Table 3 Total Medical Expenditures per Person Grouped by Number of Cardiovascular Risk Factors, Stratified by Having Overweight (BMI ≥25.0) or Not After 10-Year Follow-up From 1990 to 2001, in National Health Insurance in Shiga, Japan

Risk status category	*No. of participants*	*Total medical costs per person per month*	
		Arithmetic mean	*Adjusted geometric mean*
None			
BMI<25.0	*1,849*	*15,377 yen (130 dollars)*	*6,985 yen (59 dollars)*
BMI≥25.0	*286*	*23,011 yen (195 dollars)*	*9,168 yen† (78 dollars)*
1 risk factor			
BMI<25.0	*1,336*	*24,245 yen (206 dollars)*	*9,091 yen† (77 dollars)*
BMI≥25.0	*430*	*19,143 yen (162 dollars)*	*10,703 yen† (91 dollars)*
2 or 3 risk factors			
BMI<25.0	*351*	*24,002 yen (203 dollars)*	*10,263 yen† (90 dollars)*
BMI≥25.0	*226*	*26,782 yen (227 dollars)*	*12,048 yen† (102 dollars) p<0.01**

100 Japanese yen=0.848 US dollars, at the foreign exchange rate on November 7th, 2006.
**Analysis of covariance adjusted for age, sex, smoking habit and drinking habit.*
†Significance, vs none without overweight, for multiple post-hoc comparisons with Bonferroni correction, p<0.05.
BMI, body mass index.

Table 4 Total Medical Expenditures per Person Grouped by Type of Cardiovascular Risk Factors, Stratified by Having Overweight (BMI ≥25.0) or Not After 10-Year Follow-up From 1990 to 2001, in National Health Insurance in Shiga, Japan

Risk status category	*No. of participants*	*Total medical costs per person per month*	
		*Adjusted geometric mean (Model 1)**	*Adjusted geometric mean (Model 2)***
Hypertension			
BMI<25.0	*1,098*	*9,045 yen (77 dollars)*	*11,407 yen (97 dollars)*
BMI≥25.0	*519*	*11,026 yen† (94 dollars)*	*12,991 yen (110 dollars)*
Hypercholesterolemia			
BMI<25.0	*803*	*9,252 yen (78 dollars)*	*9,210 yen (78 dollars)*
BMI≥25.0	*312*	*10,420 yen† (88 dollars)*	*10,551 yen (89 dollars)*
Diabetes			
BMI<25.0	*153*	*15,308 yen (130 dollars)*	*15,139 yen (128 dollars)*
BMI≥25.0	*63*	*18,974 yen (161 dollars)*	*19,497 yen (165 dollars)*

100 Japanese yen=0.848 US dollars, at the foreign exchange rate on November 7th, 2006.
**Model 1, analysis of covariance adjusted for age, sex, smoking habit and drinking habit.*
***Model 2, analysis of covariance adjusted for age, sex, smoking habit, drinking habit and other risk factors except for categorized risk factor: for example, in hypertension, hypercholesterolemia and diabetes were adjusted.*
†Significance, between normal weight and overweight, p<0.05.
Abbreviation see in Table 3.

the number of risk factors and adjusted geometric means of medical expenditures in both the normal weight and overweight groups was positively graded. The increase in the rate of medical expenditures according to the number of risk factors was not parallel; however, the interaction term between the number of cardiovascular risk factors and overweight criteria did not reach statistical significance (p=0.351). Individual medical expenditures per month were higher in overweight individuals, than in the normal weight group when the number of other cardiovascular risk factors was consistent.

Table 4 shows the medical expenditures between overweight and normal weight participants with hypertension, hypercholesterolemia and diabetes. The medical expenditures per person in all 3 groups were higher in the overweight group than in the normal weight group. The difference in medical expenditures between overweight and normal weight were largest in diabetics.

