Lipoprotein-associated phospholipase A_{2} (Lp-PLA_{2}) is an enzyme that hydrolyzes the sn-2 ester bond of oxidized phospholipids in a Ca^{2+}-independent manner. It is produced and secreted by monocytes and macrophages. In human plasma, Lp-PLA_{2} is primarily found on low-density lipoprotein (LDL) particles. Lp-PLA_{2} is involved in cardiovascular disease through its action on oxidized LDL (oxLDL). OxLDL aggravates the formation of atherosclerotic plaques and Lp-PLA_{2} potentially affects the amount of oxLDL through the hydrolysis of oxidized phospholipids, a major component of oxLDL. Strong Lp-PLA_{2} activity was actually proven to reduce the level of oxLDL in a transgenic mouse that over-expressed human Lp-PLA_{2}. Concurrently, however, the enzymatic cleavage of oxidized phospholipids generates a potent atherogenic substance, lysophosphatidylcholine. Consistent with this in vitro activity of Lp-PLA_{2}, epidemiological studies in Caucasians have indicated that increased Lp-PLA_{2} activity was a risk for coronary artery diseases and cerebral infarction.

Japanese are one of the best populations in which to explore the effects of Lp-PLA_{2} in vivo, because they have a high incidence of a single-nucleotide polymorphism (SNP), G994T, in the Lp-PLA_{2} gene, which causes a complete loss of the enzyme activity. According to the allele frequency, 3–4% of Japanese are homozygous for 994T and have no Lp-PLA_{2} activity. Furthermore, Lp-PLA_{2} activity is under the codominant control of the single-nucleotide polymorphism, resulting in a greater interindividual variation in Japanese than in Caucasians who have no 994T allele.

In the present cross-sectional study in a Japanese population, we thus examined whether G994T in the Lp-PLA_{2} gene influences the oxidation of LDL and carotid intima-media thickness (IMT). Although IMT is widely recognized as a sensitive indicator for early atherosclerotic changes, no population-based studies have so far been performed on the direct effects of the G994T genotype on IMT. The clinical and epidemiological significance of our observations was discussed.
METHODS

Study population. Participants were separately recruited at health examinations held in consecutive years in two discrete communities, Kakeya (377 men and 506 women) in 2006 and Mitoya (143 men and 281 women) in 2007. The two communities are located in a rural region of Shimane prefecture. Although the background of the two communities was similar to each other, they were analyzed separately to test the reproducibility of the observations. Histories of smoking, alcohol consumption, diabetes mellitus, and hypertension were obtained through the interview at the health examinations. Subjects with diabetes mellitus (under therapy or with a fasting blood glucose >100 mg/dl) or under treatment for dyslipidemia were excluded from the study. The remaining 846 subjects (273 men and 311 women of Kakeya, and 80 men and 182 women of Mitoya) were employed for further analysis. Smoking status was defined as smokers, those currently smoking one cigarette/day or more; exsmokers, those quit smoking; and nonsmokers, those never smoke. Drinkers were defined as those consuming 27 g/day alcohol or more. Venous blood was collected after overnight fasting to measure biochemical parameters.

The study protocol was approved by the ethics committee of Shimane University School of Medicine and written informed consent was obtained from all participants.

Laboratory measurements. Total cholesterol, LDL-cholesterol and high-density lipoprotein cholesterol, and triglyceride levels were determined by standard laboratory procedures. The plasma oxLDL level was measured using an enzyme immunoassay kit (Kyowa Medix, Tokyo, Japan) following the manufacturer’s instructions. In this enzyme immunoassay, a monoclonal antibody recognizing oxidized phospholipids was used. The results were expressed in arbitrary units (U/l), with one unit is equal to 250 ng of copper-oxLDL.18 The ratio of oxLDL/LDL, an accurate estimation of the oxidation of LDL in vivo,19 was used in the current study.

The Lp-PLA2 activity was measured in 25 subjects with the TT genotype and 32 subjects randomly selected from each of the GG and GT genotypes in the Kakeya population using a commercial kit (Cayman Chemical, Ann Arbor, MI).

Genotype determination. Genomic DNA was isolated from peripheral blood samples. The genotype of G994T was determined by an allele-specific PCR as described previously15 in the Kakeya population and by the TaqMan method20 in the Mitoya population. In all, 24 samples from the Kakeya population were genotyped by the TaqMan method as well and the results were consistent with those of the allele-specific PCR.

