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ORIGINAL ARTICLE

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A promoter SNP (–1323T>C) in G-substrate gene (*GSBS*) correlates with hypercholesterolemia

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Abstract Factors predisposing to the phenotypic features of higher total cholesterol (TC) have not been clearly defined. Here we report an association between a promoter SNP (−1323T > C) in G-substrate gene (*GSBS*) and TC levels in 368 adult individuals from an east-central area of Japan. Age and gender-adjusted levels of LDL-cholesterol, TG, TC, and HDL-cholesterol were analyzed. When we separate the subjects into two genotypic groups regarding T allele, those who bear the T allele had significantly higher plasma TC levels than the others who lack the T allele (mean; 239.6 mg/dl vs. 210.6 mg/dl; $p = 0.003$; Mann–Whitney test). Of the 341 individuals with the T allele, approximately 80% individuals presented with hypercholesterolemia, whereas only 44% were hypercholesterolemic among the 27 individuals without the T allele ($p = 0.0001$). These results indicate a significant elevating effect of plasma TC levels by a SNP in the putative regulatory region of the G-substrate gene in our studied population. These data suggest that genetic variation at the G-substrate gene may be one of the determinants for plasma lipoprotein levels.

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Introduction

Accumulating evidences derived from clinical, epidemiological and experimental studies suggest that lipid and lipoprotein concentrations in plasma reflect the influence of complex genetic loci, at least in part (Zannis and Breslow 1985); defects are known only for some classical types of hyperlipoproteinemia that affect members of families following Mendelian traits. However, the genetic mechanisms responsible for most types of familial dyslipoproteinemia appear to be complex, not monogenic.

Hyperlipoproteinemia is one of the most important predictors of cardiovascular disease, determined by genetic risk factors as well as environmental factors (Hegele, 2001; Cullen et al. 1997). Clarification of the genetic risk factors is essential for diagnosis, prevention, and early effective treatment of hyperlipidemias. In addition, if the relevant genes were identified, the pathogenesis of these diseases would be explained by the variation of those genes or adjacent genes. Several gene polymorphisms have previously been associated with plasma lipoprotein abnormalities (reviewed in Goldstein et al. 1995). However, those are only a part of all the determinants.

In the course of serial investigations of population genetics for hyperlipoproteinemia in a cohort from an area located in east-central Japan, we recognized a correlation between lipoprotein variations and polymorphism of *GSBS* locus. Here we focused on the analysis of the potential effect of genetic variation in a locus encoding G-substrate gene (*GSBS* gene), investigating the correlation of plasma lipoprotein profile with the genetic variation of this gene.

Materials and Methods

Subjects

Subjects were obtained from the participants of the cohort study from an area located in east-central Japan that was originally carried out spontaneously with health check screening. The entire 22,228 participants for the health check were initially screened by distinctive criteria consist of two issues that define individuals harboring hyperlipidemic risks (T-Chol ≥ 250, or HDL-C ≤ 35 mg/dl). From about 2,000 subjects satisfying these criteria, 368 individuals were randomly selected for the present study. All the selected participants were volunteers who gave their written informed consent prior to this study, which was approved by the Institutional Review Boards of the Research Consortium. Physical and clinical profiles of these subjects (ages, male to female ratios, body mass indices, and plasma lipoprotein and lipid levels) are indicated in Table 1. None of the selected participants had medical complications or was undergoing treatment for conditions known to affect plasma lipoprotein levels, such as pituitary disease, hypo- or hyperthyroidism, diabetes mellitus, liver disease, and renal disease. None was receiving anti-hyperlipidemic therapy. The selected participants visited lipid clinics for detailed examination of their lipoprotein profile and other clinical parameters. Blood was collected after 12–16 hours of fasting in each participant. Genomic DNA was extracted as previously described (Shinohara et al. 2001).

Measurement of lipoproteins

Lipid and lipoprotein concentrations were measured by procedures described previously (Hattori et al. 2002), i.e., plasma cholesterol and triglyceride concentrations were assayed enzymatically, and concentrations of HDL cholesterol were determined by the $MgCl_2$ dextran precipitation method. LDL-cholesterol concentration was calculated by subtracting HDL-cholesterol level from the fraction of both LDL-cholesterol and HDL-cholesterol as described elsewhere (Ishii et al. 2002).

Genotyping for single nucleotide polymorphism (SNP)

PCR amplification of the polymorphism at the *GSBS* locus was carried out using a condition described previously (Nakazawa et al. 2001; Harada et al. 2001); primer sequences used are as follows: forward primer: 5′-TGCGTGGCTTCAAATGATTA C-3′; reverse primer: 5′-ACAGGTCCAG TCCTGCTGAC-3′. The surrounding sequence of the amino acid-substituting SNP in the *GSBS* gene, i.e., polymorphic nucleotides T/C substitution at the −1323 position as well as primer sequences and experimental conditions were obtained from published reports (Haga et al. 2002). SNP genotyping was performed by Invader assay (Third Wave Technologies. Madison, WI) using PCR products of the flanking sequence and probes of the Invader assay designed and synthesized by the supplier (Ohnishi et al. 2001).

