Original Article

Comprehensive Evaluation of Genetic and Environmental Factors Influencing the Plasma Lipoprotein-Associated Phospholipase A_2 Activity in a Japanese Population

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The lipoprotein-associated phospholipase A_2 (Lp-PLA_2) metabolizes oxidized phospholipids, generating lysophosphatidylcholine. The activity of the enzyme is known to be influenced largely by a single-nucleotide polymorphism, G994T, in the Lp-PLA_2 gene. Interestingly, this polymorphism is much more prevalent in Japanese than Caucasians. The purpose of the current study was to evaluate the effects of the G994T, several environmental factors, and their interactions on the Lp-PLA_2 activity in a large Japanese cohort. Participants (1,110 males and 908 females) of a health-screening examination were recruited for this study. Genotyping of the G994T was done using allele-specific polymerase chain reaction (PCR). The Lp-PLA_2 activity was measured using commercial kits. The minor allele (994T) frequency of the polymorphism was 0.17 in this study, which was consistent with previous reports. According to the multivariate linear regression analysis, the G994T was the most potent factor influencing the enzyme activity (standardized $\beta = 0.76$), followed by the low-density lipoprotein cholesterol (LDL-C) level (standardized $\beta = 0.32$) and the sex (standardized $\beta = 0.13$). The LDL-C level showed a significant interaction with the G994T genotype. By contrast, no significant interaction was observed between the LDL-C level and the sex. These observations should provide useful information for future clinical and epidemiological evaluations of the Lp-PLA_2 activity in cardiovascular diseases in Japanese. (*Hypertens Res* 2007; 30: 403–409)

***Key Words*: lipoprotein-associated phospholipase A_2, platelet-activating factor acetylhydrolase, single nucleotide polymorphisms, gene-environment interaction, oxidative stress**

Introduction

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is an enzyme that shows Ca^{2+}-independent phospholipase A_2 activity and that is synthesized mainly by monocytes and macrophages. Initially known as the platelet-activating factor (PAF) acetylhydrolase, Lp-PLA_2 inactivates the pro-inflammatory substance PAF (*1*, *2*). In addition to it, this enzyme was found to hydrolyze oxidized phospholipids, releasing lysophosphatidylcholine (lyso-PC) (*1*). Because lyso-PC has atherogenic properties, the net effect of Lp-PLA_2 on the atherogenesis is still controversial; it may aggravate atherosclerosis through the production of lyso-PC, or it may inhibit

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S.-Y. Z. was a visiting fellow supported by the Japan-China Medical Association. This study was partly supported by a grant from the Research Project Promotion Institute of Shimane University and the Shimane Institute of Health Science.

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Received October 12, 2006; Accepted in revised form December 26, 2006.

Table 1. Demographic Data of the Population Studied

	Genotype			
	GG	GT	TT	*p**
N	1,374	583	61	
M/F	743/631	332/251	35/26	0.47
Age (years)	57.8 [57.4–58.2]	58.0 [57.5–58.6]	58.0 [55.8–60.1]	0.83
BMI (kg/m^2)	23.4 [23.2–23.5]	23.3 [23.1–23.5]	23.1 [22.3–23.9]	0.69
SBP (mmHg)	127 [126–128]	128 [126–130]	123 [119–127]	0.16
DBP (mmHg)	74 [73–75]	75 [74–76]	73 [71–76]	0.30
FBG (mmol/L)	5.99 [5.91–6.07]	5.87 [5.76–5.98]	6.00 [5.66–6.35]	0.21
TC (mmol/L)	5.47 [5.43–5.52]	5.52 [5.44–5.59]	5.60 [5.39–5.81]	0.35
HDL-C (mmol/L)	1.59 [1.57–1.61]	1.61 [1.58–1.65]	1.67 [1.54–1.79]	0.23
LDL-C (mmol/L)	3.26 [3.22–3.30]	3.28 [3.20–3.35]	3.33 [3.13–3.52]	0.77
TG (mmol/L)	1.17 [1.14–1.20]	1.16 [1.12–1.21]	1.21 [1.07–1.36]	0.86
Lp(a) (mg/dL)	11.8 [10.7–12.6]	12.0 [10.7–13.5]	8.3 [5.8–12.3]	0.20
Smoking status (%)				
Nonsmokers	59.9	55.4	55.2	0.04
Mild smokers	3.6	6.8	5.2	
Heavy smokers	36.5	37.8	39.7	
Lp-PLA$_2$ act (IU/L)	444 [438–450]	244 [239–249]	46 [41–51]	<0.001

