ORIGINAL CONTRIBUTION

Allele Frequency of Apolipoprotein Gene Polymorphisms and Association between Genotype and Serum Lipid and Apolipoprotein Levels

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The rare allele frequency of the restriction fragment length polymorphism (RFLP) with Xba I (X+) at the apolipoprotein B (apo B) gene locus was 0.041 in men and 0.026 in women in H-Y district, Shiso County, Hyogo Prefecture, Japan: EcoR I at apo B gene (E−)=0.073 and 0.076, Msp I at apo Al-CIII gene (M−)=0.423 and 0.430, and Sac I at apo Al-CIII gene (S+)=0.309 and 0.349. There was no marked age- or sex-difference in the frequencies. The frequencies of X+ and E− were lower and those of S+ and M− were higher in H-Y district than in Caucasian populations.

In this population, according to the ANOVAs, the genotype for EcoR I was significantly (p<0.05) associated with serum total cholesterol, LDL cholesterol and apo B in women, and the genotype for Sac I with serum triglycerides and Msp I with serum apo CIII in men. The absolute values of Spearman correlation coefficients between genotypes and serum lipids or apolipoproteins were less than 0.2 after adjustment for age. J Epidemiol, 1995; 141-151.

allele frequency, genetic association study, serum lipid, serum apolipoprotein, restriction fragment length polymorphism

Serum lipids and apolipoproteins are considered to be influenced by both environmental and genetic factors. In many epidemiological and experimental studies, it has been shown that several lifestyle factors are related to the level of serum lipids: e.g. a positive or inverse relation of total fat, saturated fat, cholesterol and polyunsaturated fat to serum total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C), and a positive relation of physical activity and alcohol consumption to serum high density lipoprotein cholesterol (HDL-C). Although, as for the genetic factors, there have been some family and twin studies as well as studies on the association of family history with hyperlipidemia, they have not only advantages but also disadvantages, e.g. “family aggregation” in family studies, “shared environment” in family and twin studies, and recall bias in family histories. In contrast to these studies, “candidate gene” approach by identifying genotypes related to inter-individual variations of serum lipids and apolipoproteins in populations has recently been proved highly effective with development of the polymerase chain reaction (PCR) method.

One of the two aims of the present study is to epidemiologically describe the allele frequencies of four restriction fragment length polymorphisms (RFLPs) with Xba I and EcoR I at the apolipoprotein B (apo B), and Msp I and Sac I at the apo Al-CIII gene loci in a rural Japanese population, H-Y district, Shiso County, Hyogo Prefecture, Japan. The other aim is to estimate the association between the genotype and serum TC, LDL-C, HDL-C, triglycerides (TG), apo B, apo AI and apo CIII levels in the population.
SUBJECTS AND METHODS

Study District

H-Y district is located in the north-western part of Hyogo Prefecture, Japan. Most of the residents are salaried employees of minor companies, who supplement their income with part-time jobs in forestry and agriculture.

Subjects

All residents of H-Y district aged 40 years or older, 2,510 men and 2,621 women, were invited to undergo an examination in 1992, which was conducted under a Japanese law for the prevention of cardiovascular disease. Of these, 73% (1,832/2,510) of the men and 76% (1,992/2,621) of the women responded to the invitation. As for the persons aged 20-39 years, only those who desired to be examined were recruited.

Not only to genotype four RFLPs but also to measure serum lipids and apolipoproteins, a total of 893 unrelated subjects were randomly selected from the respondents. The sample size was determined, taking into account the statistical power to detect the significant difference in the allele frequencies between the Japanese and other populations. Individuals who refused to be genotyped, who took lipid-lowering medication, and who suffered from diabetes mellitus, chronic renal and hepatic diseases and chronic alcohol abuse were excluded. Finally, 673 subjects were recruited for the present analyses.

We obtained informed consent from all of the subjects. Ethical approval for the present study was granted by the Ethical Review Committee, Medical Research Institute, Tokyo Medical and Dental University.

Measurement of Serum Lipids and Apolipoproteins

Using venous blood drawn in overnight fasting state, determination was made of serum total cholesterol and triglycerides by enzymatic methods6,7, and serum HDL-C by dextran sulfate/Mg²⁺ precipitation8. The level of serum LDL-C was calculated using the Friedewald's formula9. As to the control over accuracy and precision of serum lipid measurement, the standardization was achieved by participation in the CDC Lipid Standardization Program through Osaka Prefectural Center for Adult Diseases, Japan.

Serum apo B, apo AI and apo CIII concentrations were measured by the method of turbidimetric immunoassay (TIA).

DNA Analyses

The subjects were genotyped for four RFLPs with Xba I and Eco R I at the apo B, and Msp I and Sac I at the apo AI-CIII gene loci.

Total genomic DNA was prepared from the leukocytes of five milliliter venous blood collected in vacuum tubes containing ethylene diamine tetraacetate (EDTA), using the Nucleic Acid Extraction Kit-IsoQuick (MicroProbe Corp.). All polymerase chain reactions (PCRs) were carried out on the Programmable Thermal Controller PTC-100 (MJ Research Inc.) in a final volume of 200 µl including 0.5 unit of Taq polymerase (Perkin Elmer Cetus), 3.2 µl of 1.25 mM dNTPs, 2.0 µl of 10 mM each primer, 2.0 µl of 10% reaction buffer and 1.0 µg of template DNA. The oligonucleotide primers and conditions for PCRs are shown in Table I. Ten µl of amplification product was incubated with the equal volume of digestion mixture including restriction endonuclease (Takara Biomedicals). The digestion products were electrophoresed on 4.5% polyacrylamide gel at 180 volt for 1.5 hour.

