_{1c} (%)	Therapy (%)		
							Diet	OHA	Insulin
ε2	23	51 ± 11	8.8 ± 6.8	22.2 ± 4.1	146 ± 58	9.5 ± 2.4	39.1	26.1	34.8
ε3	174	53 ± 9	9.8 ± 7.0	22.1 ± 3.8	145 ± 46	9.4 ± 2.0	41.4	35.6	23.0
ε4	45	52 ± 11	9.3 ± 7.3	22.4 ± 3.5	142 ± 43	9.2 ± 1.8	33.3	40.0	26.7

Data are means ± SD. ε2, ε3, and ε4 groups consist of subjects with E2/2 and E2/3, subjects with E3/3, and subjects with E3/4 and E4/4, respectively.

cholesterol level in the ε4 group was significantly higher than in the ε2 and ε3 groups (145 ± 42 vs. 128 ± 36 mg/dl, $P < 0.05$, and 131 ± 34 mg/dl, $P < 0.01$, respectively). Mean plasma apoE level in the ε2 group was greater than that in the ε3 group (5.3 ± 1.0 vs. 4.4 ± 1.2 mg/dl, $P < 0.01$), and that in the ε4 group was least (4.1 ± 1.2 mg/dl, $P < 0.01$ compared with those with ε2). The mean plasma apoB level in the ε3 group was significantly lower than that in the ε4 group (107 ± 27 vs. 115 ± 32 mg/dl, $P < 0.05$). Plasma apoA-I levels were not significantly different among the three groups.

After 7 days of treatment of NIDDM with caloric restrictions, FBG level, BMI, plasma triglyceride, cholesterol, apoA-I, apoB, and apoE levels were all improved (Tables 3–5). Plasma cholesterol, V plus L cholesterol, and apoB levels in the ε4 group were significantly greater than those in other groups, and the plasma apoE level in the ε4 group was significantly less than in the ε2 group at day 7 (Tables 4,5). No significant differences were seen in fasting plasma glucose, triglyceride, or apoA-I levels or BMI among the three groups.

We calculated the change in each metabolic parameter during the diet therapy for 7 days as follows: % metabolic change = 100 × (data after the treatment − data before the treatment)/(data before the treatment). We found a significant difference in metabolic improvement in plasma cholesterol levels. In the ε2 group, the mean plasma cholesterol level decreased 10.3%, whereas it decreased 6.8 and 3.8% in the ε3 and ε4 groups, respectively (Table 4). Plasma cholesterol levels were resistant to therapy in subjects with ε4 compared with other subjects when similar treatment was given to subjects with each phenotype. The improvement of plasma cholesterol level was observed in both males and females, although in females, it was not statistically significant. Plasma triglyceride levels were significantly improved after diet therapy in all three groups. Improvements of FBG level and BMI were not significantly different among the three groups. Furthermore, we analyzed the results obtained from patients with diet therapy only and from patients without insulin therapy. In both subgroups, plasma cholesterol levels were significantly improved in patients with ε2 compared with patients with ε4. When we eliminated the data obtained from subjects with E2/2 and E4/4, the results were similar to those obtained from data including subjects with E2/2 and E4/4.

We evaluated the calcification of the aorta in chest and abdominal X rays and found less aortic calcification in the ε2 group than in the ε3 and ε4 groups (22.2 vs. 46.9 and 39.4%, respectively, $P < 0.01$; Fig. 1). The average age of the subjects with aortic calcification, including 69 males and 25 females, was 56.1 ± 8.0 yr; and the average age of subjects without calcification, including 106 males and 42 females, was 50.7 ± 10.2 yr.

CONCLUSIONS— In this study, we found significant effects of apoE polymorphism on plasma cholesterol level before and during the diet therapy in NIDDM. Prevalence of apoE polymor-

Table 3—*FBG level, BMI, and plasma triglyceride level before and after the treatment, and the percentage of changes during the treatment*

	FBG (mg/dl)			BMI (kg/m^2)			Triglyceride (mg/dl)		
	Before	After	Change (%)	Before	After	Change (%)	Before	After	Change (%)
ε2	145 ± 47	108 ± 32	−19.4 ± 42.8	22.2 ± 4.1	21.8 ± 4.0	−2.0 ± 1.4	150 ± 99	92 ± 37	−25.1 ± 42.8
ε3	145 ± 46	106 ± 30	−19.0 ± 49.0	22.1 ± 3.8	21.7 ± 3.7	−1.8 ± 1.6	143 ± 79	93 ± 47	−27.9 ± 27.9
ε4	142 ± 43	109 ± 41	−22.1 ± 18.8	22.4 ± 3.5	22.0 ± 3.4	−1.8 ± 1.3	127 ± 59	89 ± 36	−23.5 ± 27.9

Data are means ± SD. ε2, ε3, and ε4 groups consist of subjects with E2/2 and E2/3, subjects with E3/3, and subjects with E3/4 and E4/4, respectively.

