with whom informed content for genotyping was sought for. Of 12,839 subjects, 2267 (17.7%) with no lipid-altering medication were randomly selected for the present study. In some institutes the information on sex was not disclosed.

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 were measured enzymatically by 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 [24]. DNA was extracted by QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Detection of gene mutations by Invader® assay

We applied the Invader® assay for screening three known mutations of the *CETP* gene, one of the *LIPC* genes, one of the *LPL* genes, and one of the *APOC3* genes, as previously described [25]. 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 microliter of primary probe/Invader®/MgCl2 mixture was added into 96 well plate. Seven microliters of 5 fmol/l

synthetic target oligonucleotides, 10 μg/ml yeast tRNA (no target blank), and genomic DNA samples (15 ng/μl) were added, which were denatured by incubation at 95°C for 10 min. After 15 μl of mineral oil (Sigma, St. Louis, MO) were overlayed into all reaction wells, the plate was incubated isothermally at 63°C for 4 h in the DNA thermalcycler (PTC-200; MJ Research, Watertown, MA) and then kept at 4°C until fluorescence measurements. The fluorescent intensities were measured by the fluorescence microtiter plate reader (Cytoflour 4000; Applied Biosystems) with excitation, 485 nm/20 nm (Wavelength/Bandwidth) and emission, 530 nm/25 nm for FAM; excitation, 560 nm/20 nm and emission, 620 nm/40 nm for RED. The genotyping was analyzed by calculation with the ratios 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 by analysis of variance. The χ^2-square test was used to compare the incidence of each genotype. The analysis was performed by SPSS software (ver. 11.5)(Chicago, IL).

Results

We investigated the frequency and phenotypic association of the common polymorphisms of *CETP*, *LPL*, *LIPC*, and *APOC3* genes at the population level in 2,267 subjects. Table 1 summarizes the mean serum lipid levels in the participants in this study. The mean age, total

cholesterol, TG, HDL-cholesterol, and LDL-cholesterol levels in this population were similar to the level of all the participants of 12,839 people in the Serum Lipid Survey 2000. 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 for the gene analysis are representative of the general Japanese population.

Table 2 summarizes the association of the gene polymorphisms with serum lipid levels in all the participants. Tables 3 and 4 show the analysis in male and female participants, respectively. We tested and found that Hardy-Weinberg equilibrium was the case for all the SNPs, supporting the assumptions of random mating in this population except *CETP* Int14 +1 G_A, in which no homozygote was found in this population.

The incidence of heterozygote mutations of D442G and Int14 +1 G→A of the *CETP* gene was 8.1 and 0.6 %, respectively. These mutations were associated with higher HDL-cholesterol. The heterozygous mutation of D442G was also associated with lower TG level only in men. Although the incidence of homozygous mutation of D442G and heterozygous mutation of Int14 +1 G→A was quite low and the difference was not significant, the TG levels tended to be higher. The incidence of B1B1, B1B2, and B2B2 genotypes the *CETP* TaqIB polymorphism was 35.8, 48.4, and 15.8%, respectively. The B2 allele of the *CETP* TaqIB polymorphism was associated with higher HDL-cholesterol levels in all the participants, men, and women. Although the difference was not statistically significant, the participants with B2 allele tended to have lower TG levels, which is

different from the results with homozygous mutation of D442G and heterozygous mutation of Int14 +1 G→A.

We then determined the polymorphism of *LPL* S447X mutations in this population. The incidence of heterozygous and homozygous mutations in the *LPL* gene was 20.7 and 1.3%, respectively. The mutation in the *LPL* S447X site was associated with higher HDL-cholesterol and lower TG levels, although the difference of HDL-cholesterol in men or that of TG in women was not statistically significant, possibly due to the small sample number.

The incidence of CC, CT, and TT genotype of *LIPC* in the Japanese was 24.9, 50.4, and 24.7%, respectively. Overall, the T allele was associated with an increase in HDL-cholesterol levels. However, the statistical significance was not found in men. The TG levels do not seem to be affected by this SNP.

The incidence of S1S1, S1S2, and S2S2 genotypes of the *APOC3* SstI polymorphism was 42.0, 45.8, and 12.2%, respectively. Although the HDL and LDL-cholesterol levels were similar in all the genotypes, the S2 allele was associated with higher TG levels in all the participants and in men, but not in women. Among the SNPs studied, no polymorphism was found to affect the LDL-cholesterol levels.

To determine the contribution of CETP and LPL gene polymorphism to hyperalphacholesterolemia (2.58 mmol/l or over) and hypoalphacholesterolemia (1 mmol/l or under), we divided all the participants into 3 groups according to HDL-cholesterol levels; 1 mmol/l

or under, 1 to 2.58 mmol/l, and 2.58 mmol/l or over. We then assessed the incidence of each genotype. The incidence of hyper- and hypoalphacholesterolemia was 8.3 and 1.8%, respectively. Among the genes studied we found 3 gene polymorphisms were associated with the incidence of high HDL-cholesterol (2.58 or over) (Table 5). Participants with B2B2 of *CETP* TaqIB had a higher incidence of high HDL-cholesterol levels than the others. Heterozygotes of *CETP* D442G polymorphism had a higher incidence of higher HDL-cholesterol levels than wild type. Homozygotes of *LPL* S447X polymorphism had a higher incidence of higher HDL-cholesterol levels than the others.