Values in brackets represent 95% confidence intervals of the mean. *Either by ANOVA or χ^2 test. M, male; F, female; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; Lp(a), lipoprotein(a).

atherosclerosis through the degradation of oxidized lipids and PAF (*1*, *3*). Epidemiological studies were recently performed to address this issue. They indicated that the increased Lp-PLA$_2$ activity was an independent risk for coronary heart diseases and ischemic stroke, supporting the atherogenic role of Lp-PLA$_2$ (*4–15*).

In this context, it is of interest that Japan has a substantial population with no Lp-PLA$_2$ activity; a single-nucleotide polymorphism (SNP), G994T, in exon 9 of the Lp-PLA$_2$ gene was found to cause Val-to-Phe substitution of the mature protein (Val279Phe), which is in turn responsible for the loss of catalytic activity (*16*). This report further indicated that the prevalence of this SNP was substantially greater in Japanese than Caucasians (*16*). This SNP can therefore be used as a natural "inhibitor" for Lp-PLA$_2$ to obtain information on the effect of this enzyme on cardiovascular events.

In addition to the SNP above, however, the Lp-PLA$_2$ activity was reported to correlate with various phenotypes, such as total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), body mass index (BMI), smoking status, age and sex (*4–14*, *17*, *18*). Despite this, the interaction between the G994T genotype and such "environmental" factors has not yet been sufficiently evaluated because the majority of studies were performed on Caucasian populations. Further, to our knowledge, all of these studies used univariate analyses to evaluate the factors influencing the Lp-PLA$_2$ activity, so that there is no comprehensive information available on factors with real independent effects. Such information would be quite important for the Japanese population, as there is a high prevalence of the 994T allele of the Lp-PLA$_2$ gene in Japanese, and the potential role of the enzyme on cardiovascular diseases is of concern.

In this communication, our goal was to evaluate the genetic and environmental factors influencing the plasma Lp-PLA$_2$ activity using a multivariate analysis in a large cohort in Japan.

Methods

Subjects

Two thousand and eighteen consecutive participants (1,110 males and 908 females) who voluntarily visited the Shimane Institute of Health Science for a health screening examination between 1995 and 2003 were recruited for the study. Their smoking history was obtained through an interview. The smoking status was categorized into 3 groups based on the smoking index (number of cigarettes per day $\times$ years): 1) nonsmokers with a smoking index of 0; 2) mild smokers with a smoking index <200; and 3) heavy smokers with a smoking index ≥ 200 (*19*). Serum was collected after overnight fasting to measure the fasting blood glucose (FBG), TC, HDL-C, TG and lipoprotein(a) (Lp(a)) levels. Serum LDL-C was calculated using the Friedewald formula. Blood pressure was measured 3 times after at least 15 min of rest, and the mean of the 3 measurements was taken as the blood pressure value. All participants gave their informed consent to participate, and the study protocol was approved by the local ethics committee.