The calculated excess medical expenditures attributable to normal weight individuals with 1 risk factor, those who were of normal weight with 2–3 risk factors, only overweight, overweight with 1 other risk factor and overweight with 2–3 other risk factors were 11,847,648 yen, 3,027,375 yen, 2,183,324 yen, 1,619,380 yen and 2,577,530 yen, respectively. Fig 1 shows the share of each excessive medical cost of the total medical expenditures of the entire population. The excess medical expenditures of the 2 normal weight categories combined (16.5%) were higher than

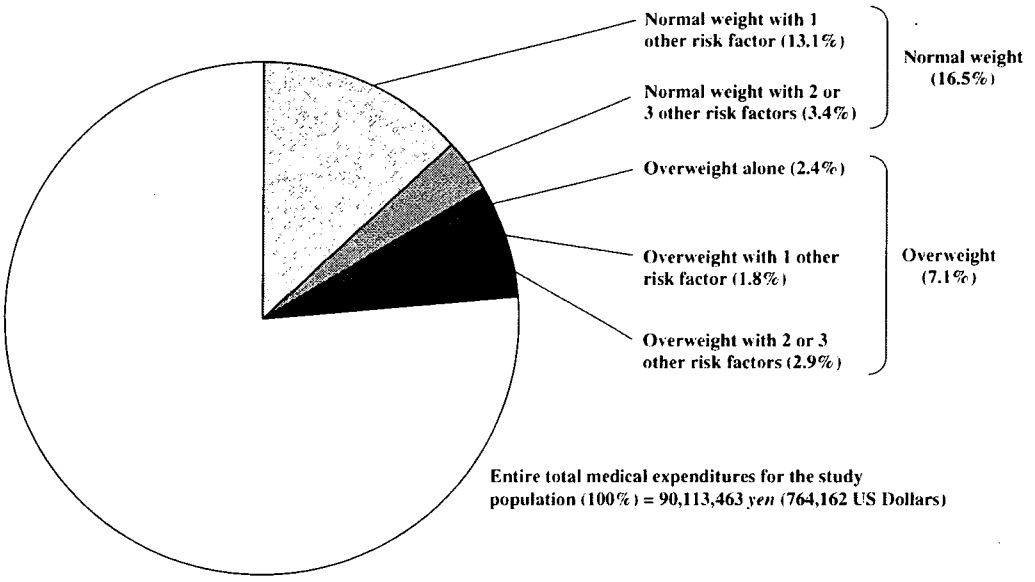

Fig 1. Ratio (%) of excess medical expenditures related to number of cardiovascular risk factors stratified by body mass index (25 kg/m^2) in whole population after 10-year follow-up, from 1990 to 2001, in National Health Insurance in Shiga, Japan (men and women combined). White area represents predicted medical expenditures if all participants were of normal weight without risk factors.

those of 3 overweight categories combined (7.1%).

Discussion

We performed a follow-up study of a Japanese community between 1990 and 2001 and found a positive graded relationship between clustering of cardiovascular risk factors and personal medical expenditures irrespective of being overweight. The mean personal medical cost was higher in overweight, than in normal weight individuals when the number of other risk factors was consistent. Furthermore, the total medical expenditures were the highest in overweight individuals with 2–3 risk factors. Nevertheless, the excess medical expenditures in these participants in entire population were only a few percent and the excess expenditures observed in normal weight categories were rather higher than those in overweight categories.

Findings from the Framingham study have already shown that the risk of atherosclerotic disease increases with combinations of risk factors, such as hypertension, glucose intolerance and hypercholesterolemia[25] Japanese epidemiological studies have also found similar results in community[6] and occupational[26] settings. However, few studies to our knowledge have investigated the association between cardiovascular risk clustering or metabolic syndrome and medical expenditures[13,14] Most other studies have focused on the effect of hypertension combined with diabetes on medical economics[22,27,28]

The continuous increase in medical expenditures is an important concern in most developed countries[29] Furthermore, the effect of cardiovascular diseases on medical economics is a major concern. For example, the medical expenditures for cardiovascular disease including hypertension was 20.4% of the total national medical expenditures in the Japanese population aged 45–69 years, which was larger than any other disease groups during 2001[30] The effective way to control medical expenditures incurred by cardiovascular diseases is to detect those at high risk and provide intensive health and lifestyle guidance or opportunities for early clinical visits for primary care. The present findings showed that overweight people with cardiovascular risk clustering should be detected as priority targets for a high-risk strategy[31] and that overweight people with cardiovascular risk factors such as hypertension, hypercholesterolemia and diabetes can also be potential targets for high-risk strategies that could significantly affect individual medical expenditures. If an individual has accumulated visceral fat or impaired glucose tolerance, which is now classified as a metabolic syndrome, then their medical expenditures should be reduced by implementing appropriate dietary measures and by increasing physical activity.

