Effect of Apolipoprotein E Genotype on Association of Menopausal Status with Lipid Level in Japanese Women

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Serum lipid level increases during hormonal transition before and after menopause. In Caucasians, changes in lipid level after menopause across apolipoprotein E (apo E) genotypes were studied. We investigated 159 Japanese healthy female workers (age range, 35–63). The apo E genotypes were determined by the polymerase chain reaction single-strand conformation polymorphisms method for the entire apo E-coding region. The low-density lipoprotein cholesterol (LDL-cholesterol) level significantly varied according to the apo E genotype, but not according to the menopausal status after adjusting for age and body mass index ($p < 0.001$). The effect of menopause on LDL-cholesterol level significantly varied depending on the apo E genotype ($p < 0.05$). Apo E2 [APOE, R158C] carriers in the senium period had a lower LDL-cholesterol level than those in the fertile period (81.5 ± 12.0 mg/dl *vs.* 122.3 ± 12.9 mg/dl). In contrast, apo E3 homozygotes in the senium period showed a higher LDL-cholesterol level than those in the fertile period (140.9 ± 7.0 mg/dl *vs.* 134.1 ± 6.1 mg/dl). The LDL-cholesterol levels of apo E4 [APOE, C112R] carriers were the similar for the two periods. These results indicate that the serum lipid control in perimenopausal women should be customized according to the apo E genotype for the promotion of women's health in Japan.

Key words ——— menopause, lipid levels, apolipoprotein E genotype, polymerase chain reaction-single strandard conformation polymorphism, Japanese women, low-density lipoprotein cholesterol

INTRODUCTION

The risk for coronary heart disease increases in menopausal women, which is considered to be caused by changes in blood lipid levels after sex-hormone transition.[1–4] It was reported that menopause causes increases in the levels of serum total cholesterol, low-density lipoprotein cholesterol (LDL-cholesterol), triglyceride and apolipoprotein B, as well as decreases in the levels of high-density lipoprotein cholesterol (HDL-cholesterol) and apolipoprotein AI.[5–6] A recent survey in Japan, where the diet and lifestyles are different from those in North America, has also showed that the serum total cholesterol level (< 220 mg/dl) in 50.9% of women in their fifties exceeded the recommended level.[7] However, the data were derived from a cross-sectional survey, and the results may be confounded by age and factors other than menopause. Indeed, some women are normolipidemic even after menopause, which is not fully explained by the menopausal status and lifestyles. Genetic property would be another factor, but this is a poorly examined issue in studies of women and serum lipid levels.

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Some genetic factors affect lipid levels differentially across age and gender. Previous studies indicated that the frequency of the ε4 allele of apolipoprotein E (apo E) among octogenarians is lower than that among younger subjects (mean age, 36 years) and that this difference is statistically significant only in women.[8] Similar findings were reported for other populations of Caucasian women.[9,10]

Apo E plays important roles in the metabolism of lipoproteins as ligands of LDL receptors and lipoprotein remnant receptors. There are three common apo E isoforms, namely, E3, E4, and E2.[11–14] Apo E2 and apo E4 differ from the most frequently observed apo E3 isoform by a single amino acid substitution caused by a single point mutation. A study of women showed that the association between apo E phenotype and LDL-cholesterol level is stronger in postmenopausal women than in premenopausal women or in men.[9] Schaefer *et al*.[9] reported that in general the ε2 allele could lower plasma LDL-cholesterol level by 8.2 mg/dl on average in premenopausal Caucasian women and by 20.4 mg/dl in postmenopausal women. On the other hand, the ε4 allele is associated with a plasma LDL-cholesterol level increase by 1.6 mg/dl in premenopausal women and by 7.1 mg/dl in postmenopausal women. However, another study found no such difference between pre- and postmenopausal Caucasian women.[15] Because the apo E allele frequency is highly heterogeneous among populations,[16] it is worth investigating whether such a difference can be observed among ethnically different populations.