Table 2. Univariate Analysis on Factors Potentially Influencing the Plasma Lp-PLA$_2$ Activity

	GG		GT		TT	
	r (or *F*)	*p*	*r* (or *F*)	*p*	*r* (or *F*)	*p*
Sex*	28.5	<0.001	21.0	<0.001	0.29	0.59
Age	0.004	0.89	−0.060	0.16	0.10	0.46
BMI	0.09	0.002	0.10	0.027	0.08	0.53
SBP	0.03	0.35	0.10	0.01	0.03	0.81
DBP	0.05	0.07	0.08	0.06	−0.04	0.78
TC	0.39	<0.001	0.46	<0.001	0.13	0.34
HDL-C	−0.26	<0.001	−0.27	<0.001	0.17	0.20
LDL-C	0.46	<0.001	0.45	<0.001	0.03	0.85
log FBG	0.09	<0.001	0.07	0.09	−0.02	0.893
log TG	0.27	<0.001	0.34	<0.001	0.04	0.78
log Lp(a)†	0.03	0.52	0.03	0.68	0.02	0.93
Smoking*	4.68	0.01	4.51	0.01	0.37	0.69

*Analyzed by ANOVA. In this case, *F* values are indicated instead of Pearson's correlation (*r*). †Nine hundred and seventy samples with the information of Lp(a) concentration were used in the analysis. Lp-PLA$_2$, lipoprotein-associated phospholipase A$_2$. Other abbreviations are the same as in Table 1.

Genotype Analysis

DNA was extracted from peripheral blood samples. The genotype of the G994T in the Lp-PLA$_2$ gene was determined using an allele-specific PCR as described in a previous report (*19*). The sequences of the primers were as follows: T1 (5′-TTCTTTTGGTGGAGCAACAT-3′), G1 (5′-GTCTGATCA TCACTAAGAGTCTGAATAGC-3′), PAFAH 1 (5′-TAT TTACCATCCCCATGAAATGAAC -3′), and PAFAH 2 (5′-AGGAGAGGAGATGTTTTAGCCTAGG-3′). PCR was performed in a total volume of 20 μL containing 50 ng of genomic DNA, 0.5 μmol/L of the PAFAH 1 and PAFAH 2 primers, 2 μmol/L of the T1 and G1 primers, 1.5 mmol/L of $MgCl_2$ and 0.4 U of Taq polymerase (Promega, Madison, USA). After a hot start at 96°C, amplification was achieved by 35 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 20 s. The G and T allele gave a 186- and a 237-bp product, respectively, with a 396-bp common product. The PCR products were analyzed by electrophoresis on 1.5% agarose gel.

In order to confirm the accuracy of genotyping, 60 DNA samples were randomly selected and the PCR products amplified with the PAFAH 1 and PAFAH 2 were subjected to Mae II digestion (Fermentas, Burlington, Canada).

Measurement of the Lp-PLA$_2$ Activity

The Lp-PLA$_2$ activity in the plasma was measured using a commercial kit (Azwell Inc., Osaka, Japan) that employed 1-myristoyl-2-(4-nitrophenyl succinyl) phosphatidylcholine as a substrate. The enzymatic activity was expressed as IU/L.

Statistical Analysis

Since the distribution of TG, Lp(a) and FBG was highly skewed, log-transformation was carried out before the analysis. A contingency table and analysis of variance (ANOVA) were used to evaluate the clinical parameters in the studied population. Analysis of covariance (ANCOVA) was used to evaluate the interaction between the Lp-PLA$_2$ genotype and LDL-C, and between sex and LDL-C. All univariate analyses and a multiple linear regression analysis were performed with the SPSS package (version 11.0). The difference was considered statistically significant when $p<0.05$.

Results

The demographic data of the studied population are shown in Table 1. Of the 2,018 subjects, the frequencies of the GG, GT and TT genotypes of the G994T were 68.1%, 28.9% and 3.0%, respectively. The minor allele frequency was 0.17, which was consistent with a previous observation in a Japanese population (*17*). The genotype frequency was well within the Hardy-Weinberg equilibrium ($\chi^2=0.01$). The plasma Lp-PLA$_2$ activity was associated with the G994T genotype, as reported previously (*16*, *17*). Except for a slight difference in the smoking status, the parameters were not significantly different among the three genotype groups.