By contrast, irrespective of high individual medical expenditures, the proportion of excess medical expenditures in the normal weight categories with 1 or more other risk factors was higher than those of all overweight categories combined. The low proportion of excess medical expenditures incurred by overweight individuals is a result of relatively small number of overweight participants identified in the present study. The 1989 to 1991 baseline survey defined only 21% of participants as being overweight (25 kg/m^2 or more). Accordingly, from the viewpoint of an entire population and a population strategy[31] regardless of being overweight, the presence of other cardiovascular risk factors such as hypertension, diabetes and hypercholesterolemia significantly effects medical expenditures. Normal weight people with other risk factors, especially in non-Western populations with a low prevalence of obesity, should be carefully considered.

The present study has several limitations. First, the public medical insurance system in Japan differs from that in other countries. Therefore, absolute values of medical expenditures for the participants in the present study might not be directly relevant to other populations. Second, we clustered risk factors from a single measurement at the baseline survey, which generated a regression dilution bias. Third, we did not have values for fasting blood glucose,

triglycerides or HDL-cholesterol, which are important components of metabolic syndrome![5] We used BMI as an indicator of being overweight. One report indicates that waist circumference predicts visceral fat accumulation (which plays a major role on atherosclerosis) better than BMI![32] Accordingly, we might have underestimated or misclassified obesity or being overweight by the BMI method. Finally, details of medical diagnoses, medical treatment status (eg, prescriptions), clinical condition and cause of mortality were not available. Thus, further studies are required to clarify the effects of these variables.

In conclusion, cardiovascular risk clustering and being overweight can be a useful predictor of medical expenditures. On the contrary, the sum of excess medical expenditures because of risk factor clustering in normal weight individuals is larger than that in overweight individuals because of the relatively small ratio of overweight individuals in Japan. However, the obesity epidemic is not restricted to Western countries. Furthermore, mean BMI is rapidly increasing in Asian countries such as Japan. Accordingly, being overweight might increase population medical expenditures in the future.

Acknowledgements

The present study was part of the research of the Health Promotion Research Committee of the Shiga NHI Organizations, to whom we express our gratitude. This study was funded by a research grant from the Ministry of Health, Labor and Welfare (H17-kenko-007, H18-Seishuu-012).