Discussion

In this study we have demonstrated the frequency of six common gene polymorphisms of the four genes related to lipid metabolism and its incidence and association with serum lipid levels in the general Japanese population. Because this is the largest analysis in the Japanese population, these data would be useful for the future analysis in the general Japanese population.

The prevalence of the D442G and Int14 +1 G_A mutations is very high in the general Japanese population, with heterozygote frequencies of 7 and 1 %, respectively [9, 10, 26, 27]. Our large scaled study showed similar frequencies of these mutations, with 8.1 and 0.6 %, respectively, indicating that our study population represents the general Japanese population and confirmed that the frequency of these mutations is quite frequent in our population. Because these mutations are associated with lower CETP activities [26], the plasma level of HDL-cholesterol is higher in

heterozygotes and homozygotes. We have also confirmed that the incidence of D442G mutation is higher in people with hyperalphalipoproteinemia (2.58 mmol/l or over).

Genetic *CETP* deficiency is the most important and common cause of hyperalphalipoproteinemia in Japanese and the *CETP* deficiency contributes to 60% of hyperalphacholesterolemia [28]. However, the role of CETP in atherogenesis is still under debate. Study in the Japanese Omagari area has shown a relatively increased incidence of coronary atherosclerosis in CETP deficiency [29]. In Copenhagen City Heart Study, increased HDL-cholesterol levels caused by mutations in *CETP* are associated with an increased risk of CAD in white women [30]. In contrast, the B2 allele of TaqIB polymorphism is associated with low CETP mass, higher HDL-cholesterol levels, and a decreased risk for coronary artery disease [16]. The reason for this discrepancy is unknown. It might be due to dose effects of CETP mass or another genetic abnormality can be involved in this difference to explain the risk for CAD. Hirano et al showed that the people with low LIPC activity had a higher incidence of CAD [31]. Therefore, it is possible that the LIPC activity is involved in these differences. Further studies are necessary to determine the role of CETP in CAD in various populations with difference genetic background.

Our study is consistent with other studies in terms of allele frequency of the S447X polymorphism of *LPL* gene [18, 19, 32]. Recent studies show that the X447 mutation is associated with a favorable lipid profile, with lower TG and higher HDL-cholesterol levels, and that it may confer protection against coronary artery disease [18, 19, 33]. We also found similar tendency in

men and women. However, the statistical significance was found in HDL-cholesterol levels of the total population and women, but not in men. Because the X447 mutation is associated with higher LPL activity, the TG levels were lower in heterozygotes and homozygotes as expected, although the significance was not noted in women. Homozygotes seem to have lower TG levels than heterozygotes, which reflects the gene dosage effect. Because the carriers of the S447X have favorable lipid profile in terms of HDL-cholesterol and TG, and several studies have shown a decreased risk of CAD in carriers of the S447X [34, 35], we should examine whether carriers of the S447X have less coronary artery events in the future.

In terms of *LIPC* gene polymorphism, our data clearly indicate that the frequency of the TT genotype is significantly higher in the Japanese than that in Caucasians [36, 37]. However, the higher frequency of the TT genotype is also found in Koreans and Japanese [38-40]. Therefore, this difference might partly explain higher HDL-cholesterol levels in Asians.

Our results on allele frequency of the SstI polymorphism of the *APOC3* gene were almost comparable to the data on Asian Indians [41], but not on Caucasians [42]. Caucasians seem to have less allele frequency of S2. Although the association of higher TG levels with S2 allele has been reported in studies carried out in Caucasians [43-45] and Asians [46-48], our data show that the association was found in the total population and in men, but not in women. Few other studies, however, did not find any significant association between SstI polymorphism and hypertriglyceridemia [49-51]. The linkage disequilibrium between this polymorphism and the

causative mutation might be weakened or absent in some populations [43].

Our data clearly showed that the heterozygotes of D442G mutation, homozygote of LPL S447X mutation, and people with TaqIB2B2 genotype had a higher incidence of hyperalphalipoproteinemia with HDL-cholesterol levels of 2.58 mmol/l or over. Alcohol consumption and smoking can also affect the levels of HDL-cholesterol. Corbex et al showed that the HDL levels of the people with certain polymorphisms of the *CETP* gene are modulated by alcohol consumption [52]. Therefore, it might be necessary to taking account of the environmental effects on the effect of gene polymorphism on HDL-cholesterol levels as well as on the risk of cardiovascular events.

In summary we have provided a largest database of gene polymorphism related to lipid metabolism in the general Japanese population. Prospective study is now under way to determine the contribution of these gene polymorphisms to cardiovascular risk in Japanese.

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Appendix

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Table 1.

Lipid profile and age of all the participants.

	all	men	women
T-Cho (mmol/l)	5.18 (0.021)	5.23 (0.046)	5.15 (0.046)
TG (mmol/l)	1.31 (0.024)	1.58 (0.050)*	1.11 (0.039)*
HDL-c (mmol/l)	1.53 (0.010)	1.38 (0.020)*	1.65 (0.017)*
LDL-c (mmol/l)	3.00 (0.020)	3.08 (0.044)*	2.93 (0.039)*
Age (years)	47.1 (0.58)	49.5 (0.87)*	45.3 (0.76)*
Men (%)	43		

Data are expressed as mean (SEM). * $p<0.01$, men vs. women.