In the subsequent univariate analysis (Table 2), we examined the correlation between the clinical parameters and the plasma Lp-PLA$_2$ activity in each genotype of G994T. The Lp-PLA$_2$ activity was strongly correlated with sex, TC, LDL-C, HDL-C and log TG in the GG and GT subpopulation. Smoking status was also positively correlated with the Lp-PLA$_2$ activity. Although Lp-PLA$_2$ activity showed correlations with BMI, FBG and systolic blood pressure (SBP) in both the GG and GT subpopulations, these relationships were nevertheless quite weak ($r\leq0.1$). None of the factors showed a significant correlation with the Lp-PLA$_2$ activity in the TT subpopula-

Table 3. Factors Independently Correlated with the Lp-PLA$_2$ Activity

	β±SEM	Standardized β	t	p
G944T genotype	201±3.4	0.76	59.5	<0.001
LDL-C	56.2±2.3	0.32	23.7	<0.001
Male sex	36.3±3.9	0.13	9.2	<0.001
log TG	80.6±10.4	0.11	7.7	<0.001
HDL-C	−22.3±5.0	−0.07	−4.5	<0.001
BMI	−2.6±0.68	−0.05	−3.9	<0.001

Total cholesterol was excluded from the analysis because of the co-linearity with other lipids. Lp-PLA$_2$, lipoprotein-associated phospholipase A$_2$; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; BMI, body mass index.

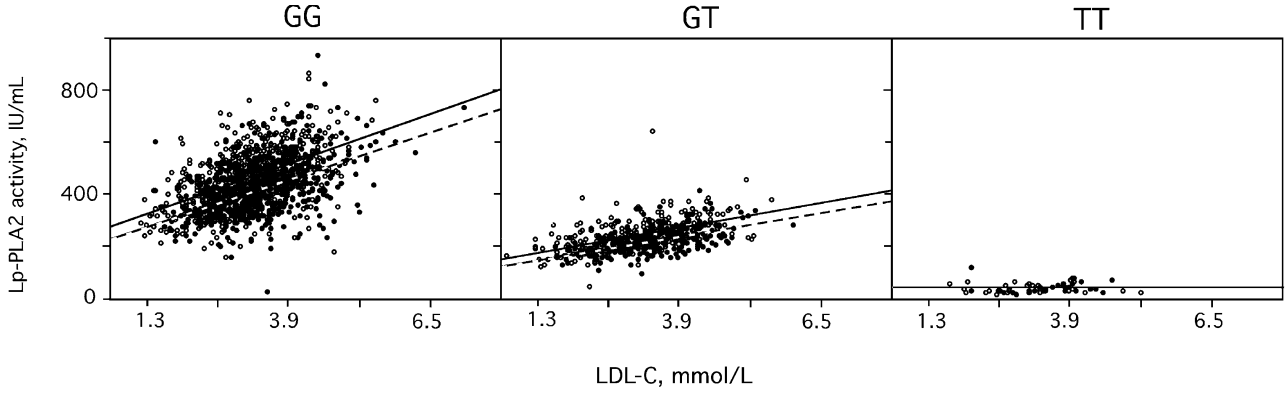

Fig. 1. *Correlation between the LDL-C level and the Lp-PLA$_2$ activity in the three genotypes of the G994T. Open circles and closed circles indicate men and women, respectively. Solid and dashed lines show regression lines for men and for women, respectively.*

tion, indicating that the activity observed in this subpopulation was "background" noise of the measurement. The effect of the Lp(a) concentration on the Lp-PLA$_2$ activity was not significant.

In the present study, the univariate analysis was performed on the three Lp-PLA$_2$ genotypes separately. Separate analyses were employed to eliminate the large effect of the genotype in an attempt to identify environmental factors with weaker effects. The factors identified above showed significant effects on the Lp-PLA$_2$ activity even when the analysis was done on the whole population (data not shown).