References

1. Bhatt DL, Steg PG, Ohman EM, Hirsch AT, Ikeda Y, Mas JL, et al. International prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. *JAMA* 2006; **295:** 180–189.
2. Reaven GM. Banting lecture role of insulin resistance in human disease. *Diabetes* 1988; **37:** 1595–1607.
3. Kaplan NM. The deadly quartet: Upperbody obesity, glucose intolerance, hyper-triglyceridemia, and hypertension. *Arch Intern Med* 1989; **149:** 1514–1520.
4. DeFronzo RA, Ferrannini E. Insulin resistance: A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 1991; **14:** 173–194.
5. Fujioka S, Matsuzawa Y, Tokunaga K. Contribution of intraabdominal visceral fat accumulation to the impairment of glucose and lipid metabolism in human obesity. *Metabolism* 1987; **36:** 54–59.
6. Nakamura Y, Yamamoto T, Okamura T, Kadowaki T, Hayakawa T, Kita Y, et al. Combined cardiovascular risk factors and outcome: NIPPON DATA80, 1980-1994. *Circ J* 2006; **70:** 960–964.
7. Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR, et al. Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. *Circulation* 2004; **110:** 1245–1250.
8. Hebel JR, McCarter RJ, Sexton M. Health care costs for employed hypertensives. *Med Care* 1990; **28:** 446–457.
9. Nakamura K, Okamura T, Kanda H, Hayakawa T, Kadowaki T, Okayama A, et al. Impact of hypertension on medical economics: A 10-year follow-up study of national health insurance in Shiga, Japan. *Hypertens Res* 2005; **28:** 859–864.
10. Daviglus ML, Liu K, Yan LL, Pirzada A, Manheim L, Manning W, et al. Relation of body mass index in young adulthood and middle age to Medicare expenditures in older age. *JAMA* 2004; **292:** 2743–2749.
11. Kuriyama S, Tsuji I, Ohkubo T, Anzai Y, Takahashi K, Watanabe Y, et al. Medical care expenditure associated with body mass index in Japan: The Ohsaki Study. *Int J Obes Relat Metab Disord* 2002; **26:** 1069–1074.
12. Krop JS, Saudek CD, Weller WE, Powe NR, Shaffer T, Anderson GF. Predicting expenditures for Medicare beneficiaries with diabetes: A prospective cohort study from 1994 to 1996. *Diabetes Care* 1999; **22:** 1660–1666.
13. Daviglus ML, Liu K, Greenland P, Dyer AR, Garside DB, Manheim L, et al. Benefit of a favorable cardiovascular risk-factor profile in middle age with respect to Medicare costs. *N Engl J Med* 1998; **339:** 1122–1129.
14. Schauffler HH, D'Agostino RB, Kannel WB. Risk for cardiovascular disease in the elderly and associated Medicare costs: The Framingham Study. *Am J Prev Med* 1993; **9:** 146–154.
15. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel). *JAMA* 2001; **285:** 2486–2497.
16. Alberti KGMM, Zimmet P, Shaw J. Metabolic syndrome–a new world-wide definition: A Consensus Statement from the International Diabetes Federation. *Diabetes Med* 2006; **23:** 469–480.
17. Katzmarzyk PT, Janssen I, Ross R, Church TS, Blair SN. The importance of waist circumference in the definition of metabolic syndrome: Prospective analyses of mortality in men. *Diabetes Care* 2006; **29:** 404–409.
18. Yoon YS, Lee ES, Park C, Lee S, Oh SW. The new definition of metabolic syndrome by the international diabetes federation is less likely to identify metabolically abnormal but non-obese individuals than the definition by the revised national cholesterol education program: The Korea NHANES Study. *Int J Obes* 2007; **31:** 528–534.
19. Health and Welfare Statistics Association. 2004 Kokumin Eisei no Doko (Trend for National Health and Hygiene, Japan). Tokyo: Health and Welfare Statistics Association; 2004 (in Japanese).
20. Health and Welfare Statistics Association. 2004 Hoken to Nenkin no Doko (Trend for Insurance and Pension, Japan). Tokyo: Health and Welfare Statistics Association; 2004 (in Japanese).
21. Nakamura K, Okamura T, Kanda H, Hayakawa T, Okayama A, Ueshima H; Health Promotion Research Committee of the Shiga National Health Insurance Organizations. The value of combining serum alanine aminotransferase levels and body mass index to predict mortality and medical costs: A 10-year follow-up study of National Health Insurance in Shiga, Japan. *J Epidemiol* 2006; **16:** 15–20.
22. Nakamura K, Okamura T, Kanda H, Hayakawa T, Okayama A, Ueshima H; Health Promotion Research Committee of the Shiga National Health Insurance Organizations. Medical costs of patients with hypertension and/or diabetes: A 10-year follow-up study of National Health Insurance in Shiga, Japan. *J Hypertens* 2006; **24:** 2305–2309.
23. The Ministry of Health and Welfare. Manual for Health Check-ups under Medical Service Law for the Aged. Tokyo: Japan Public Health Association; 1987 (in Japanese).
24. World Health Organization. Obesity: Preventing and managing the global endemic. WHO Technical Report Series no 894. Geneva: WHO, 2000.
25. Kannel WB, Castelli WP, Gordon T. Cholesterol in the prediction of atherosclerotic disease: New perspectives based on the Framingham study. *Ann Intern Med* 1979; **90:** 85–91.
26. Nakamura T, Tsubono Y, Kameda-Takemura K, Funahashi T, Yamashita S, Hisamichi S, et al. Magnitude of sustained multiple risk factors for ischemic heart disease in Japanese employees: A case-control study. *Jpn Circ J* 2001; **65:** 11–17.
27. French MT, Mundt MP, Fleming M, Zavala SK. The cost of medical care for patients with diabetes, hypertension and both conditions: Does alcohol use play a role? *J Intern Med* 2005; **258:** 45–54.
28. Amin SP, Mullins CD, Duncan BS, Blandford L. Direct health care costs for treatment of diabetes mellitus and hypertension in an IPA-group-model HMO. *Am J Health Syst Pharm* 1999; **56:** 1515–1520.
29. Forti EM, Johnson JA, Graber DR. Aging in America: Challenges and strategies for health care delivery. *J Health Hum Serv Adm* 2000; **23:** 203–213.
30. Health and Welfare Statistics Association: 2003 Kokumin Eisei no Doko (Trend for National Health and Hygiene, Japan). Tokyo: Health and Welfare Statistics Association, 2003 (in Japanese).
31. Okamura T, Tanaka T, Babazono A, Yoshita K, Chiba N, Takebayashi T, et al. The high-risk and population strategy for occupational health promotion (HIPOP-OHP) study: Study design and cardiovascular risk factors at the baseline survey. *J Hum Hypertens* 2004; **18:** 475–485.
32. Ohnishi H, Saitoh S, Takagi S, Katoh N, Chiba Y, Akasaka H, et al. Incidence of type 2 diabetes in individuals with central obesity in a rural Japanese population. *Diabetes Care* 2006; **29:** 1128–1129.