Table 1. Oligonucleotide primer and PCR condition.

| RFLP and Primer | Enzyme site Location | Sequence | PCR condition | Fragment Size |
|-----------------|----------------------|----------|---------------|--------------|
| Xba I Primer 1  | Exon 26 of the B gene| 5'-CCGTGAGGTGACTCAGAGAC-3' 5'-AGGCAGGCATGGCTCCAAGG-3' | Initial denaturation 96°C for 2 min. 30 cycles: 96°C for 0.5 min., 62°C for 1 min., 72°C for 4 min. | 217,253 bp |
| Xba I Primer 2  | Exon 26 of the B gene|  | | |
| EcoR I Primer 3 | Exon 29 of the B gene| 5'-AACAACAGTAGACTTTTATAA-3' 5'-ATCTCTTTTACGTGAATTATG-3' | 35 cycles: 94°C for 1 min., 55°C for 1 min., 72°C for 1 min. | 418,637 bp |
| EcoR I Primer 4 | Exon 29 of the B gene|  | | |
| Sac I Primer 5  | 3' non-coding region of the CHI gene| 5'-CTGACTGTTGCTGCAGTGCAGT-3' 5'-CCAGAAGGTGGATAGACGCGC-3' | Same as the condition for Xba I | 264,270 bp |
| Sac I Primer 6  | 3' non-coding region of the CHI gene|  | | |
| Msp I Primer 7  | Intron 3 of the AI gene| 5'-CAGCGCAGAGACTATGTGT-3' 5'-CCGTTGTCAGCTGGAGCGA-3' | Initial denaturation 96°C for 2 min. 30 cycles: 96°C for 0.5 min., 58°C for 1 min., 72°C for 3 min. | 190,304 bp |
| Msp I Primer 8  | Intron 3 of the AI gene|  | | |
The gel samples were stained with ethidium bromide. The presence or absence of cutting sites were designated to ‘+’, or ‘−’ respectively.

Different 20 samples were measured twice for each of Xba I, EcoR I, Sac I and Msp I polymorphisms to assess the reproducibility, which was found to be 100%.

The allele frequency was estimated by the gene-counting method.

Statistical Analyses
SAS software (Version 6.08) was used for all statistical analyses.

The frequency distributions of values for serum lipids and apolipoproteins were examined whether they followed the normal distribution or not. Since, of them, serum TG and apo CIII concentrations appeared to be approximately log-normally distributed, the values were logarithmically transformed for statistical analyses, although the non-transformed means are presented in the tables.

ANOVA was performed using the general linear model procedure (PROC GLM) to estimate the association between each of the RFLPs and each of serum lipids or apolipoproteins. Age was included as an independent variable in the model to adjust its effect. Least-Square means, the values for genotype class means after adjustment for age, were also calculated.

Since persons homozygous for the X+ allele (X+X+) of Xba I and E− allele (E−E−) of EcoR I were very few in H-Y district, they were also combined with those heterozygous for the corresponding allele, X + X − or E+ E −, for ANOVAs.

In addition, Spearman correlation coefficients were calculated between RFLPs and serum traits, adjusting age effect, in order to estimate the strength of association.

RESULTS
Serum Lipids and Apolipoproteins
The means and standard deviations of serum lipids and apolipoproteins are presented in Table 2. The mean of serum apo B-related trait (TC, LDL-C or apo B) increased with age, reaching the maximum at the age group of 50-59 in men and at the age group of 60-69 in women. Then, afterwards, it decreased. Women showed lower levels than men until the age of 49 years, but their levels surpassed men’s at the age of 50 years and over.

The mean or median of serum TG showed the same change with age as that of serum TC in both sexes.

No age-dependent change was observed in the mean of serum HDL-C, while the levels in women were higher than those in men. The mean of serum apo Al had a peak at the age group of 50-59 in both men and women, while women showed higher means than men after the age of 50 years. The mean or median of serum apo CIII tended to decrease with age in men, but to increase in women.

Allele and Genotype Frequencies
The gender- and age-specific allele frequencies of four RFLPs for apo B and Al-CIII genes are shown in Table 3. The rare allele frequencies of Xba I RFLP were in the range of 0.020 to 0.051 in men and 0.014 to 0.038 in women: 0.031-0.122 and 0.050-0.153 for EcoR I, 0.277-

Table 2. Means and standard deviations of serum lipid (mmol/L) and apolipoprotein (mg/dL) levels in H-Y district, Shiso County, Hyogo Prefecture, Japan.

| Age | Number | TC ± SD | LDL-C ± SD | TG ± SD | HDL-C ± SD |
|-----|--------|---------|------------|--------|------------|
| Men |        |         |            |        |            |
| <40 | 44     | 4.81 ± 0.97 | 2.82 ± 0.91 | 3.26 ± 4.19 | 1.34 ± 0.48 |
| 40- | 50     | 5.14 ± 0.86 | 3.24 ± 0.92 | 3.35 ± 2.11 | 1.21 ± 0.25 |
| 50- | 56     | 5.43 ± 0.86 | 3.41 ± 0.86 | 3.89 ± 2.95 | 1.29 ± 0.28 |
| 60- | 94     | 4.81 ± 0.92 | 2.97 ± 0.89 | 2.55 ± 1.40 | 1.35 ± 0.31 |
| ≥70 | 49     | 5.02 ± 0.96 | 3.33 ± 0.92 | 2.56 ± 1.10 | 1.16 ± 0.28 |
| Women |        |         |            |        |            |
| <40 | 49     | 4.59 ± 0.78 | 2.84 ± 0.73 | 1.57 ± 0.59 | 1.43 ± 0.24 |
| 40- | 72     | 5.09 ± 0.99 | 3.25 ± 0.87 | 2.26 ± 1.48 | 1.37 ± 0.27 |
| 50- | 90     | 5.56 ± 1.02 | 3.59 ± 0.90 | 2.77 ± 1.30 | 1.41 ± 0.34 |
| 60- | 93     | 5.84 ± 0.97 | 3.80 ± 0.92 | 3.29 ± 2.08 | 1.38 ± 0.34 |
| ≥70 | 76     | 5.61 ± 0.99 | 3.74 ± 0.83 | 2.70 ± 1.28 | 1.35 ± 0.31 |