We next performed a multiple linear regression analysis, including the G994T genotype as well as the eight parameters that showed significant effects on the Lp-PLA$_2$ activity in the univariate analysis (Table 3). Based on the results shown in Table 1, the G994T was assumed to have a codominant effect on the Lp-PLA$_2$ activity. TC was excluded from the analysis because of its co-linearity with other lipids. In addition to the genotype, LDL-C, sex, log TG, HDL-C and BMI showed independent effects on the Lp-PLA$_2$ activity. The effect of the genotype was the strongest, followed by the effects of LDL-C and sex (see standardized β values shown in Table 3). Although HDL-C and BMI showed a negative correlation, the physiological significance was not clear, as the correlation was quite weak. The smoking status, SBP and FBG were not significant independent factors in the linear regression analysis.

In the next analysis, the interactions among the G994T genotype, sex and LDL-C were evaluated by ANCOVA. While the interactions between the genotype and sex (F=0.26, p=0.77), and between sex and LDL-C (F=0.08, p=0.78) were not significant, the interaction between the genotype and LDL-C was highly significant (F=30.6, p<0.001). Figure 1 illustrates the regression lines between LDL-C and the Lp-PLA$_2$ activity in the subpopulations with the three genotypes. The regression coefficient for the GG subpopulation was twice as large as that for GT (63.1±3.4 and 33.3±2.5 IU/mmol for GG and GT, respectively).

Discussion

To our knowledge, this is the first comprehensive, multivariate analysis of the genetic and environmental factors influencing Lp-PLA$_2$ activity. The results clearly indicated that the plasma Lp-PLA$_2$ activity was influenced by both genetic and environmental factors; the most potent factor was the G994T

genotype of the Lp-PLA$_2$ gene, followed by the LDL-C level and sex. Several additional factors, but not the smoking status or age, showed independent effects as well (Table 3). The effects of such factors as age, BMI, smoking status and blood pressure were controversial in previous univariate analyses (*5*, *8*, *10*, *11*, *17*, *18*). The present study therefore provided new insight on this issue. By using multivariate analysis, we were able to provide additional information on the relative intensity of each factor independently (see the standardized β values in Table 3), and these data will be useful for designing future epidemiological and clinical studies.

In the present study, we simultaneously evaluated the effects of both G994T and LDL-C in a general population. The regression coefficient for the GG was shown to be twice as large as that for the GT (Fig. 1). This result was quite reasonable if only one allele was translated to the active form of the enzyme in individuals with GT, while both alleles were active in those with GG. The same amount of low-density lipoprotein (LDL) particles in individuals with the GG thus was associated with twice the amount of active Lp-PLA$_2$ seen in individuals with the GT. No matter what roles Lp-PLA$_2$ plays in atherogenesis, this observation has an important pathophysiological implication, because people with the same level of LDL-C had quite different levels of Lp-PLA$_2$ according to their genotypes.

In contrast to the G994T genotype, sex did not show a significant interaction with the LDL-C level (Fig. 1). This implied that a greater proportion of LDL particles was associated with Lp-PLA$_2$ molecules in men than in women at all LDL-C levels. It is natural to assume that estrogen was responsible for this sex difference. To obtain a hint as to the estrogen effect, we compared the Lp-PLA$_2$ activity between the younger (age$<$50 years) and the older (age$>$55 years) female subpopulations in the three genotypes separately. The results showed that there was no significant difference between the younger and the older subpopulations (data not shown). Another potential factor confounding to sex was smoking; although the effects of smoking on the Lp-PLA$_2$ activity were excluded in the multiple regression analysis, the apparent sex difference might have been due to a large difference in the number of smokers between men and women (the proportion of nonsmokers was 27% in men and 73% in women). We therefore performed a multiple linear regression analysis on non-smokers. The sex difference was still significant, even in the subpopulation of nonsmokers (standardized β=0.16, t=9.0, $p$$<$0.0001). Hence, we have no adequate causal explanation for this sex difference at the moment.