Measurements of the carotid IMT. Carotid IMT was evaluated with the Vivid i portable echo system (GE Yokogawa Medical System, Tokyo, Japan), using a 9.0-MHz linear-array transducer and a duplex scanner. The IMT was measured sequentially at three 1.5-cm segments of the common carotid artery immediately proximal to the bifurcation, and at a 1.5-cm segment of the internal carotid artery immediately distal to the bifurcation.21 Maximum thickness was taken as the maximal IMT and a maximal IMT over 1 mm was considered indicative of atherosclerosis.21 The IMT data of 517 participants (263 and 254 of Kakeya and Mitoya population, respectively) were available.

Statistical analysis. Statistical analyses were performed with the SPSS package (version 13.0; SPSS, Chicago, IL). Results are represented as the mean and 95% confidence interval of the mean, or as percent frequency. The normality of the distribution was examined and log-transformed values for significantly skewed data were used in the analysis. For descriptive purposes, the means given are untransformed and unadjusted values. An analysis of variance and \( \chi^2 \)-test were used for continuous and dichotomous variables, respectively. As there were no gender-specific effects on oxLDL/LDL across the genotypes (data not shown), we included samples from both sexes in the analysis of oxLDL/LDL by Lp-PLA2 genotype. Spearman’s single linear correlation was subsequently applied to assess contributors to the variance of oxLDL/LDL, followed by a multiple linear or logistic regression analysis to make adjustments for confounding factors. \( P < 0.05 \) was considered statistically significant.

RESULTS

The frequencies of the GG, GT, and TT genotypes of the G994T polymorphism in the two populations are listed in Table 1. The minor allele frequency was 0.187 and 0.198 for the Kakeya and the Mitoya population, respectively, which was in agreement with previous observations in Japanese.15,22 The genotype distribution was within the Hardy–Weinberg equilibrium: Kakeya, \( \chi^2 = 2.71, P = 0.10 \); Mitoya, \( \chi^2 = 0.07, P = 0.80 \).

Table 1 shows the demographic data for the study populations as well. The anthropometries, lifestyle factors, and serum lipid profiles did not differ significantly among the three genotypes in the Kakeya population. The high-density lipoprotein cholesterol, oxLDL levels and smoking status were significantly different in the Mitoya population across the G994T genotypes. Additionally, the Mitoya population is older, having a higher oxLDL level and a higher female ratio and less smokers and drinkers.

The oxLDL/LDL ratios in the two populations are depicted in the Figure 1. In both populations, the ratio was significantly greater (by Dunnet’s post hoc test) in subjects with the TT genotype than in those with the other two genotypes, which implied a recessive effect of the T allele on the ratio. By contrast, a codominant effect of the G994T genotype on Lp-PLA2 activity was confirmed in the Kakeya subjects extracted from the three genotypes; the activity was 22.6 ± 1.1 nmol/min/ml in GG (mean ± s.e.m., \( n = 32 \)), 13.8 ± 0.8 nmol/min/ml in GT (\( n = 32 \)), and 0.7 ± 0.1 nmol/min/ml in TT (\( n = 25 \)) (\( P < 0.01 \) by analysis of variance). The significance remained after adjusting for sex, age, body mass index, lipid profiles, and lifestyle factors and was consistent with our previous observation.15 Due to the dominant nature of the G allele’s effect on the oxLDL/LDL ratio, the G allele carriers (GG and GT) were pooled together in the subsequent analyses.
In this cross-sectional study, a functional single-nucleotide polymorphism in the Lp-PLA₂ gene was indicated to have a significant effect on plasma LDL oxidation represented by the oxLDL/LDL ratio. Further, our data suggested that the T allele had a recessive effect on the oxidation of LDL, although it had a codominant effect on the activity of Lp-PLA₂.

**DISCUSSION**

The independent association of age and of the G994T genotype with the oxLDL/LDL ratio was then confirmed in the multiple linear regression analysis (Table 2).

Effects of the three genotypes on IMT in the two populations are listed in Table 3. The G994T genotype did not show significant effects on the maximal IMT in either population or after they were combined.