TC=total cholesterol; LDL-C=low density lipoprotein cholesterol; HDL-C=high density lipoprotein cholesterol; TG=triglycerides; Apo=apolipoprotein.

Table 2. Continued

| Age | Number | Apo B ± SD | Apo Al ± SD | Apo CIII ± SD |
|-----|--------|------------|-------------|---------------|
| Men |        |            |             |               |
| <40 | 44     | 94.52 ± 33.03 | 150.95 ± 28.62 | 14.26 ± 7.51 |
| 40- | 50     | 110.35 ± 26.26 | 155.41 ± 25.91 | 15.16 ± 5.05 |
| 50- | 56     | 110.75 ± 26.02 | 158.84 ± 29.93 | 15.39 ± 5.40 |
| 60- | 94     | 88.29 ± 24.92 | 150.84 ± 27.58 | 12.70 ± 3.86 |
| ≥70 | 49     | 95.39 ± 22.12 | 132.94 ± 20.27 | 12.28 ± 3.44 |
| Women |        |            |             |               |
| <40 | 49     | 75.92 ± 17.86 | 148.41 ± 19.90 | 10.19 ± 1.93 |
| 40- | 72     | 90.75 ± 22.75 | 154.32 ± 25.78 | 11.27 ± 3.74 |
| 50- | 90     | 102.94 ± 23.40 | 162.19 ± 25.75 | 13.17 ± 3.96 |
| 60- | 93     | 113.20 ± 28.47 | 160.40 ± 24.35 | 14.50 ± 5.51 |
| ≥70 | 76     | 104.04 ± 22.24 | 150.07 ± 25.11 | 12.70 ± 3.28 |
### Table 3. Allele frequency of Apo B and Apo Al-CIII gene RFLPs in H-Y district, Shiso County, Hyogo Prefecture, Japan

| Gene Region and RFLP | Age (years) | —— | —+ | ++ | Rare Allele Frequency | —— | —+ | ++ | Rare Allele Frequency |
|----------------------|-------------|----|----|----|----------------------|----|----|----|----------------------|
| Apo B Xba I          | <40         | 41 | 3  | 0  | 0.034                | 47 | 2  | 0  | 0.020                |
|                      | 40-         | 45 | 3  | 1  | 0.051                | 70 | 2  | 0  | 0.014                |
|                      | 50-         | 51 | 5  | 0  | 0.045                | 86 | 4  | 0  | 0.022                |
|                      | 60-         | 87 | 7  | 1  | 0.047                | 87 | 5  | 1  | 0.038                |
|                      | ≥70         | 47 | 2  | 0  | 0.020                | 71 | 5  | 0  | 0.033                |
|                      | total       | 271| 20 | 2  | 0.041                | 361| 18 | 1  | 0.026                |
| EcoR I               | <40         | 0  | 5  | 39 | 0.057                | 1  | 13 | 35 | 0.153                |
|                      | 40-         | 0  | 3  | 46 | 0.031                | 1  | 6  | 65 | 0.056                |
|                      | 50-         | 0  | 8  | 48 | 0.071                | 1  | 7  | 82 | 0.050                |
|                      | 60-         | 0  | 15 | 80 | 0.079                | 0  | 11 | 82 | 0.059                |
|                      | ≥70         | 3  | 6  | 40 | 0.122                | 0  | 15 | 61 | 0.099                |
|                      | total       | 3  | 37 | 253| 0.073                | 3  | 52 | 325| 0.076                |
| Apo Al-CIII Sac I    | <40         | 19 | 24 | 1  | 0.295                | 18 | 24 | 7  | 0.388                |
|                      | 40-         | 19 | 27 | 3  | 0.337                | 24 | 39 | 9  | 0.396                |
|                      | 50-         | 28 | 25 | 3  | 0.277                | 39 | 42 | 9  | 0.333                |
|                      | 60-         | 47 | 41 | 7  | 0.289                | 41 | 39 | 13 | 0.349                |
|                      | ≥70         | 18 | 26 | 5  | 0.367                | 34 | 39 | 3  | 0.296                |
|                      | total       | 131| 143| 19 | 0.309                | 156| 183| 41 | 0.349                |
| Msp I                | <40         | 4  | 29 | 11 | 0.420                | 10 | 23 | 16 | 0.439                |
|                      | 40-         | 14 | 18 | 17 | 0.469                | 18 | 36 | 18 | 0.500                |
|                      | 50-         | 10 | 24 | 22 | 0.393                | 13 | 47 | 30 | 0.406                |
|                      | 60-         | 13 | 51 | 31 | 0.405                | 18 | 44 | 31 | 0.430                |
|                      | ≥70         | 10 | 24 | 15 | 0.449                | 9  | 41 | 26 | 0.388                |
|                      | total       | 51 | 146| 96 | 0.423                | 68 | 191| 121| 0.430                |

n = number of subjects. Apo = apolipoprotein. Frequency = [Hom × 2 + Het]/([Hom + Het + Hoc] × 2). Hom = homozygosity with rare allele; Het = heterozygosity; Hoc = homozygosity with common allele.