Recent epidemiological studies indicated that increased Lp-PLA$_2$ activity was a risk factor for coronary heart diseases (*4*–*6*, *8*–*14*) and cerebral stroke (*7*) in Caucasian populations. It was hypothesized that the increased activity of Lp-PLA$_2$ *in vivo* generated more lyso-PC and oxidized fatty acids, which exacerbated atherosclerosis (*1*, *15*). A recent study in Korea supported these observations, by showing that the minor allele of the G994T (the "loss-of-function" allele) was less frequent in patients with coronary heart diseases (*20*). In contrast, studies on Japanese subjects indicated that those with the minor allele were more susceptible to coronary heart diseases (*17*, *21*) and cerebral stroke (*22*). Although the sizes of the populations employed in these genetic studies might not have been large enough to ensure analytical power, the results of an *in vitro* study support the notion of a protective role of Lp-PLA$_2$ against atherosclerosis (*3*).

Based on these discrepant observations, one can draw two hypotheses: 1) a modest level of Lp-PLA$_2$ activity is necessary for the enzyme to play a beneficial role against atherosclerosis; a level of activity that is either too high or too low has deleterious effects. 2) The increase in the Lp-PLA$_2$ activity is a secondary event in response to various pro-inflammatory and atherogenic stimuli. Hence, in spite of its anti-atherogenic role, the Lp-PLA$_2$ activity is increased in subjects with a high risk of cardiovascular disease, even before the manifestation of clinical symptoms. In addition, there is the possibility that, among a number of mechanisms reducing oxidative stresses *in vivo*, Lp-PLA$_2$ may not be important enough to influence the clinical outcomes (*23*, *24*). To obtain a definite answer to this issue, a prospective study is essential.

According to the database of the HapMap project (http://www.hapmap.org/), the prevalence of the 994T allele was zero in a Caucasian as well as in an African population. Even in a Chinese population, the allele frequency of the 994T was much lower than in the Japanese population (0.03 *vs*. 0.17). The Japanese may thus be unique with respect to their high frequency of this allele, and if so, would be a natural choice for future cohort studies. For example, it would be of interest to evaluate the effects of the G994T in several ongoing prospective studies that employ coronary events as an endpoint (*25*, *26*).

In addition to the G994T, several polymorphisms (and mutations) in the Lp-PLA$_2$ gene have been reported recently. The Ala379Val is a functional SNP found commonly in the Caucasian population, and its minor allele 379Val has been shown to give the enzyme a lower affinity for the substrates and a greater V_{max} (*27*). The following study, however, indicated that the effect of the 379Val on the Lp-PLA$_2$ activity *in vivo* was weak; nevertheless, the Ala379Val polymorphism was associated with coronary heart diseases (*28*). Furthermore, a recent study on Korean subjects failed to reproduce the association of the SNP with coronary heart diseases (*20*). These results suggest that the physiological roles of the Ala379Val were less convincing from both the biochemical and the epidemiological point of view. Ishihara *et al*. identified the 189insA and I317N mutations through the screening of 295 Japanese hyperlipidemic/diabetic patients (*29*). Although these mutations had a strong effect on the Lp-PLA$_2$ activity, their prevalence seemed too low to influence disease susceptibility in the general population. For all of these reasons, we focused on the G994T, which was highly prevalent in Japanese and showed a clear effect on the activity of Lp-PLA$_2$.

This study had several limitations. First, LDL-C was estimated rather than measured directly. Further, it would have been quite useful if information on the oxidized LDL-C level was available. The lack of these parameters was partly due to the use of old samples (as old as 1995) included in the study. Second, because the study design was based on a health screening, it was difficult to obtain accurate information on the medication for hyperlipidemia. The interview revealed that 110 participants were under treatment for hyperlipidemia. The analysis was then performed after excluding those subjects to give the same results as above (data not shown). Nonetheless, clinical trials may be needed to comprehensively evaluate the effects of the medication.

In summary, several genetic and environmental factors had independent effects on the Lp-PLA_2 activity *in vivo*. The information provided by the present study should be useful for the design of a large scale epidemiological study in the future.

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