Table 4—*Plasma cholesterol levels before and after the treatment and the percentage of changes during the treatment*

	CHOLESTEROL (MG/DL)			V PLUS L CHOLESTEROL (MG/DL)			LDL CHOLESTEROL (MG/DL)			HDL CHOLESTEROL (MG/DL)		
	BEFORE	AFTER	CHANGE (%)	BEFORE	AFTER	CHANGE (%)	BEFORE	AFTER	CHANGE (%)	BEFORE	AFTER	CHANGE (%)
ε2	211 ± 42	185 ± 25	−10.3 ± 14.2	164 ± 47	141 ± 26	−9.2 ± 19.0	128 ± 36	122 ± 26	−3.2 ± 23.2	47 ± 15	45 ± 14	−2.3 ± 12.4
ε3	206 ± 41	193 ± 38*	−6.1 ± 11.0†	163 ± 42	149 ± 38	−7.2 ± 13.7	131 ± 34‡	129 ± 33	−1.2 ± 20.8	44 ± 13	44 ± 11	1.1 ± 24.1
ε4	215 ± 49	205 ± 45†	−3.8 ± 9.6§	170 ± 45	161 ± 37†	−3.8 ± 13.4	145 ± 42†	144 ± 39§	−0.2 ± 15.6	45 ± 20	44 ± 18	0.1 ± 11.7

Data are means ± SD. ε2, ε3, and ε4 groups consist of subjects with E2/2 and E2/3, subjects with E3/3, and subjects with E3/4 and E4/4, respectively.
*$P < 0.05$, ε3 group compared with ε4 group.
†$P < 0.05$ compared with ε2 group.
‡$P < 0.01$, ε3 compared with ε4 group.
§$P < 0.01$ compared with ε2 group.

phism in diabetes mellitus was similar to that in Japanese nondiabetic subjects (7; Table 1), indicating no specific deviation of apoE polymorphism attributable to diabetes mellitus. As reported previously, plasma LDL-cholesterol levels were significantly higher in subjects with ε4 than with ε2 (7–10). ApoE polymorphism has been proposed to influence intestinal cholesterol absorption, hepatic uptake of chylomicrons, and the resulting hepatic content of cholesterol that regulates the LDL-receptor activity and finally determines plasma LDL-cholesterol levels (16,17). Chylomicrons containing apoE2 cannot be efficiently taken up by the liver because of low affinity to chylomicron remnant receptors and LDL receptors, whereas chylomicrons containing apoE4 with a high affinity to liver receptors are efficiently removed by the liver. Thereafter, dietary cholesterol taken up by the liver can suppress LDL-receptor activity in the liver, resulting in relatively high plasma LDL-cholesterol levels in subjects with apoE4. Furthermore, we found higher plasma apoE levels in subjects with ε2 than with ε3 and ε4. Retarded clearance of lipoproteins containing apoE2 through either the LDL receptor or the chylomicron remnant receptor may cause high plasma apoE levels in subjects with ε2. In fact, subjects with E2/2, dysbetalipoproteinemia, have very high plasma apoE levels (18).

After 7-day diet therapy for diabetes mellitus, metabolic states such as FBG level, BMI, plasma lipids levels, and plasma apolipoprotein levels were all improved. These improvements after the treatment may be mainly attributable to strict caloric restriction by hospitalization because other medications were not altered during the 7 days. Diet therapy, of course, is the most fundamental therapy to reduce plasma glucose, lipoprotein levels, and BMI in diabetes. This study demonstrated greater improvements in plasma cholesterol, V plus L cholesterol, and apoB levels in subjects with ε2 than with either ε3 or ε4, in particular, improvement of plasma cholesterol levels was significantly influenced by the apoE polymorphism (Table 4). On the other hand, improvement of blood glucose levels did not appear to be significantly different among the three groups. Thus, metabolic background influenced by apoE polymorphism might play a significant role in reducing plasma cholesterol levels during the metabolic control of diabetes. It is well known that diabetic subjects often have impaired plasma VLDL metabolism, which is improved by diabetic control. Diet therapy may first improve VLDL metabolism and then reduce plasma LDL cholesterol levels as a product of VLDL. It is apparent from our data that the major change in

Table 5—*Plasma apolipoprotein levels before and after the treatment, and the percentage of changes during the treatment*

	apoA-I (MG/DL)			apoB (MG/DL)			apoE (MG/DL)		
	BEFORE	AFTER	CHANGE (%)	BEFORE	AFTER	CHANGE (%)	BEFORE	AFTER	CHANGE (%)
ε2	124 ± 20	112 ± 16	−9.2 ± 11.5	106 ± 31	94 ± 24	−8.5 ± 18.3	5.3 ± 1.0	4.7 ± 1.5	−9.6 ± 22.8
ε3	123 ± 26	110 ± 22	−9.1 ± 16.3	107 ± 27*	98 ± 26†	−5.6 ± 20.6	4.4 ± 1.2‡	3.8 ± 0.9†‡	−10.8 ± 22.7
ε4	123 ± 27	111 ± 19	−8.0 ± 14.8	115 ± 32	104 ± 25	−6.1 ± 19.3	4.1 ± 1.2‡	3.3 ± 0.9‡	−14.4 ± 23.3