### Table 4. Association of genotypes with serum lipids (mmol/L) or apolipoprotein (mg/dL) according to the ANOVAs, age-adjusted.

| RFLP | Genotype | n     | TC   | LDL-C | Apo B | TG    | HDL-C | Apo Al | Apo CIII |
|------|----------|-------|------|-------|-------|-------|-------|--------|----------|
| Xba I| ——       | 271   | 4.98±0.06 | 3.13±0.06 | 97.52±1.67 | 8.52±1.07 | 1.31±0.02 | 149.04±1.66 | 12.82±1.02 |
|      | —+       | 20    | 5.40±0.21 | 3.33±0.21 | 108.22±6.15 | 12.59±1.35 | 1.33±0.08 | 155.94±6.11 | 15.17±1.08 |
|      | ++       | 2     | 5.46±0.66 | 3.94±0.62 | 108.58±19.45 | 5.37±2.45 | 1.10±0.23 | 138.32±19.34 | 11.61±1.29 |
|      | p        | 0.130 | 0.275 | 0.146 | 0.357 | 0.375 | 0.314 | 0.080 |          |
| EcoR I| ——       | 3     | 4.99±0.55 | 2.98±0.52 | 94.62±16.14 | 8.71±2.14 | 1.41±0.19 | 154.58±16.07 | 14.16±1.21 |
|      | —+       | 37    | 4.89±0.16 | 3.07±0.15 | 98.67±4.56 | 8.89±1.23 | 1.20±0.06 | 143.34±4.54 | 12.79±1.06 |
|      | ++       | 253   | 5.03±0.06 | 3.16±0.06 | 98.71±1.75 | 8.71±1.10 | 1.29±0.02 | 151.10±1.74 | 12.97±1.02 |
|      | p        | 0.701 | 0.829 | 0.971 | 0.984 | 0.235 | 0.270 | 0.874 |          |
| Sac I| ——       | 131   | 4.97±0.08 | 3.11±0.08 | 95.12±2.40 | 7.24±1.12 | 1.38±0.03 | 151.77±2.41 | 12.36±1.03 |
|      | —+       | 143   | 5.05±0.08 | 3.17±0.08 | 101.31±2.31 | 10.47±1.12 | 1.25±0.03 | 149.50±2.32 | 13.53±1.03 |
|      | ++       | 19    | 5.01±0.22 | 3.23±0.21 | 99.74±6.33 | 10.53±1.35 | 1.09±0.08 | 143.87±6.35 | 13.76±1.08 |
|      | p        | 0.812 | 0.777 | 0.177 | 0.048 | 0.070 | 0.471 | 0.073 |          |
| Msp I| ——       | 51    | 5.14±0.14 | 3.31±0.13 | 105.99±3.85 | 10.96±1.20 | 1.24±0.05 | 148.09±3.87 | 14.37±1.05 |
|      | —+       | 146   | 5.02±0.08 | 3.16±0.07 | 97.53±2.28 | 8.91±1.12 | 1.26±0.03 | 148.34±2.29 | 12.74±1.03 |
|      | ++       | 96    | 4.93±0.10 | 3.04±0.09 | 95.77±2.80 | 7.41±1.15 | 1.33±0.03 | 153.46±2.82 | 12.62±1.03 |
|      | p        | 0.461 | 0.231 | 0.087 | 0.215 | 0.197 | 0.355 | 0.048 |          |
Table 4. Continued

| RFLP | Genotype | n   | TC   | LDL-C | Apo B | TG   | HDL-C | Apo Al | Apo CIII |
|------|----------|-----|------|-------|-------|------|-------|--------|----------|
| Xba I | - -      | 361 | 5.44±0.05 | 3.52±0.05 | 99.76±1.30 | 6.76±1.07 | 1.39±0.02 | 159.15±1.32 | 12.13±1.02 |
|      | - +      | 18  | 5.25±0.23  | 3.33±0.21  | 99.98±5.81  | 10.00±1.29 | 1.30±0.07 | 152.13±5.91 | 11.78±1.08 |
|      | + +      | 1   | 6.02±0.98  | 3.43±0.87  | 103.39±24.65 | 16.38±2.95 | 1.11±0.31 | 142.54±25.05 | 17.22±1.34 |
|      | p        |     | 0.423 | 0.460  | 0.334  | 0.313  | 0.081  | 0.054  | 0.445    |
| EcoR I | - -      | 3   | 4.53±0.57  | 2.68±0.50  | 79.59±14.21 | 6.03±1.91 | 1.39±0.18 | 158.59±14.49 | 11.86±1.18 |
|      | - +      | 52  | 5.13±0.14  | 3.25±0.12  | 93.40±3.41  | 6.92±1.17 | 1.36±0.04 | 150.53±3.47 | 11.75±1.04 |
|      | + +      | 325 | 5.49±0.06  | 3.57±0.05  | 101.10±1.36 | 6.92±1.07 | 1.39±0.02 | 156.92±1.39 | 12.19±1.02 |
|      | p        |     | 0.015 | 0.014  | 0.041  | 0.962  | 0.790  | 0.230  | 0.706    |
| Sac I | - -      | 156 | 5.49±0.08  | 3.58±0.07  | 100.91±1.98 | 6.92±1.10 | 1.38±0.03 | 156.67±2.01 | 11.85±1.02 |
|      | - +      | 183 | 5.41±0.07  | 3.42±0.07  | 98.54±1.83  | 6.76±1.10 | 1.40±0.02 | 156.38±1.86 | 12.08±1.02 |
|      | + +      | 41  | 5.33±0.16  | 3.28±0.14  | 101.93±3.88 | 7.94±1.17 | 1.40±0.05 | 152.30±3.94 | 13.36±1.05 |
|      | p        |     | 0.634 | 0.079  | 0.579  | 0.707  | 0.740  | 0.597  | 0.065    |
| Msp I | - -      | 68  | 5.32±0.12  | 3.39±0.11  | 98.65±3.01  | 7.24±1.15 | 1.37±0.04 | 153.83±3.04 | 12.61±1.04 |
|      | - +      | 191 | 5.43±0.07  | 3.53±0.06  | 99.75±1.79  | 6.92±1.07 | 1.38±0.02 | 154.10±1.81 | 12.12±1.02 |
|      | + +      | 121 | 5.49±0.09  | 3.57±0.08  | 100.78±2.25 | 6.76±1.10 | 1.40±0.03 | 158.14±2.28 | 11.85±1.03 |
|      | p        |     | 0.549 | 0.392  | 0.847  | 0.909  | 0.835  | 0.311  | 0.372    |

