Original Article

Polymorphisms of Apolipoprotein E and Methylenetetrahydrofolate Reductase in the Japanese Population

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Aim: The aim of this study is to analyze the effect of apolipoprotein E (apo E) and methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms on serum lipid and homocysteine levels in the general Japanese population.

Methods: We analyzed the polymorphisms in individuals randomly selected from among participants of Serum Lipid Survey 2000.

Results: The frequency of the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles of *APOE* was 4.2, 85.3, and 10.5%, respectively. Individuals with the genotype $\epsilon 4/\epsilon 4$ had the highest total and low-density lipoprotein (LDL) cholesterol levels, while those with $\epsilon 2/\epsilon 2$ had the lowest. Individuals with the $\epsilon 2/\epsilon 2$ and $\epsilon 2/\epsilon 4$ genotypes had higher remnant-like particles (RLP)-cholesterol levels than those with $\epsilon 2\epsilon 3$, $\epsilon 3\epsilon 3$, and $\epsilon 3\epsilon 4$. There was a trend for individuals with the $\epsilon 2/\epsilon 4$ and $\epsilon 2/\epsilon 2$ genotypes to have higher triglyceride levels, although the difference was not significant. The presence of the T allele in a *MTHFR* polymorphism (C667T) was associated with higher homocysteine levels, which is more prominent in men than in women.

Conclusion: Thus in our large-scale analysis we have shown that RLP-cholesterol is better associated with *APOE* genotype than triglyceride and the effect of the T allele on *MTHFR* polymorphism (C667T) homocysteine levels is more prominent in men than in women among Japanese.

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Key words; Hyperlipidemia, Polymorphism, Apolipoprotein E, *MTHFR*, Homocysteine

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Introduction

Apolipoprotein E (apo E) is an important structural constituent of serum chylomicrons, very low-density lipoproteins, and high-density lipoproteins (HDL) and plays a critical role in lipoprotein metabolism, where it can facilitate the clearance of remnant lipoprotein and cellular efflux of cholesterol¹⁾. Apo E has three polymorphisms, $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$, which affect lipoprotein metabolism and atherosclerosis²⁾. The $\epsilon 4$ allele is associated with higher low-density lipoprotein (LDL) cholesterol levels than the other alleles and with a higher incidence of coronary heart disease³⁾. Apo E4 is also shown to be involved in the development of Alzheimer's disease⁴⁾, while homozygosity for apo E2 is associated with the development of type III hyperlipidemia⁵⁾.

We also studied the *MTHFR* gene because its polymorphisms affect serum homocysteine levels and homocysteine is also associated with cardiovascular disease and Alzheimer's disease⁶⁻⁹⁾. An elevated homocysteine level is associated with coronary heart disease and the C677T polymorphism in the *MTHFR* gene results in reduced *MTHFR* enzyme activity and reduced methylation of homocysteine to methionine resulting in mild hyperhomocysteinemia¹⁰⁾. Although several studies have examined the incidence of *APOE* and *MTHFR* polymorphisms^{8, 11)}, there has been no large-scale study to determine the incidence of *APOE* and *MTHFR* polymorphisms and their association with lipoprotein profiles and homocysteine levels in the general Japanese population. In 2000, we conducted a lipid survey in the Japanese population, 12,839 people all over the country. In this survey, we examined *APOE* and *MTHFR* gene polymorphisms to determine the incidence of each and its relationship with lipid profiles and homocysteine levels in the Japanese.

Methods

Design and Data Collection

This work is part of Serum Lipid Level Survey 2000 from various parts of Japan. The Ethics committee, Graduate School and Faculty of Medicine, Kyoto University approved the study protocol and all subjects provided written informed consent for participation in the gene analysis. The handling of DNA samples followed the guidelines from the Ministry of Health, Labor, and Welfare. In Serum Lipid Survey 2000, a total of 12,839 subjects were recruited at 36 hospitals across the country. The subjects in the present study were participants in the survey at 9 hospitals from whom informed content for genotyping was sought. Of the 12,839 subjects, 2,267 (17.7%) with no lipid-

altering medication were randomly selected for the present study. Among the 2,267 participants, we examined serum homocysteine levels and *MTHFR* gene polymorphisms in 505 participants.