Appendix 1

The Health Promotion Research Committee of the Shiga NHI Organizations

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Secretariat: Yukio Tobita, Kanehiro Okamura, Kiminobu Hatta, Takao Okada, Michiko Hatanaka (Shiga NHI Organizations).

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 $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ alleles of *APOE* was 4.2, 85.3, and 10.5%, respectively. Individuals with the genotype $\varepsilon 4/\varepsilon 4$ had the highest total and low-density lipoprotein (LDL) cholesterol levels, while those with $\varepsilon 2/\varepsilon 2$ had the lowest. Individuals with the $\varepsilon 2/\varepsilon 2$ and $\varepsilon 2/\varepsilon 4$ genotypes had higher remnant-like particles (RLP)-cholesterol levels than those with $\varepsilon 2\varepsilon 3$, $\varepsilon 3\varepsilon 3$, and $\varepsilon 3\varepsilon 4$. There was a trend for individuals with the $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ 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.

J Atheroscler Thromb, 2007; 14:167-171.

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

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Received: January 22, 2007
Accepted for publication: March 14, 2007

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 metablism, where it can facilitate the clearance of remnant lipoprotein and cellular efflux of cholesterol[1]. Apo E has three polymorphisms, ε2, ε3, and ε4, which affect lipoprotein metabolism and atherosclerosis[2]. The ε4 allele is associated with higher low-density lipoprotein (LDL) cholesterol levels than the other alleles and with a higher incidence of coronary heart disease[3]. Apo E4 is also shown to be involved in the development of Alzheimer's disease[4], while homozygosity for apo E2 is associated with the development of type Ⅲ hyperlipidemia[5].

We also studied the *MTHFR* gene because its polymorphisms affect serum homocysteine levels and homocysteine is also associated with cardiovascular disease and Alzheimer's disease[6-9]. 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 methioneine resulting in mild hyperhomocysteinemia[10]. Although several studies have examined the incidence of *APOE* and *MTHFR* polymorphisms[8, 11], these 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 Program[12]. 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 μL of primary probe/Invader® mix and 5 μL of 22.5 mM MgCl2 per reaction. The primary probes/Invader® mixture contained 3.5 μmol/L wild primary probe, 3.5 μmol/L mutant primary probe, 0.35 μmol/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 μg/mL yeast tRNA (no target blank), and genomic DNA (15 ng/μL) were added, and denatured by incubation at 95℃ for 10 min. After 15 μL of mineral oil (Sigma, St. Louis, MO) was overlayed into all reaction wells, the plate was incubated isothermally at 63℃ for 4 h in a DNA thermalcycler (PTC-200; MJ Research, Watertown, MA) and then kept at 4℃ until fluorescence were measured. The intensity of the fluorescence was measured with a fluorescence microtiter plate reader (Cytoflour 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 2000[14]. 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 ε2, ε3, and ε4 alleles was 4.2, 85.3, and 10.5%, respectively. As in other studies, the genotypes ε2ε2, ε2ε4, and ε4ε4 were quite rare. High frequencies of the ε3 allele are also found in Chinese, but the frequency is lower in Caucasians[15].