ANOVA = analysis of variance. RFLP = restriction fragment length polymorphism. SE = standard error. n = number of subject. TC = total cholesterol; LDL-C = low density lipoprotein cholesterol; HDL-C = high density lipoprotein cholesterol; TG = triglycerides; Apo = apolipoprotein. Differences of serum levels among the 3 genotypes were tested by ANOVA controlling for age.

Table 5. Spearman correlation coefficient of RFLPs with serum lipids or apolipoproteins, age-adjusted.

| Independent | Men (n = 293) | Women (n = 380) |
|-------------|--------------|-----------------|
|             | TC | LDL-C | TG | Apo B | HDL-C | Apo Al | Apo CIII | TC | LDL-C | TG | Apo B | HDL-C | Apo Al | Apo CIII |
| Xba I       | 0.094 | 0.061 | 0.023 | 0.095 | 0.081 | 0.075 | -0.043 | 0.050 | 0.010 | 0.005 | -0.102 | -0.150 | 0.007 |
| EcoR I      | 0.033 | 0.027 | -0.002 | 0.023 | 0.077 | 0.108 | 0.005 | 0.146 | 0.147 | 0.018 | 0.118 | 0.019 | 0.066 | 0.036 |
| Sac I       | 0.015 | 0.047 | 0.103* | 0.105* | -0.167 | -0.078 | 0.100* | -0.064 | -0.105* | 0.001 | -0.039 | 0.005 | -0.009 | 0.082 |
| Msp I       | -0.033 | -0.083 | -0.092 | -0.067 | 0.188 | 0.083 | -0.084 | 0.043 | 0.066 | -0.003 | 0.021 | 0.031 | 0.015 | -0.068 |

*: p < 0.010; †: 0.010 ≤ p < 0.050; ††: 0.050 ≤ p < 0.100. n = number of subject.

RFLP = restriction fragment length polymorphism. TC = total cholesterol; LDL-C = low density lipoprotein cholesterol; HDL-C = high density lipoprotein cholesterol; TG = triglycerides; Apo = apolipoprotein.

0.367 and 0.296–0.396 for Sac I and 0.393–0.469 and 0.388–0.500 for Msp I RFLPs. No marked gender- and age-difference was observed in the allele frequency of any RFLP.

As for all of the four RFLPs, the genotype frequency distribution in each age group by sex was close to or not significantly different from the Hardy-Weinberg prediction (all of χ² < 6.00, d.f. = 2, p > 0.05)

Associations of RFLPs with Serum Lipids and Apolipoproteins

The results of the ANOVA are shown in Table 4. Xba I RFLP was not associated with variation in any biochemical trait, but suggestively (0.05 ≤ p < 0.10) associated with serum HDL-C and apo A1 in women. In women, the genotype for EcoR I RFLP was significantly associated with serum TC, LDL-C, and apo B. The genotype for Sac I RFLP was significantly associated with serum TG and suggestively associated with serum HDL-C and apo CIII in men and with serum LDL-C and apo CIII in women. Msp I RFLP was significantly related to serum apo CIII and suggestively to serum apo B in men. (Another ANOVA was done for the association of Xba I or EcoR I RFLPs with serum traits, in which the minor and heterozygous genotypes were combined. As presented in Appendix 1, the results were almost similar to those of non-combined analyses, Table 4).

As shown in Table 5, Spearman correlation coefficients were relatively small between RFLPs and serum traits with the absolute value less than 0.2.
Table 6. Allele frequencies of RFLPs at the apo B and Al-CIII gene loci among different populations (reviews).