Laboratory Methods

All serum and blood samples were obtained in the fasting state. All lipid and other analyses were conducted on venous blood samples within one week of collection at BML (Saitama, Japan). Serum cholesterol and TG levels were measured by enzymatic assay. HDL-cholesterol and LDL-cholesterol levels were measured enzymatically with a kit from Daiichi Kagaku Co. Ltd. (Tokyo, Japan). The results of lipid analyses in the four surveys were indirectly standardized according to the criteria of the CDC Lipid Standardization Program¹²⁾. The serum homocysteine level was assayed by high performance liquid chromatography with fluorescent detection as described by Ubbink *et al.*¹³⁾. DNA was extracted with a QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Detection of gene Mutations by Invader[®] Assay

We used the Invader[®] assay to screen for mutations of the *APOE* and *MTHFR* genes, as previously described. In brief, the probe/Invader[®]/MgCl₂ mixture was prepared by combining 3 μ L of primary probe/Invader[®] mix and 5 μ L of 22.5 mM MgCl₂ per reaction. The primary probes/Invader[®] mixture contained 3.5 μ mol/L wild primary probe, 3.5 μ mol/L mutant primary probe, 0.35 μ mol/L Invader[®] oligonucleotide, and 10 mmol/L MOPS. Eight microliters of primary probe/Invader[®]/MgCl₂ mixture was added per well of a 96-well plate. Seven microliters of 5 fmol/L synthetic target oligonucleotides, 10 μ g/mL yeast tRNA (no target blank), and genomic DNA (15 ng/ μ L) were added, and denatured by incubation at 95°C for 10 min. After 15 μ L of mineral oil (Sigma, St. Louis, MO) was overlaid into all reaction wells, the plate was incubated isothermally at 63°C for 4 h in a DNA thermocycler (PTC-200; MJ Research, Watertown, MA) and then kept at 4°C until fluorescence were measured. The intensity of the fluorescence was measured with a fluorescence microtiter plate reader (Cytofluor 4000; Applied Biosystems) with excitation at 485 nm/20 nm (Wavelength/Bandwidth) and emission at 530 nm/25 nm for FAM; and excitation at 560 nm/20 nm and emission at 620 nm/40 nm for RED. The genotyping was analyzed by calculating the ratio of net counts with wild primary probe to net counts with mutant primary probe. The probes used in this study were designed and synthesized by Third Wave Technologies, Inc (Madison, WI).

Data Analyses

Differences in means were evaluated with an analysis of variance. The analysis was performed with the statistical Package for Social Sciences (SPSS Japan Inc. ver. 11.5, Tokyo, Japan).

Results

We investigated the frequency and phenotypic association of *APOE* gene polymorphisms of 2,267 subjects. We found that the SNPs were in Hardy-Weinberg equilibrium. As previously described, the mean age, total cholesterol, TG, HDL-cholesterol, and LDL-cholesterol levels in this population were similar to the levels for all 12,839 patients in Serum Lipid Survey 2000¹⁴. We also found that the medians of total, LDL-, and HDL-cholesterol levels did not differ appreciably from the means, thereby excluding gross right-hand tailing of the distribution (data not shown). These data indicate that the participants in the gene analysis are representative of the general Japanese population.

The genotype and allelic frequency of *APOE* polymorphisms are presented in **Table 1**. The frequency of the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles was 4.2, 85.3, and 10.5%, respectively. As in other studies, the genotypes $\epsilon 2\epsilon 2$,

$\epsilon 2\epsilon 4$, and $\epsilon 4\epsilon 4$ were quite rare. High frequencies of the $\epsilon 3$ allele are also found in Chinese, but the frequency is lower in Caucasians¹⁵.