---

**Table 1** | Demographic data of the study populations sorted by the G994T genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Kakeya</th>
<th>Mitoya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>237/311</td>
<td>149/219</td>
</tr>
<tr>
<td>Genotype frequency, %</td>
<td>100</td>
<td>67.2</td>
</tr>
<tr>
<td>Age</td>
<td>63.7 (62.4, 64.8)</td>
<td>63.7 (62.1, 65.2)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.5 (22.2, 22.8)</td>
<td>22.5 (22.1, 22.8)</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>127.4 (126.0, 128.8)</td>
<td>127.3 (125.1, 129.0)</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>76.2 (75.3, 77.1)</td>
<td>76.5 (75.3, 77.2)</td>
</tr>
<tr>
<td>Smoker, %</td>
<td>15.5</td>
<td>14.1</td>
</tr>
<tr>
<td>Exsmoker, %</td>
<td>12.4</td>
<td>12.0</td>
</tr>
<tr>
<td>Nonsmoker, %</td>
<td>72.1</td>
<td>73.9</td>
</tr>
<tr>
<td>Alcohol drinker, %</td>
<td>35.7</td>
<td>35.0</td>
</tr>
<tr>
<td>Antihypertensive therapy, %</td>
<td>25.5</td>
<td>25.5</td>
</tr>
</tbody>
</table>

Values in parentheses are 95% confidence intervals of the mean. The differences between the genotypes within each population and between the populations were tested by analysis of variance, *t*-test or unpaired *t*-test when deemed appropriate.

BMI, body mass index; DBP, diastolic blood pressure; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; oxLDL, oxidized low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.

**Table 2** | Multiple regression analysis of variables independently affected the oxLDL/LDL levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>β ± s.e.m.</th>
<th>Standard β</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kakeya</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.004 ± 0.001</td>
<td>0.181</td>
<td>3.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Genotype (TT vs. GG + GT)</td>
<td>0.117 ± 0.062</td>
<td>0.132</td>
<td>2.26</td>
<td>0.024</td>
</tr>
<tr>
<td><strong>Mitoya</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.002 ± 0.002</td>
<td>0.070</td>
<td>0.98</td>
<td>0.033</td>
</tr>
<tr>
<td>Genotype (TT vs. GG + GT)</td>
<td>0.195 ± 0.076</td>
<td>0.159</td>
<td>2.57</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Adjusted for gender, BMI, lipid profiles, cigarette smoking, alcohol consumption, and medication.

---

**Figure 1** | Effect of the Lp-PLA₂ genotype on oxLDL/LDL levels. The open and shaded columns represent the Kakeya and the Mitoya population, respectively. Error bars represent standard error of the mean. *P < 0.05 compared with the values for GG and GT using Dunnet’s post hoc test.

Age (r = 0.19, P < 0.001 by Spearman’s correlation analysis) and the G994T genotype (F = 6.1, P = 0.014 by analysis of variance) were positively correlated with the oxLDL/LDL ratio. The independent association of age and of the G994T genotype with the oxLDL/LDL ratio was then confirmed in the multiple linear regression analysis (Table 2).
By contrast, the G994T genotype did not affect the IMT, suggesting that the Lp-PLA₂ activity was not a major contributor to the development of atherosclerosis. The results were confirmed in the two independent populations, which provided convincing evidence. Further, as the Lp-PLA₂ activity in our population was under the genetic influence that was large (the lowest activity was zero) and long-lasting (from the birth), the present result was a very strong evidence against the major role of this enzyme in athrogenesis.

The oxLDL/LDL ratio was comparable between the subjects with the GT genotype and those with the GG genotype in spite that the level of Lp-PLA₂ activity was significantly decreased by half in the former group. This apparent discrepancy was probably due to a number of environmental and serological factors affecting the oxidation of LDL in vivo. In fact, we found that aging was independently correlated with the oxLDL/LDL ratio. As such, many oxidative and antioxidative substances were expected to influence the level of LDL oxidation. It is therefore notable that a significant increase in the oxLDL/LDL ratio was observed in those with the TT genotype, suggesting that other antioxidation systems could not compensate for the complete loss of Lp-PLA₂ activity. This further implies a particular significance for the TT genotype in the clinical and epidemiological evaluation of the pathological role of Lp-PLA₂.