| Gene | RFLP | Frequency (cutting) | Population (n) | Age (years) | Author | Reference |
|------|------|---------------------|----------------|-------------|--------|-----------|
| B    | Xba I| 0.49 (+)            | Caucasians (404) | 52–58       | Marshall, HW; et al. | 13       |
|      | Xba I| 0.50 (+)            | Caucasians (84)  | 58±10       | Hegele, RA; et al.   | 14       |
|      | Xba I| 0.53 (+)            | Caucasians (122) | 73±6*       | Genest, JJ, Jr; et al. | 15       |
|      | Xba I| 0.52 (+)            | French (309)     | 3–18        | Hallman, DM; et al.  | 16       |
|      | Xba I| 0.41–0.44 (+)       | Finns (307)      |             | Aalto-Setala, K; et al. | 17       |
|      | Xba I| 0.29 (+)            | South Asians (107) | 40–69*      | Renges, HH; et al.   | 18       |
|      | Xba I| 0.10 (+)            | Javanese (205)   | 1–80        | Gajra, B; et al.     | 19       |
|      | Xba I| 0.09 (+)            | Chinese (221)    | 25–60       | Saha, N; et al.      | 20       |
|      | Xba I| 0.04 (+)            | Japanese (107)   | 19–80       | Aburatani, H; et al. | 21       |
|      | EcoRI| 0.11 (−)            | Caucasians (84)  | 58±10       | Hegele, RA; et al.   | 14       |
|      | EcoRI| 0.18 (−)            | Caucasians (404) | 52–58       | Marshall, HW; et al. | 13       |
|      | EcoRI| 0.21 (−)            | Caucasians (122) | 73±6*       | Genest, JJ, Jr; et al. | 15       |
|      | EcoRI| 0.20 (−)            | French (309)     |             | Hallman, DM; et al.  | 16       |
|      | EcoRI| 0.11 (−)            | South Asians (46) | 40–69*      | Renges, HH; et al.   | 18       |
|      | EcoRI| 0.06 (−)            | Javanese (205)   | 1–80        | Gajra, B; et al.     | 19       |
|      | EcoRI| 0.08 (−)            | Chinese (221)    | 25–60       | Saha, N; et al.      | 20       |
|      | Al-CIII| 0.03 (−)         | Caucasians (52)  | 20–50       | Paul, H; et al.      | 22       |
|      | Al-CIII| 0.08 (−)          | Caucasians (145) | 50±7        | Ordovas, JM; et al.  | 23       |
|      | Al-CIII| 0.08 (−)          | Caucasians (404) | 52–58       | Marshall, HW; et al. | 13       |
|      | Al-CIII| 0.11 (−)          | Caucasians (231) |             | Thompson, EA; et al. | 24       |
|      | Al-CIII| 0.05 (−)          | Mediterranean (129) |            | Antonarakis, SE; et al. | 25       |
|      | Al-CIII| 0.04 (−)          | U.S. Blacks (75) |             | Antonarakis, SE; et al. | 25       |
|      | Al-CIII| 0.13 (−)          | Blacks (62) (U.S.) |           | Thompson, EA; et al. | 24       |
|      | Al-CIII| 0.26 (−)          | Blacks (27)      | 20–50       | Paul, H; et al.      | 22       |
|      | Al-CIII| 0.46 (−)          | Indian Asians (23) | 20–50       | Paul, H; et al.      | 22       |
|      | Al-CIII| 0.22 (−)          | Chinese (53)     |             | Meng, XW; et al.     | 26       |
|      | Al-CIII| 0.40 (−)          | Japanese (82)    | 20–62       | Satoh, J; et al.     | 27       |
|      | Al-CIII| 0.44 (−)          | Japanese (68)    |             | Thompson, EA; et al. | 24       |
|      | Al-CIII| 0.45 (−)          | Japanese (75)    |             | Rees, A; et al.      | 28       |
|      | Al-CIII| 0.56 (−)          | Japanese (27)    | 20–50       | Paul, H; et al.      | 22       |
|      | Al-CIII| 0.01 (+)          | Caucasians (92)  | 20–50       | Paul, H; et al.      | 22       |
|      | Al-CIII| 0.07 (+)          | Caucasians (404) | 52–58       | Marshall, HW; et al. | 13       |
|      | Al-CIII| 0.08 (+)          | Caucasians (145) | 50±7        | Ordovas, JM; et al.  | 23       |
|      | Al-CIII| 0.09 (+)          | Caucasians (366) |             | Thompson, EA; et al. | 24       |
|      | Al-CIII| 0.09 (+)          | Mediterranean (129) |             | Antonarakis, SE; et al. | 25       |
|      | Al-CIII| 0.06–0.11 (+)      | Finns (307)      | 3–18        | Aalto-Setala, K; et al. | 17       |
|      | Al-CIII| 0.05 (+)          | U.S. Blacks (75) |             | Antonarakis, SE; et al. | 25       |
|      | Al-CIII| 0.13 (+)          | Blacks (53) (U.S.) |           | Thompson, EA; et al. | 24       |
|      | Al-CIII| 0.27 (+)          | Blacks (28)      | 20–50       | Paul, H; et al.      | 22       |
|      | Al-CIII| 0.19 (+)          | Indian Asians (24) | 20–50       | Paul, H; et al.      | 22       |
|      | Al-CIII| 0.17 (+)          | Chinese (45)     |             | Meng, XW; et al.     | 26       |
|      | Al-CIII| 0.33 (+)          | Japanese (75)    |             | Rees, A; et al.      | 28       |
|      | Al-CIII| 0.34 (+)          | Japanese (82)    | 20–62       | Satoh, J; et al.     | 27       |
|      | Al-CIII| 0.35 (+)          | Japanese (34)    | 20–50       | Paul, H; et al.      | 22       |
|      | Al-CIII| 0.37 (+)          | Japanese (68)    |             | Thompson, EA; et al. | 24       |

*: Only male subjects were employed.
DISCUSSION

The levels of serum total cholesterol for both men and women in H-Y district are almost the same as those of the whole of Japan according to the Japanese National Survey of Circulatory Disorders, 1990\(^{29}\). For women, the average values of serum HDL-C in H-Y district were 0.078 mmol/L (3 mg/dL) lower than those in whole Japan, although there was no difference for men. Therefore, the subjects of the present study did not appear to differ markedly from the average Japanese.