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

genotype	*n*	%	alleles	*n*	%
ε2/ε2	9	0.4	ε2	192	4.2
ε2/ε3	155	6.8	ε3	3,868	85.3
ε2/ε4	19	0.8	ε4	474	10.5
ε3/ε3	1,653	72.9			
ε3/ε4	407	18.0			
ε4/ε4	24	1.1			

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 ε2ε2 than ε4ε4 and genotype ε2ε3 than ε3ε3, ε2ε4, or ε4ε4. The HDL-cholesterol level was significantly higher for ε2ε3 than ε2ε4. The LDL-cholesterol level was significantly lower for genotypes ε2ε2 and ε2ε3 than for ε3ε3, ε3ε4, and ε4ε4. The RLP-cholesterol level was significantly higher for ε2ε2 than ε2ε3, ε3ε3, ε3ε4, or ε4ε4 and for genotype ε2ε4 than ε2ε3, ε3ε3, or ε3ε4, 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 2. Mean of serum lipid levels and blood glucose in each genotype of *APOE* in Japanese.

	ε2/ε2	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4	total	*p* value
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	
T-cho	165.0 ± 23.8	189.7 ± 3.00	202.9 ± 12.6	201.8 ± 0.92	206.8 ± 1.95	223.3 ± 9.18	202.1 ± 0.81	<0.0001
TG	171.4 ± 52.8	118.8 ± 8.55	189.0 ± 53.3	117.0 ± 2.41	128.0 ± 5.16	127.9 ± 18.9	119.8 ± 2.13	0.023
HDL-c	51.2 ± 9.51	63.6 ± 1.92	53.0 ± 3.07	59.8 ± 0.41	58.0 ± 0.82	61.9 ± 3.48	59.7 ± 0.36	0.007
LDL-c	70.5 ± 5.63	101.9 ± 2.75	117.2 ± 8.07	118.5 ± 0.91	120.5 ± 1.93	131.5 ± 7.97	117.7 ± 0.79	<0.0001
RLP-c	22.9 ± 1.15	4.4 ± 0.37	12.5 ± 7.59	4.7 ± 0.17	5.2 ± 0.33	4.1 ± 0.58	4.8 ± 0.15	<0.0001
FBS	121.3 ± 19.5	104.7 ± 3.27	110.6 ± 9.37	103.9 ± 0.94	103.3 ± 2.17	88.6 ± 2.54	103.9 ± 0.83	0.461
age	52.8 ± 10.1	49.5 ± 2.11	50.8 ± 53.2	46.7 ± 0.69	47.4 ± 1.30	43.2 ± 4.61	47.1 ± 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	*n*	%	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	*n*	%	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	*n*	%	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 heterogenous among various populations. Epidemiological data indicate that the frequency of the ε3 allele is higher in Japanese and Chinese than in Caucasians, while the frequency of the ε4 allele is lower in Asians than Caucasians[3, 16]. Our data indicate that the frequency of the ε3 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 population[15].

Our study confirmed that the ε4 allele is associated with higher, and the ε2 allele is associated with lower, LDL cholesterol levels. Although there was a trend for individuals with the genotypes ε2/ε4 and ε2/ε2 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 ε2/ε4 and ε2/ε2 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 ε2/ε2 homozygotes, it would be intriguing to know whether individuals with the ε2/ε4 and ε2/ε2 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.

Acknowledgements

We thank Shizuya Yamashita (Osaka University) and Hideaki Bujo (Chiba University) for critical reading of the manuscript. This study was supported by research grants for health sciences from the Japanese Ministry of Health and a grant from the Japan Atherosclerosis Society. We also thank Osaka Pharmaceutical Manufactures Association for supporting our work.