We next examined the association of the *APOE* genotype and lipid profiles in these participants. As shown in **Table 2**, all the lipid parameters and blood glucose differed significantly among these genotypes by ANOVA. Total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, and RLP-cholesterol levels were different among the groups. The *p* values are shown in the right column. According to the post-hoc analysis, the total cholesterol level was significantly lower for genotype $\epsilon 2\epsilon 2$ than $\epsilon 4\epsilon 4$ and genotype $\epsilon 2\epsilon 3$ than $\epsilon 3\epsilon 3$, $\epsilon 2\epsilon 4$, or $\epsilon 4\epsilon 4$. The HDL-cholesterol level was significantly higher for $\epsilon 2\epsilon 3$ than $\epsilon 2\epsilon 4$. The LDL-cholesterol level was significantly lower for genotypes $\epsilon 2\epsilon 2$ and $\epsilon 2\epsilon 3$ than for $\epsilon 3\epsilon 3$, $\epsilon 3\epsilon 4$, and $\epsilon 4\epsilon 4$. The RLP-cholesterol level was significantly higher for $\epsilon 2\epsilon 2$ than $\epsilon 2\epsilon 3$, $\epsilon 3\epsilon 3$, $\epsilon 3\epsilon 4$, or $\epsilon 4\epsilon 4$ and for genotype $\epsilon 2\epsilon 4$ than $\epsilon 2\epsilon 3$, $\epsilon 3\epsilon 3$, or $\epsilon 3\epsilon 4$, although there was no significant difference in triglyceride levels according to the post-hoc analysis. Blood glucose or age did not differ significantly among the groups.

We next examined the association of the *MTHFR* C667T polymorphism with serum homocysteine levels in 505 samples randomly selected from 2,267 samples. As shown in **Table 3**, the incidence of the CC, CT, and TT genotypes was 33.9, 46.1, and 20.0%, respectively. The TT genotype was significantly associated with higher homocysteine levels in men and women, and statistical significance was found between CC and TT and between CT and TT by a post-hoc analysis. However, the difference was more prominent in men.

Discussion

There, we have shown in a large-scale study, the

Table 1. Genotype and allele frequency of *APOE* gene in Japanese.

genotype	<i>n</i>	%	alleles	<i>n</i>	%
$\epsilon 2/\epsilon 2$	9	0.4	$\epsilon 2$	192	4.2
$\epsilon 2/\epsilon 3$	155	6.8	$\epsilon 3$	3,868	85.3
$\epsilon 2/\epsilon 4$	19	0.8	$\epsilon 4$	474	10.5
$\epsilon 3/\epsilon 3$	1,653	72.9			
$\epsilon 3/\epsilon 4$	407	18.0			
$\epsilon 4/\epsilon 4$	24	1.1			

Table 2. Mean of serum lipid levels and blood glucose in each genotype of *APOE* in Japanese.

	$\epsilon 2/\epsilon 2$	$\epsilon 2/\epsilon 3$	$\epsilon 2/\epsilon 4$	$\epsilon 3/\epsilon 3$	$\epsilon 3/\epsilon 4$	$\epsilon 4/\epsilon 4$	total	<i>p</i> value
	mean $\pm$ SEM	mean $\pm$ SEM	mean $\pm$ SEM	mean $\pm$ SEM	mean $\pm$ SEM	mean $\pm$ SEM	mean $\pm$ SEM	
T-cho	165.0 $\pm$ 23.8	189.7 $\pm$ 3.00	202.9 $\pm$ 12.6	201.8 $\pm$ 0.92	206.8 $\pm$ 1.95	223.3 $\pm$ 9.18	202.1 $\pm$ 0.81	<0.0001
TG	171.4 $\pm$ 52.8	118.8 $\pm$ 8.55	189.0 $\pm$ 53.3	117.0 $\pm$ 2.41	128.0 $\pm$ 5.16	127.9 $\pm$ 18.9	119.8 $\pm$ 2.13	0.023
HDL-c	51.2 $\pm$ 9.51	63.6 $\pm$ 1.92	53.0 $\pm$ 3.07	59.8 $\pm$ 0.41	58.0 $\pm$ 0.82	61.9 $\pm$ 3.48	59.7 $\pm$ 0.36	0.007
LDL-c	70.5 $\pm$ 5.63	101.9 $\pm$ 2.75	117.2 $\pm$ 8.07	118.5 $\pm$ 0.91	120.5 $\pm$ 1.93	131.5 $\pm$ 7.97	117.7 $\pm$ 0.79	<0.0001
RLP-c	22.9 $\pm$ 1.15	4.4 $\pm$ 0.37	12.5 $\pm$ 7.59	4.7 $\pm$ 0.17	5.2 $\pm$ 0.33	4.1 $\pm$ 0.58	4.8 $\pm$ 0.15	<0.0001
FBS	121.3 $\pm$ 19.5	104.7 $\pm$ 3.27	110.6 $\pm$ 9.37	103.9 $\pm$ 0.94	103.3 $\pm$ 2.17	88.6 $\pm$ 2.54	103.9 $\pm$ 0.83	0.461
age	52.8 $\pm$ 10.1	49.5 $\pm$ 2.11	50.8 $\pm$ 53.2	46.7 $\pm$ 0.69	47.4 $\pm$ 1.30	43.2 $\pm$ 4.61	47.1 $\pm$ 0.58	0.659