The rare allele frequencies of Xba I (X+) and EcoR I RFLPs (E-) in H-Y district (Table 3) were much lower than those in American and European populations (Table 6\(^{13-28}\)). The median value of the frequencies (the range from the lowest to the highest values) of X+ from the Caucasian populations was 0.49 (0.41–0.53)\(^{13-17}\) and that of E- was 0.18 (0.11–0.21)\(^{13-16}\), although the frequencies observed in the present study were almost the same as those from another Japanese population\(^{21}\), 0.04 for X+ and a Chinese population\(^{29}\), 0.09 for X+ and 0.08 for E-.

On the other hand, the frequencies of Sac I (S+) and Msp I RFLPs (M-) were four times as high as those in Caucasian populations, 0.08 (0.01–0.11) for S+ and 0.08 (0.03–0.11) for M-\(^{13,17,22-25}\) and 0.08 (0.00–0.11) for M-\(^{13,22-25}\) and American black populations, 0.09 (0.05–0.13)\(^{24,25}\) and 0.08 (0.04–0.13)\(^{24,25}\). The frequencies in other Japanese populations, 0.35 (0.33–0.37) for S+\(^{22,24,27,28}\), and 0.45 (0.44–0.56) for M-\(^{22,24,27,28}\), and those in a Chinese population, 0.17 and 0.22\(^{29}\), were not different from ours.

According to the Japanese National Nutrition Survey\(^{29}\), fat intake was 20–30 g/day and the P/S ratio of the diet (P: polyunsaturated fat, S: saturated fat) was 1.0–1.5 during the national privation period before 1960. Fat intake increased during the high economic growth period from 1960 to 1975, reached the level of 55–60 g/day in 1975, and was stabilized during the low economic growth period from 1975 to 1990. Although the level of fat intake is still much lower and the P/S ratio is much higher in current Japanese than in Europeans and Americans\(^{20-32}\), there is no marked difference in the average level of serum cholesterol between Japan\(^{12}\) and the US\(^{20}\) recently. Thus, from an ecological point of view, this fact might be partially explained by genetic susceptibility of serum cholesterol to dietary fat exposures. Xba I and EcoR I RFLPs, of which allele frequencies are extremely different between these two populations, are likely to be related to this kind of genetic susceptibility. There are some dietary manipulation studies which support this hypothesis\(^{24-30}\). The frequencies of some genotypes, e.g. X−X− which might be associated with the high sensitivity to high saturated fatty acids and cholesterol diet\(^{37}\), were extremely high in the Japanese population.

Xba I RFLP was not significantly correlated to any serum trait in both sexes in the present study, but fourteen\(^{17,18,20,39-49}\) of the sixteen studies\(^{17,18,20,21,39-50}\) reported the significant relationship with serum TC, LDL-C, TG, apo B, HDL-C or apo AI in non-Japanese populations. Our result agreed with the other study in the Japanese population\(^{51}\). EcoR I RFLP was significantly correlated to serum TC, LDL-C and apo B only in women. On the other hand, the positive, negative and non-association took the nearly equal share in the number of the literatures\(^{20,43,45,51-55}\) (Appendix 2).

Sac I and Msp I RFLPs were significantly or suggestively associated with serum TG, HDL-C, apo B, LDL-C or apo CIII in men or women. Some studies suggested an association of Sac I with TG positively\(^{23,56-58}\) and with HDL-C inversely\(^{57,58}\), but others did not\(^{59}\). The persons with M+M+ genotype of Msp I RFLP tended to have higher level of serum HDL-C or apo AI than those with M−M− genotype\(^{57,60}\) (Appendix 2).

Thus, we can not conclude that the four RFLPs selected in the present study are genetically strong factors for the levels of serum lipids and apolipoproteins, although the statistical associations of some RFLPs with serum traits were observed.

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### Appendix 1. Association of genotypes with serum lipids (mmol/L) or apolipoprotein (mg/dL) according to the ANOVAs, age-adjusted, minor homozygosity and heterozygosity combined.

| RFLP | Genotype | n  | TC      | LDL-C   | Apo B   | TG       | HDL-C   | Apo A I   | Apo C III |
|------|----------|----|---------|---------|---------|----------|---------|-----------|-----------|
| Xba I|        |    |         |         |         |          |         |           |           |
|      | - -      | 271| 4.98±0.06| 3.13±0.06| 97.52±1.67| 8.51±1.07| 1.31±0.02| 149.09±1.66| 12.82±1.02|
|      | - + / + + | 22 | 5.41±0.20| 3.39±0.20| 109.53±5.85| 11.75±1.32| 1.27±0.07| 152.25±5.85| 14.79±1.07|
|      | p        |    | 0.044   | 0.193   | 0.050   | 0.264   | 0.301   | 0.222     | 0.049     |
| EcoR I|        |    |         |         |         |          |         |           |           |
|      | - - / + + | 40 | 4.89±0.15| 3.07±0.14| 98.37±4.39| 8.91±1.23| 1.21±0.05| 144.15±4.38| 12.88±1.05|
|      | + +      | 253| 5.03±0.06| 3.16±0.06| 98.71±1.74| 8.71±1.10| 1.29±0.02| 151.11±1.74| 12.97±1.02|
|      | p        |    | 0.409   | 0.557   | 0.989   | 0.860   | 0.177   | 0.141     | 0.904     |