On the basis of previous studies cited above, we hypothesize that different apo E genotypes may differentially modify the effect of menopausal transition on serum lipid levels among Japanese women, even after adjusting for other known factors such as body mass index (BMI) and age.

MATERIALS AND METHODS

Subjects —— Japanese female workers (n = 183) in retail service in Tokyo and Yokoyama, Japan were recruited during a regular health checkup. An annual health checkup for employees is mandatory and provided by employers for health protection/promotion in Japan. For the study purpose, female workers aged 35 years and older were selectively recruited to participate upon obtaining their oral or written informed consent.

Participants were requested to answer a self-administered questionnaire to obtain basic information regarding their medical history of hysterectomy/oophorectomy, current status of menstruation, smoking habit, and treatments such as hormone replacement therapy or anti-hyperlipidemic medication. To confirm the response to the questionnaire, particularly on the regularity of their menstrual cycle in the past and present, the first author conducted a face-to-face interview of each participant. Those who had treatment for hyperlipidemia, hormone replacement therapy, previous gynecological surgery, and irregular menstrual period since puberty were excluded from the study. Consequently, 159 female workers (age range, 35–63; average age ± S.D., 48.3 ± 7.8) were available for further analysis.

Lipid Analysis —— Peripheral blood samples during fasting were collected in ethylenediaminetetraacetic acid-coated tubes at a final concentration of 0.15%. The samples were refrigerated immediately and assayed within 24 hr. Serum total cholesterol, triglyceride and HDL cholesterol levels were measured by enzymatic assay. LDL-cholesterol level was directly measured by a homogeneous method based on an innovative detergent technology (Cholestest LDL, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Identification of Menopausal Status —— Using the classification method established by the World Health Organization,[17] we classified the subjects into three groups, namely, those in the senium period, fertile period, and climacteric period, according to this current status of menstruation, and levels of serum lutenizing hormone (LH) and follicular stimulating hormone (FSH). LH and FSH levels were measured by immuno-radiometric assay (SPAC-S LH kit, SPAC-S FSH kit, Daiichi Radio Isotope Labs., Ltd., Tokyo, Japan). The senium period, or definitely postmenopausal period, was designated when all of the following conditions were satisfied: menstruation ceased more than one year ago; LH ≥ 10 mIU/ml; and FSH ≥ 30 mIU/ml. Women who currently have regular periodic menstrual cycles were classified as being in the fertile period. The climacteric period was a transitional period between fertile and senium periods. Subjects were classified as climacteric when this menstruation had been absent for less than one year, or when the level of LH or FSH did not reach the criterion for the senium period even when menstruation had ceased for more than one year, or when the menstrual cycle had been irregular for 10 years. Because the number of subjects in the climacteric period was small (n = 25),

and the subjects of this group were heterogenous by definition, we excluded them from further statistical analysis and we focused on subjects in the fertile period and senium period in the comparison of premenopausal and postmenopausal statuses.

Identification of apo E Genotype ——— Although most of the previous studies relied on the analyses of plasma based on phenotypes using isoelectric focusing (IEF), we determined apo E genotypes by DNA analysis using the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method because it allows the determination of the complete base sequence of the region encoding apo E proteins and genotypes with a high accuracy. The five pairs of PCR primers spanned all of the apo E protein-coding sequences.[18] Three common alleles, namely apo E3 [APOE, C112 AND R158], apo E4 [APOE, C112R], and apo E2 [APOE, R158C], were the focus of our analysis.[19] Seven individuals found to have other apo E variants, namely, E2/4 and E3/5, and mutants were excluded. We classified individuals according to the following apo E genotypes: 1) apo E2 carriers including apo E2/3 and E2/2, 2) apo E3 homozygotes, and 3) apo E4 carriers including apo E4/4 and apo E3/4.