T-cho: total cholesterol (mg/dL), TG: triglyceride (mg/dL), HDL-c: HDL-cholesterol (mg/dL), LDL-c: LDL-cholesterol (mg/dL), RLP-c: remnant-like particles cholesterol (mg/dL), FBS: fasting blood sugar (mg/dL), SEM: standard error of the mean

Table 3. Genotype frequency of the *MTHFR* gene and its association with serum homocysteine levels in Japanese.

total					
genotype	<i>n</i>	%	mean	SEM	
CC	171	33.9	10.9	0.3	$p < 0.001$
CT	233	46.1	11.6	0.24	
TT	101	20.0	15.7	1.23	
total	505	100	12.2	0.29	
male					
genotype	<i>n</i>	%	mean	SEM	
CC	92	33.6	10.7	0.36	$p < 0.001$
CT	132	48.2	12.9	0.35	
TT	50	18.2	19.8	2.41	
total	274	100	13.4	0.52	
female					
genotype	<i>n</i>	%	mean	SEM	
CC	79	34.2	10.2	0.43	$p = 0.005$
CT	101	43.7	10.1	0.27	
TT	51	22.1	11.9	0.57	
total	231	100	10.5	0.23	

SEM: standard error of the mean

frequency of the *APOE* genotype in the Japanese and its association with serum lipid levels. Frequencies of *APOE* genotypes are highly heterogeneous among various populations. Epidemiological data indicate that the frequency of the $\epsilon 3$ allele is higher in Japanese and Chinese than in Caucasians, while the frequency of the $\epsilon 4$ allele is lower in Asians than Caucasians^{3, 16}. Our data indicate that the frequency of the $\epsilon 3$ allele is quite consistent with previous reports in Japanese^{8, 11, 16, 17}, and is slightly higher than that of Icelandic and Hungarian populations and much higher than that in the Finnish population¹⁵.

Our study confirmed that the $\epsilon 4$ allele is associated with higher, and the $\epsilon 2$ allele is associated with lower, LDL cholesterol levels. Although there was a trend for individuals with the genotypes $\epsilon 2/\epsilon 4$ and $\epsilon 2/\epsilon 2$ to have higher triglyceride levels, it was not statistically significant by a post-hoc analysis, probably because triglyceride levels are highly variable. However, individuals with $\epsilon 2/\epsilon 4$ and $\epsilon 2/\epsilon 2$ had significantly higher RLP-cholesterol levels than did those with the other genotypes, indicating that RLP-cholesterol might be better correlated with *APOE* genotype. Although in this study we could not compare the body

mass index of $\epsilon 2/\epsilon 2$ homozygotes, it would be intriguing to know whether individuals with the $\epsilon 2/\epsilon 4$ and $\epsilon 2/\epsilon 2$ genotypes have metabolic abnormalities, such as abdominal obesity and insulin resistance, because they have higher triglyceride, RLP-cholesterol, and blood glucose levels.

Elevated levels of homocysteine have been considered a risk for cardiovascular disease. Our study is consistent with other studies that show higher homocysteine levels in people with the TT genotype. However, the relationship between the C677T *MTHFR* polymorphism and cardiovascular disease is still controversial. Because our study population is made up of healthy volunteers, a prospective study is necessary to determine which genotype is associated with cardiovascular risk.

In summary, we have provided the largest database of gene polymorphisms related to lipid metabolism and homocysteine in the general Japanese population. A prospective study is necessary to determine the contribution of these gene polymorphisms to cardiovascular risk in Japanese.

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