### Appendix 1. Continued

| RFLP | Genotype | n  | TC      | LDL-C   | Apo B   | TG       | HDL-C   | Apo A I   | Apo C III |
|------|----------|----|---------|---------|---------|----------|---------|-----------|-----------|
| Xba I|        |    |         |         |         |          |         |           |           |
|      | - -      | 361| 5.44±0.05| 3.52±0.05| 99.76±1.30| 6.76±1.07| 1.39±0.02| 159.15±1.32| 12.13±1.02|
|      | - + / + + | 19 | 5.39±0.23| 3.38±0.20| 102.10±5.68| 10.47±1.29| 1.19±0.07| 146.25±5.76| 12.02±1.07|
|      | p        |    | 0.853   | 0.698   | 0.689   | 0.104   | 0.042   | 0.039     | 0.891     |
| EcoR I|        |    |         |         |         |          |         |           |           |
|      | - - / + + | 55 | 5.10±0.13| 3.22±0.12| 92.65±3.31| 6.76±1.15| 1.36±0.04| 150.97±3.37| 11.75±1.05|
|      | + +      | 325| 5.49±0.06| 3.57±0.05| 101.10±1.36| 6.92±1.07| 1.39±0.02| 156.92±1.39| 12.30±1.02|
|      | p        |    | 0.007   | 0.007   | 0.019   | 0.856   | 0.508   | 0.103     | 0.405     |

ANOVA = analysis of variance. RFLP = restriction fragment length polymorphism. SE = standard error. n = number of subject. TC = total cholesterol; LDL-C = low density lipoprotein cholesterol; HDL-C = high density lipoprotein cholesterol; TG = triglycerides; Apo = apolipoprotein.
Appendix 2  Associations of RFLPs with serum lipids and apolipoproteins in selected populations (reviews).

| Gene | RFLP (site) | Association | Population (age) | Author | Reference |
|------|-------------|-------------|------------------|--------|-----------|
| B    | Xba I(+)    | LDL-C ↑     | Fins (9–21)      | Aalto-Setala, K; et al. | 17 |
| Xba I(+) | HDL-C ↓, HDL-C/TC ↓ | South Asian men (40–69) | Renges, HH; et al. | 18 |
| Xba I(+) | HDL-C ↓, apoAI ↓, apoAII ↓ | Chinese (25–60) | Saha, N; et al. | 20 |
| Xba I(+) | TG ↑, TC ↑ | White men (50–69) | Law, A; et al. | 39 |
| Xba I(+) | TC ↑, apoB ↑ | Norwegian (309) | Berk, K; et al. | 40 |
| Xba I(+) | TC ↑ | White | Talmud, P; et al. | 41 |
| Xba I(+) | TC ↑, apoB ↑ | Danish men (45) | Hansen, PS; et al. | 42 |
| Xba I(+) | LDL-C ↑, apoB ↑ | Swedish (35–45) | Peacock, R; et al. | 43 |
| Xba I(+) | TC ↓, LDL-C ↓, apoB ↑ | Israeli (25–64) | Friedlander, Y; et al. | 44 |
| Xba I(+) | TC ↑, LDL-C ↑, apoB ↑ | Austrian | Paulweber, B; et al. | 45 |
| Xba I(+) | TC ↑, LDL-C ↑, apoB ↑ | Spanish (19–65) | Villeva, E; et al. | 46 |
| Xba I(+) | TC ↑, Fins (3–18) | Finnish | Lehtimaki, T; et al. | 47 |
| Xba I(+) | TC ↑, LDL-C ↑ | White | Houlston, RS; et al. | 49 |
| Xba I(+) | NS | Japanese | Aburata, H; et al. | 21 |
| Xba I(+) | NS | Swedish | Darnfors, C; et al. | 50 |
| B    | EcoR I(−)   | TC ↑, VLDL-C ↑, TG ↑ | Austrian | Paulweber, B; et al. | 45 |
| EcoR I(−) | LDL-C ↑, VLDL-C ↑, TG ↑ | Danish | Tybjaerg-Hansen, A; et al. | 52 |
| EcoR I(−) | TC ↑, apoB ↑ | White men | Pouliot, MC; et al. | 53 |
| EcoR I(−) | LDL-C ↓ | Spanish (35–49) | Houlston, RS; et al. | 54 |
| EcoR I(−) | TC ↓, LDL-C ↓, apoB ↓ | South Italian (50–60) | De Benedictis, G; et al. | 55 |
| EcoR I(−) | NS | Swedish (25–60) | Saha, N; et al. | 20 |
| EcoR I(−) | NS | Danish men (40–69) | Tybjaerg-Hansen, A; et al. | 56 |
| EcoR I(−) | NS | White | Mendis, S; et al. | 51 |
| A1-CIII | Sac I(+) | LDL-C ↑ | Fins (9–21) | Aalto-Setala, K; et al. | 17 |
| Sac I(+) | TG ↑ | White (40–59) | Ordovas, JM; et al. | 23 |
| Sac I(+) | TG ↑ | Danish men (40–69) | Tybjaerg-Hansen, A; et al. | 56 |
| Sac I(+) | HDL-C ↓, TG ↑ | White | Hegele, RA; et al. | 57 |
| Sac I(+) | HDL-C ↓, TG ↑ | White | Anderson, RA; et al. | 58 |
| Sac I(+) | NS | White and Black (45–80) | Kasturi, R; et al. | 59 |
| Sac I(+) | HDL-C ↓, TG ↑ | Hutterite Brethren | Hegele, RA; et al. | 62 |
| A1-CIII | Msp I(+) | apoAI ↑ | White | Hegele, RA; et al. | 57 |
| Msp I(+) | HDL-C ↑ | Black men (35–74) | Anderson, RA; et al. | 60 |

(1)  RFLP = restriction fragment length polymorphism.
(2)  TC = total cholesterol; VLDL-C = very low density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; HDL-C = high density lipoprotein cholesterol; TG = triglycerides; Apo = apolipoprotein.
(3)  ↑: positive association; ↓: negative association; NS: non significant association.