Ethical Issues ——— All blood samples were analyzed and the study was finished before March 2000 when the ethical guidelines for human genome/gene analysis research were established. All DNA samples were anonymous and unlinked to specific individual information, that is, no identification numbers, names or occupation. The protocol of the present study was approved by the ethics review committee of the Shinshu University, School of Medicine (protocol number 152).

Statistical Analyses ——— Age, BMI, and the levels of serum total cholesterol, LDL-cholesterol, HDL cholesterol, and triglyceride (log-translated) were compared between the two menstrual statuses using Student's *t*-test. The distribution of the three apo E genotypes and the menstrual status was shown in a cross-table using the chi-square test. Then, the analysis of covariance was conducted to isolate the main effects of menopause on serum lipid levels, after adjusting for age, BMI, and apo E genotype. Finally, to test the effect of modification owing to the genotype, or differential effects owing to different genotypes, an interaction term between genotypes and menopausal status was introduced in the analysis. All statistical analyses were performed using SAS statistical software (version 6.12, SAS Institute, Cary NC, U.S.A.). *p*-Values below 0.05 were considered as statistically significant.

RESULTS

According to the criteria defined earlier, 74 subjects were found to be in their fertile period, 60 in their senium period, and 25 in their climacteric period. Table 1 shows the comparison between the fertile period group and the senium period group in terms of age, BMI, serum lipid levels, and the frequencies of apo E genotypes. It was reasonable that age was higher in the senium period group than in the fertile period group ($p < 0.001$). No significant

Table 1. Comparison of Serum Lipid Levels and apo E Genotype (E2 Carriers, E3 Homozygote, and E4 Carriers) Frequencies between Women in their Fertile and Senium Periods

	Fertile period ($n = 74$)	Senium period ($n = 60$)	p-value
age (years)	41.4 ± 4.4	55.6 ± 3.4	<0.001[c]
BMI (kg/m · m)	20.7 ± 2.9	21.4 ± 2.9	0.151[c]
total serum cholesterol (mg/dl)	201.3 ± 27.4	226.1 ± 36.6	<0.001[c]
LDL-cholesterol (mg/dl)	128.8 ± 33.3	139.8 ± 36.1	0.070[c]
HDL-cholesterol (mg/dl)	71.4 ± 15.8	71.7 ± 13.9	0.912[c]
triglyceride (mg/dl)[a]	70.1 ± 31.7	93.7 ± 54.1	0.003[c]
apo E genotypes[b]			0.465[d]
E2 carriers (E2/3, E2/2)	7 (9%)	9 (15%)	
E3 homozygotes (E3/3)	48 (62%)	41 (64%)	
E4 carriers (E3/4, E4/4)	19 (25%)	10 (15%)	

Data are expressed as means ± S.E. BMI: body mass index; LDL-cholesterol: low-density lipoprotein cholesterol; HDL-cholesterol: high-density lipoprotein cholesterol; apo E: apolipoprotein E; *a*) Log-translated triglyceride was used for the analyses. *b*) Apo E frequencies (%) were calculated including "other apo E genotypes (E2/4, E3/5, and mutants)." *c*) Student's *t*-test; *d*) Chi-square test.

Table 2. Effects of Menopausal Status and apo E Genotypes on Serum Lipid Levels

Variable		total cholesterol		LDL-cholesterol		HDL-cholesterol		triglyceride	
	DF	*f*-value	*p*-value	*f*-value	*p*-value	*f*-value	*p*-value	*f*-value	*p*-value
age	1	0.07	0.790	0.72	0.396	4.88	0.029	0.14	0.709
BMI	1	2.13	0.147	9.12	0.003	18.77	< 0.001	16.46	<0.001
Fertile *vs.* Senium	1	6.14	0.015	0.07	0.791	5.11	0.025	0.99	0.320
apo E genotype	2	5.56	0.005	10.95	< 0.001	1.65	0.197	0.44	0.645

apo E genotype: apo E3/3, apo E2 carrier (E2/3 and E2/2), apo E4 carrier (E3/4 and E4/4). LDL-cholesterol: low-density lipoprotein cholesterol; HDL-cholesterol: high-density lipoprotein cholesterol; triglyceride: log-translated; BMI: body mass index.