Data are means ± SD. ε2, ε3, and ε4 groups consist of subjects with E2/2 and E2/3, subjects with E3/3, and subjects with E3/4 and E4/4, respectively.
*$P < 0.05$, ε3 group compared with ε4 group.
†$P < 0.01$, ε3 group compared with ε4 group.
‡$P < 0.01$ compared with ε2 group.

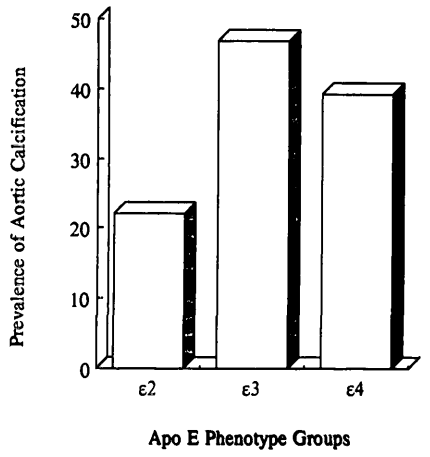

Figure 1—*Prevalence (%) of aortic calcification in subjects of each phenotype group. Prevalence was expressed as percentage of subjects with aortic calcification in total subjects of each apoE phenotype group.*

plasma cholesterol levels during the 7 days results from a reduction in VLDL cholesterol; and because subjects with ε4 had somewhat lower baseline triglycerides, the absolute fall in VLDL cholesterol is less in subjects with ε4.

The plasma cholesterol level is thought to be regulated by at least three metabolic mechanisms in humans: *1*) intestinal absorption of dietary cholesterol; 2) endogenous cholesterol synthesis in the liver; and 3) clearance of plasma LDL via the LDL-receptor pathway. In our diet therapy for diabetic subjects, the total intake of calorie was reduced, but mean daily intake of cholesterol and P/S ratio were not necessarily reduced much (290 mg cholesterol/day to 275 mg cholesterol/day and 0.93 to 0.93, respectively), because daily intakes of cholesterol and fat in our diabetic subjects before hospitalization were similar to those in the Step 1 Diet recommended by the American Heart Association, as we reported (19). Thus, the reduction in total caloric intake rather than in exogenous cholesterol intake may play an important role in reducing plasma cholesterol levels in this study. Caloric restriction may reduce both endogenous cholesterol synthesis and VLDL production in the liver. In fact, mean plasma triglyceride levels, which reflect plasma VLDL levels, were significantly reduced in all three groups after the treatment. Decreased endogenous cholesterol synthesis, which enhances hepatic LDL-receptor activity, may result in lowered plasma cholesterol level.

Effects of apoE polymorphism on plasma cholesterol levels were more clearly manifested after the treatment than before. Individual diabetic state and nutritional state might have masked the effects of apoE polymorphism on plasma cholesterol levels before the treatment. After the treatment, plasma cholesterol levels in subjects with either ε3 or ε4 were evidently greater than those with ε2, and the improvement in subjects with ε4 during the treatment with low-calorie diet was least among apoE phenotype groups. Our results suggest that total plasma cholesterol levels in subjects with ε4 fall less than other subjects in response to a 7-day calorie-restricted diet, despite plasma cholesterol levels being similar among phenotype groups before dietary intervention and when similar diet therapy without reduction in cholesterol intake is given to all subjects under strict metabolic control. This difference reflects mainly VLDL-cholesterol changes. Hepatic LDL-receptor activities in subjects with ε4 may also not be upregulated as much even after the treatment, because cholesterol intake was not decreased much compared with before the treatment. Previous studies showed that plasma cholesterol levels in hypercholesterolemic subjects with ε4 were reduced more by longer term dietary intervention such as a low-cholesterol diet than in subjects with ε2 or ε3 (20,21). In diabetic subjects with either ε3 or ε4, calorie-restricted diet therapy is not satisfactory to reduce the plasma cholesterol level in the short term; therefore, reduction in cholesterol and saturated fat intake should be included in their dietary modification.

We previously reported a high prevalence of ε4 in multi-infarct dementia (9); and in this study, we demonstrated higher incidence of aortic calcification in subjects with ε4 than in others. Thus, apoE polymorphism can be a genetic marker not only to determine plasma cholesterol level but also to predict future atherosclerosis. It is important to prevent future atherosclerosis such as coronary heart disease in diabetes mellitus, especially in subjects with E3/4 and E4/4. We recommend intensive treatment in those subjects to lower plasma cholesterol levels to prevent the progression of atherosclerosis.

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