References

1) Curtiss LK and Boisvert WA: Apolipoprotein E and atherosclerosis. Curr Opin Lipidol, 2000; 11:243-251
2) Davignon J, Gregg RE, and Sing CF: Apolipoprotein E polymorphism and atherosclerosis. Arteriosclerosis, 1988; 8:1-21
3) Wang XL, McCredie RM, and Wilcken DE: Polymorphisms of the apolipoprotein E gene and severity of coronary artery disease defined by angiography. Arterioscler Thromb Vasc Biol, 1995; 15:1030-1034
4) Katzman R: Apolipoprotein E and Alzheimer's disease. Curr Opin Neurobiol, 1994; 4:703-707
5) Rall SC Jr. and Mahley RW: The role of apolipoprotein E genetic variants in lipoprotein disorders. J Intern Med, 1992; 231:653-659
6) Boushey CJ, Beresford SA, Omenn GS, and Motulsky AG: A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA, 1995; 274:1049-1057
7) Graham IM, Daly LE, Refsum HM, Robinson K, Brattstrom LE, Ueland PM, Palma-Reis RJ, Boers GH, Sheah-

an RG, Israelsson B, Uiterwaal CS, Meleady R, McMaster D, Verhoef P, Witteman J, Rubba P, Bellet H, Wautrecht JC, de Valk HW, Sales Luis AC, Parrot-Rouland FM, Tan KS, Higgins I, Garcon D, and Andria G: Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. Jama, 1997; 277:1775-1781

8) Ou T, Yamakawa-Kobayashi K, Arinami T, Amemiya H, Fujiwara H, Kawata K, Saito M, Kikuchi S, Noguchi Y, Sugishita Y, and Hamaguchi H: Methylenetetrahydrofolate reductase and apolipoprotein E polymorphisms are independent risk factors for coronary heart disease in Japanese: a case-control study. Atherosclerosis, 1998; 137:23-28
9) Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, and Ueland PM: Folate, vitamin B12, and serum total homocysteine levels in confirmed Alzheimer disease. Arch Neurol, 1998; 55:1449-1455
10) Brattstrom L, Wilcken DE, Ohrvik J, and Brudin L: Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease: the result of a meta-analysis. Circulation, 1998; 98:2520-2526
11) Shinomiya S, Sasaki J, Kiyohara C, Tsuji E, Inoue H, Marugame T, Handa K, Hayabuchi H, Hamada H, Eguchi H, Fukushima Y, and Kono S: Apolipoprotein E genotype, serum lipids, and colorectal adenomas in Japanese men. Cancer Lett, 2001; 164:33-40
12) Johnson CL, Rifkind BM, Sempos CT, Carroll MD, Bachorik PS, Briefel RR, Gordon DJ, Burt VL, Brown CD, Lippel K, and Cleeman JI: Declining serum total cholesterol levels among US adults. The National Health and Nutrition Examination Surveys. Jama, 1993; 269:3002-3008
13) Ubbink JB, Hayward Vermaak WJ, and Bissbort S: Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr, 1991; 565:441-446
14) Arai H, Yamamoto A, Matsuzawa Y, Saito Y, Yamada N, Oikawa S, Mabuchi H, Teramoto T, Sasaki J, Nakaya N, Itakura H, Ishikawa Y, Ouchi Y, Horibe H, Egashira T, Hattori H, Shirahashi N, and Kita T: Polymorphisms in four genes related to triglyceride and HDL-cholesterol levels in the general Japanese population in 2000. J Atheroscler Thromb, 2005; 12:240-250
15) Hallman DM, Boerwinkle E, Saha N, Sandholzer C, Menzel HJ, Csazar A, and Utermann G: The apolipoprotein E polymorphism: a comparison of allele frequencies and effects in nine populations. Am J Hum Genet, 1991; 49:338-349
16) Eto M, Watanabe K, and Ishii K: Reciprocal effects of apolipoprotein E alleles (epsilon 2 and epsilon 4) on plasma lipid levels in normolipidemic subjects. Clin Genet, 1986; 29:477-484
17) Kobori S, Nakamura N, Uzawa H, and Shichiri M: Influence of apolipoprotein E polymorphism on plasma lipid and apolipoprotein levels, and clinical characteristics of type III hyperlipoproteinemia due to apolipoprotein E phenotype E2/2 in Japan. Atherosclerosis, 1988; 69:81-88