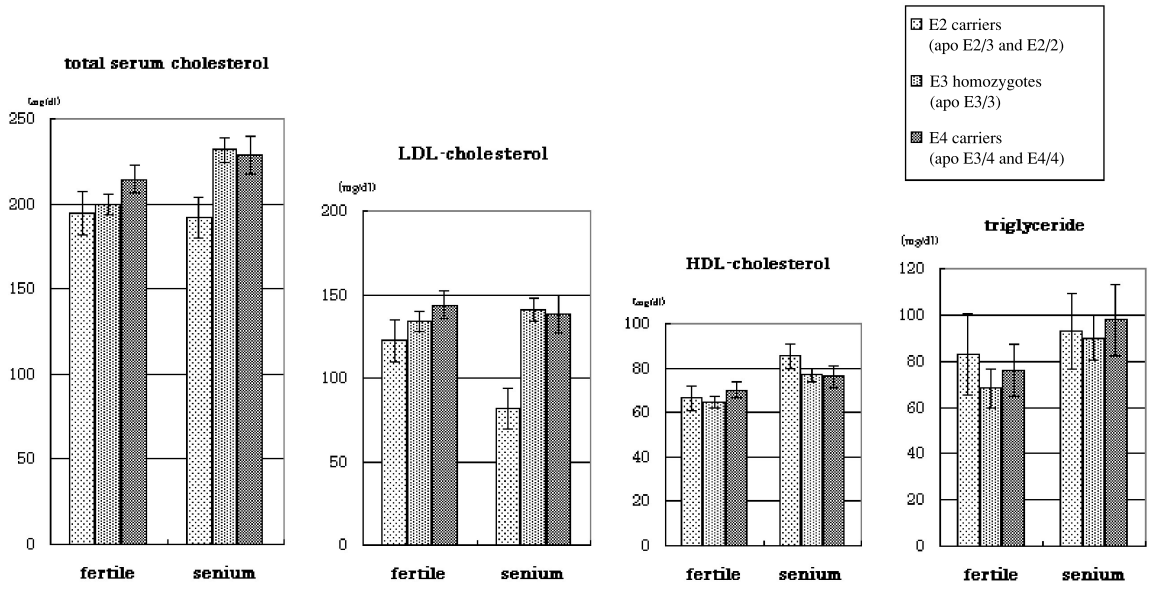

Fig 1. Average Lipid Levels Adjusted for Age and Body Mass Index in Fertile and Senium Period Groups that are Further Classified according to apo E Genotype (E2 Carriers, E3 Homozygotes, and E4 Carriers)

This analysis includes the model of interaction between menstrual status and apo E genotype. Results are expressed as means ± S.E., adjusted for age and BMI. LDL-cholesterol: low-density lipoprotein cholesterol; HDL-cholesterol: high-density lipoprotein cholesterol; LDL-cholesterol: Interaction between menstrual status and apo E was significant at $p < 0.05$.

difference in BMI was observed. However, the average serum total cholesterol level ($p < 0.001$) and triglyceride level ($p < 0.01$) were significantly higher in the senium period group than in the fertile period group. The LDL cholesterol level in the senium period group tended to be higher than that in the fertile period group, although the difference was not statistically significant. The average HDL-cholesterol level was not significantly different between the fertile and senium period groups. The apo E frequency in this study population was almost the same as that previously reported in Japanese men.[16,20] The apo E4 frequency for the senium period group (15%) was lower than that for the fertile period group (25%), but the difference was not statistically significant.