Table 1

	T (+)	T (−)	*p* value[a]
Number	341	27	–
Gender (M/F)	140/201	17/10	NS
Age (years)	60.7 ± 8.8	64.0 ± 9.5	NS
BMI (kg/m^2)	26.7 ± 3.7	23.7 ± 2.7	NS
TG (mg/dl)	178.7 ± 152.9	176.8 ± 102.9	NS
TC (mg/dl)	239.6 ± 35.1	210.6 ± 44.9	0.003
HDL-C (mg/dl)	50.5 ± 16.5	41.1 ± 21.7	0.037
LDL-C (mg/dl)	152.6 ± 31.1	134.8 ± 40.3	NS

[a] *p* value was calculated by Mann-Whitney test, except for gender. $\chi 2$ test was conducted for distribution analysis of gender. NS; not significant. Values are expressed in means ± SD

Statistical analysis

Plasma levels of lipoproteins were adjusted by gender and ages of the subjects, using standard data obtained from 11,994 individuals of a 2001 cohort study from the general Japanese population. Coefficients for skewness and kurtosis were calculated to test deviation from a normal distribution. As the clinical and biochemical traits in each genotypic group were not always distributed normally, we applied the non-parametric Mann–Whitney test or an analysis of variance (ANOVA) with linear regression analysis as a post-hoc test ($p < 0.05$) to compare those traits among groups divided by a single SNP (Ota et al. 2001). Fisher's exact test was used to compare differences in the prevalence of hypertriglyceridemia among the population. Chi-square tests were used to detect the Hardy–Weinberg equilibrium.

Results

To carry out a correlation analysis of the potential effect of the regulatory SNP (−1323T/C) in the *GSBS* gene, 368 individuals were genotyped. The analyzed subjects were selected from participants for a cohort of an area initially screened by criteria that define individuals harboring hyperlipidemic risks. Due to initial screening for the health check assessment, the basal level of each value was a little higher than that of data from the general Japanese population (Table 1). However, the average differences were less than 0.5 × SD in general. When the subjects were categorized by genotype into three groups (27 homozygous minor C allele carriers, 139 heterozygous carriers and 202 homozygous T allele carriers), no deviation of genotype frequencies from the Hardy-Weinberg equilibrium were observed ($p = 0.90$; Chi^2-test).

Distribution and mean values of TC, TG, LDL-cholesterol and HDL-cholesterol were analyzed among these groups. Although the LDL-cholesterol and TG levels among these three groups were almost the same, TC and HDL-cholesterol levels were significantly different. Plasma TC levels of homozygous minor C allele carriers ($n = 27$), heterozygous carriers ($n = 139$) and homozygous T allele carriers ($n = 202$) were 210.6 ± 44.9 mg/dl, 238.7 ± 36.6 mg/dl, and 240.1 ± 34.0 mg/dl respectively, indicating a co-dominant TC lowering effect of minor C allele ($r = 0.15$, $p = 0.004$). In contrast, plasma HDL-cholesterol levels were 41.1 ± 21.7 mg/dl, 49.43 ± 15.9 mg/dl, and 51.3 ± 16.9 mg/dl. As the subjects carrying minor C allele were rare ($n = 27$), suitable categorization of the subjects was re-considered. We thus separated the subjects into two groups, those who bear the T allele (−1323T > C) and those who lack the T allele; the former subjects had significantly higher plasma TC levels than the latter (mean ± SD 239.6 ± 35.1 mg/dl vs. 210.6 ± 44.9 mg/dl; $p = 0.003$). In contrast, the former subjects had significantly higher HDL-cholesterol levels than the latter (mean ± SD

50.5 ± 16.5 mg/dl vs. 41.1 ± 21.7 mg/dl; $p = 0.037$) (Table 1).

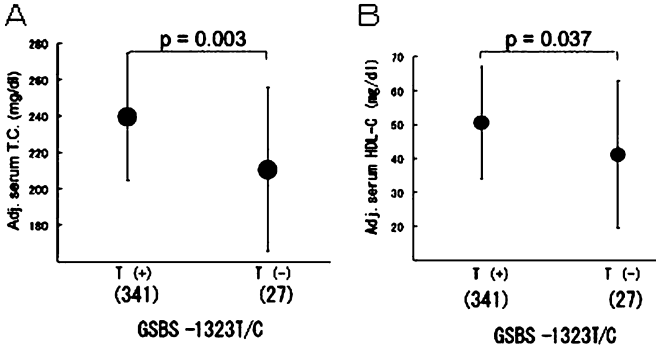
Since the data presented here suggests involvement of the *GSBS* locus, in expression of the hypercholesterolemic phenotype in this population, we correlated manifestation of hypercholesterolemia in respect of the presence or absence of T or C alleles at the −1323 position in the regulatory region of *GSBS*. Hypecholesterolemia was defined as a plasma cholesterol level above the reference values after age-, sex-adjustment (TC > 220 mg/dl). This criterion is based on a recommended pre-clinical level for hyperlipidemic individuals who need to be alerted of a high risk for ischemic heart disease. It classified 283 subjects as having hypercholesterolemia among the study population (368 individuals). Of the 341 individuals with theT allele, 271 presented with hypercholesterolemia (79%), whereas only 12 did so among 27 individuals without the T allele (44%) The difference of the distribution was significant when using the Fisher's exact test ($p = 0.0001$; Relative risk = 1.79; 95% CI: 1.17–2.74) (Fig. 1).