The association of the G994T genotype with plasma LDL oxidation further raises interesting questions about the potential pro- or antiatherosclerotic roles of the enzyme. Epidemiological evidence obtained recently in Caucasians support a deleterious role for Lp-PLA₂ in atherosclerosis. Consistent with this, subjects with the GT or TT genotype, which conferred a lower level of Lp-PLA₂ activity, were shown to have a reduced risk for cardiovascular disease among Koreans. By contrast, studies on Japanese subjects indicated that carriers of the T allele had a greater genetic risk for myocardial and cerebral infarction. Our result, indicating no difference in the oxLDL/LDL levels between the GG and GT genotypes, implies that it may not be appropriate to combine the GT and TT genotypes in such case-control studies. It is necessary to perform larger-scale case-control and/or prospective studies analyzing the three genotypes separately to evaluate the precise effect of this single-nucleotide polymorphism, and of the loss of the enzyme activity, on atherosclerotic diseases.

Two studies have examined the relationship between Lp-PLA₂ activity and oxLDL. One was conducted in Caucasians with diabetes mellitus, showing that a relatively small increase in Lp-PLA₂ activity resulted in a concomitant decrease in the oxLDL/LDL ratio. The other, conducted in Korean men, indicated that G994T did not significantly affect oxLDL either in cases of cardiovascular disease or in normal controls. Careful interpretation is, however, required when making comparisons with these studies. First, the background of the subjects was different: we employed participants of a health examination with the exclusion of diabetic subjects. Consequently, our subjects might be exposed to less oxidative stress than those with diabetes or cardiovascular diseases.

Second, the enzyme immunoassay used in the previous studies measured both copper-oxLDL and malondialdehyde-LDL, whereas the kit used in the present study was optimized to quantify the minimally modified LDL formed in the early phase of LDL oxidation. Importantly, it has now become clear that the oxidation of phospholipids, occurring mainly in minimally modified LDL, is one of the earliest triggers promoting the formation of plaques. Therefore, the oxLDL quantified as minimally modified LDL may be a more suitable marker for the evaluation of the risk of atherosclerosis.

We were unable to establish a link between the G994T polymorphism and carotid IMT. This result is consistent with previous studies in Caucasians, in which no association was found between Lp-PLA₂ activity and extracoronary IMT. These observations seem, however, contradictory to the association of the enzyme activity with cardiovascular events observed in large-scale population-based studies. Two explanations are possible for this apparent discrepancy: (i) Lp-PLA₂ activity may have a major role not in the formation of plaques per se but in the development of vulnerability as in the case of C-reactive protein; although positively correlated with coronary events, C-reactive protein was not a good predictor of the extent of atherosclerotic damage. (ii) As the formation of atherosclerotic plaques occurred “far” from Lp-PLA₂ in the pathophysiological cascade, the influence of this enzyme on IMT might be attenuated or eclipsed by other genetic and/or environmental factors.

In this context, it is of great interest that recent studies indicated that darapladib, a newly developed inhibitor for Lp-PLA₂, prevented the expansion of necrotic core of atheromatous plaques both in pigs with experimental hypercholesterolemia and in humans with coronary artery disease. These observations strongly suggested that the Lp-PLA₂ mainly influence the “stability” of atheroma plaque. In the human study, darapladib reduced the Lp-PLA₂ activity by 59%, which is comparable with the effect of the single 994T
allele (heterozygotes of the G994T have a 50% reduction of the activity when compared with the GG homozygotes). According to the high allele frequency of the 994T, about one third of Japanese have a half or less activity of Lp-PLA2 by nature than that in the other two thirds.15

It is known that Japanese still have a lower incidence of coronary artery disease even though the lifestyle has been rapidly westernized.10,14 If the effect of G994T on the Lp-PLA2 activity is considered, “the resistance to coronary artery disease” in Japanese may be partly explained by the substantial number of the GT/TT in the population. A large-scale case–control study between the G994T and the coronary event in Japan may give us a definite answer whether the (lack of) Lp-PLA2 activity influences the vulnerability of coronary plaques, which will be invaluable information in the development of the inhibitor of Lp-PLA2.

Acknowledgments: We cordially thank Satoko Mishima, Hiroshi Shibata, Yoshimoto Notsu, and Yoji Suyama for technical assistance. This study was partly supported by grants from Daiwa Health Foundation and the Research Project Promotion Institute of Shimane University.

Disclosure: The authors declared no conflict of interest.