Table 2 shows the effects of the apo E genotype and menstrual period on lipid levels after adjusting for age and BMI. LDL-cholesterol ($p < 0.001$) and total cholesterol ($p < 0.01$) levels were significantly associated with the apo E genotype; apo E2 [APOE, R158C] carriers showed significantly lower levels of total cholesterol and LDL-cholesterol than apo E3 homozygotes and E4 carriers. BMI was also significantly associated with the levels of LDL-cholesterol ($p < 0.01$), HDL-cholesterol ($p < 0.001$), and triglyceride ($p < 0.001$).

Fig. 1 shows the average lipid levels adjusted for age and BMI analyzed including the model of interaction. The effect of the modification of the apo E genotype on the association between menopause and serum lipid levels was examined by including an interaction between genotype and menopausal status (Fig. 1). The effect of menopause on the LDL-cholesterol level significantly varied depending on the apo E genotype ($p < 0.05$). On the other hand, the relationship between HDL-cholesterol level and

menopause was not influenced by the apo E genotype. Apo E2 [APOE, R158C] carriers in the senium period showed lower LDL-cholesterol levels than those in the fertile period (81.5 ± 12.0 mg/dl *vs.* 122.3 ± 12.9 mg/dl). In contrast, the apo E3 homozygotes in the senium period showed higher LDL-cholesterol levels than their counterpart in the fertile period (140.9 ± 7.0 mg/dl *vs.* 134.1 ± 6.1 mg/dl). The LDL-cholesterol levels in apo E4 [APOE, C112R] carriers in the fertile period were similar to those in the senium period (138.3 ± 11.1 mg/dl *vs.* 143.7 ± 8.1 mg/dl). The average total cholesterol level in subjects with the apo E2 genotype was less than 200 mg/dl even in those in the senium period. The difference in total cholesterol level between apo E4 carriers and apo E3 homozygotes in the fertile period was not clearly observed in the senium period. The average HDL-cholesterol levels were comparatively similar between apo E4 carriers and apo E3 homozygotes in the senium and fertile periods. However, the average HDL-cholesterol level was high in the apo E2 carriers in the senium period.

DISCUSSION

Although the cholesterol level in many postmenopausal women is high, there are some postmenopausal women with normal lipid levels. We studied the effect of apo E genotype as one of the predisposing factors affecting serum lipid levels in premenopausal and postmenopausal Japanese women.

We found that in apo E3 homozygotes, the levels of total cholesterol and LDL-cholesterol were higher in the senium period than those in the fertile period, while the opposite trend was observed among apo E2 carriers. In apo E4 carriers, LDL-cholesterol level was not significantly different between the two periods. In particular, a marked difference in the LDL-cholesterol level was observed, which agrees with the results reported for European and American populations.[5,6,21]

Apo E genotypes were determined by a highly accurate DNA analysis using the PCR-SSCP method,[18] by which the base sequence of the entire apo E-coding region and genotypes were determined, to avoid confusion caused by multiple variant genotypes. After adjustment for age and BMI, it was found that the apo E genotype significantly affected LDL-cholesterol ($p < 0.001$) and serum total cholesterol ($p < 0.01$) levels. Among the subjects in the postmenopausal group, the LDL-cholesterol level in apo E4 carriers was approximately 46.8 mg/dl higher than that in apo E2 carriers; the difference was smaller between these two genotypes in premenopausal women. Schaefer *et al*. reported on the basis of results obtained from a cross-sectional sample of the Framingham Offspring Study that the association of apo E phenotype with LDL-cholesterol level is stronger in postmenopausal women than in premenopausal women and men.[9] In this study of an American Caucasian population, the effect of the $\varepsilon 2$ allele on postmenopausal LDL-cholesterol level is shown; the level is 20.4 mg/dl lower than that in subjects with the other apo E genotypes, and the difference is only 8.2 mg/dl in premenopausal individuals. To the best of our knowledge, our study using the PCR-SSCP method provided for the first time compatible results for non-Caucasian subjects on a population study.

The heterogeneity of apo E type frequency and the mean levels of serum lipids in ethnically different populations were reported.[16] According to Hallman *et al*., Chinese and Japanese share the characteristic of having a higher frequency of the $\varepsilon 3$ allele and a lower frequency of the $\varepsilon 4$ allele than other populations. Although the distribution of the apo E allele might be different across populations, data from previous studies and our present study suggest that the association of apo E genotype with menopause and serum LDL-cholesterol level might be universal.

Among the three common genotypes (*i.e*., apo E3, E4, and E2), the total serum cholesterol level in apo E4 carriers, approximately 16% of the Japanese population, is about 10 mg/dl higher than that in apo E3 homozygotes in the Japanese male.[22] The LDL receptor binding functions in E3 homozygotes and E4 carriers are similar; the binding capability in E2 carriers is low at 2%.[23] Because the rate of catabolism of very-low-density lipoprotein (VLDL) to LDL in E4 carriers is higher than that in E3 homozygotes, the serum LDL cholesterol level in the former is considered to be higher than that in the latter.[24] It has been reported that the responsivity to statin, which is used for the treatment of patients with hypercholesteromia, is lower in E4 carriers than that in E3 homozygotes.[25] Although the detailed mechanisms underlying this difference in responsivity remain to be elucidated, it is possible that lipid metabolism, which may change as a result of menopause, differs depending on the genotype of apolipoprotein E, and that the serum LDL choles-

terol level in E4 allele carriers does not significantly change compared with that in E3 homozygotes. Note that the expression level of hepatic LDL receptors is up-regulated in the presence of estrogen.[26)]

With the recent interest in the so-called "genotype-environment interaction,"[27–29)] the data we obtained will have several implications in the treatment of hypercholesterolaemia and prevention of atherosclerosis in women. The effect of the apo E isoform and menopause on LDL-cholesterol level can be taken as one of the models of genotype-environment interaction.[30,31)] Hines *et al*. reported that moderate drinkers who are homozygous for the slow-oxidation alcohol dehydrogenase type 3 allele have higher HDL levels and a substantially decreased risk of myocardial infarction, not only in men but also in postmenopausal women.[32)] A customized approach to treating/preventing atherosclerosis in women may need to take into consideration not only individual lifestyles based on a certain cultural norm/behavior, but also the genotype and life transitions such as menopause. Tsuda et al. reported that hormone replacement therapy has little effect on the serum lipid profile in apo E4 carriers.[33)] There is a controversial report that male apo E4 carriers with a high LDL-cholesterol level are more responsive to dietary therapy than males with other apo E phenotypes.[34)] Future research should clarify gender difference in association with therapeutic effectiveness and apo E genotype.

This study was a cross-sectional study using a limited number of subjects (n = 159). Our subjects were healthy female workers in the community, who were recruited in this study during their regular health checkup. Thus, it would be less likely that we had selectively included those with a higher risk of atherosclerosis and other clinical conditions. The distribution of apo E genotypes agreed with that previously observed in Japanese men.[16,20)] However, further studies in different settings should be performed to confirm the validity of our results.

Since our study was not performed in a longitudinal manner, we could not determine whether the impact of the apo E genotype on serum lipid level in each woman will change before or after menopause. Akahoshi *et al*. reported that serum total cholesterol level in women starts to significantly increase from 3 years before natural menopause to 1 year after menopause.[35)] In our study, individuals in the climacteric period showed somewhat medium levels of LDL- and total cholesterol compared with those in the fertile and senium periods (data not shown). These observations should be further clarified in prospective studies.

Although we acknowledge some limitations of this study, our results support the hypothesis that the apo E genotype is an important predisposing factor that determines the transitional pattern of serum cholesterol levels in non-Caucasian pre- and postmenopausal women. The results should be the bases for further examinations of the association between women's life stage and lipid metabolism that might lead to genetically and environmentally customized strategies for promoting women's health.

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