Discussion

Multiple environmental and genetic factors appear to influence phenotypic variation of the plasma lipoprotein profile. Life-style variations among individuals in physical exercise, control of food-calorie intake, proper understandings and awareness to the disease and compliance for treatments including medications, should influence lipoprotein variations among individuals. In addition, unidentified genetic modifiers may cause variability among the individuals.

Hypercholesterolemia is one of the important risk factors of ischemic heart disease (Matsuzawa, 1995; Norioka, 2000). In this study, we showed a tendency that hypercholesterolemic patients had a major variant allele of *GSBS* gene (−1323 T > C). An association study revealed elevating effect of plasma total cholesterol and HDL-cholesterol by the *GSBS* −1323 T allele. The high prevalence (79%) of hypercholesterolemia (mean ± SD; 240.1 ± 34.0 mg/dl and 238.7 ± 36.6 mg/dl) among the individuals with this genotype (−1323 T/T and T/C) is in contrast to the scarceness (44%) of hypercholesterolemia (means ± SD 210.6 ± 44.9 mg/dl) among the rest of the study subjects (−1323 C/C). It might be interesting to test whether distribution of familial combined hyperlipidemia patients among the subject groups of our cohort is different, although insufficient information of the subjects did not allow us to analyze it at this point. Nevertheless, our results indicated that *GSBS* variation might modify the lipoprotein phenotype of plasma cholesterol and HDL-cholesterol.

The suspected link between functional changes of GSBS expression and plasma cholesterol metabolism was unexpected. *GSBS* was originally identified as a gene coding an endogenous substrate for cGMP-dependent protein kinase that exists in cerebellar Purkinje cells, and it is possibly involved in the induction of long-term depression (Endo et al. 1999). Phosphorylated G-substrate inhibits the catalytic subunit of native protein phosphatase-1 (PP-1) and phosphatase-2A (PP-2A). Like that, G-substrate regulates the activity of PP-1 and PP-2A and controls synaptic transmission (Hall, et al; 1999). If it would affect regulation of cholesterol metabolism in plasma, the *GSBS*-1323 T/C variation might bring about changes in the expression levels of GSBS result in considerable differences in metabolic function. The expression of *GSBS* in the hypothalamus may indicate that *GSBS* effects for food intake by the hypothalamo-pituitary-adrenal axis (Szeto, 2003). However, a possibility cannot be ruled out that this SNP marker may itself be in linkage disequilibrium with other unexamined functional variants localized in close proximity to the *GSBS*-1323 T/C. Contribution of other one non-synonymous coding SNPs in the *GSBS* gene may have to be tested that are archived in the dbSNP database, although a statistically significant contribution of rare SNPs is less likely. A true mechanistic basis for the associations should be clarified (Shastry, 2002). Functional studies would be required for ruling out the other possibilities. In addition, it would be important to confirm the association of geographically distinct populations and other ethnic groups including domestically different cohorts as well as those from other countries. Those studies would be conducted in the future.



C

GSBS -1323T>C	T-Allele (+)	T-Allele (-)	Total
High TC (percentage)	271 (79%)	12 (44%)	
Normal (percentage)	70 (21%)	15 (56%)	
Total (percentage)	341 (100%)	27 (100%)	368

Fig. 1A–C Comparisons of plasma lipoprotein levels among genotypically determined groups according to the GSBS-1323T/C variation. **A** Adjusted plasma total cholesterol levels were compared. **B** Adjusted plasma HDL-C levels were compared by Mann–Whitney test ($p < 0.01$). Closed circles represent mean values. Error bars represent the standard deviation (SD). **C** Distribution analysis of hypercholesterolemic patients among GSBS −1323 T/C classified-genotypes; −1323 T(+)/(−). The 2×2 table was analyzed by Fisher's exact test ($p < 0.05$). Clinical phenotypes were defined by adjusted plasma total cholesterol (TC) levels above the 220 mg/dl

In summary, we noted association between a putative regulatory region variation of the G-substrate (*GSBS*) gene and hypercholesterolemia in 368 subjects from an area of east-central region of Japan. Given our genetic results, we expect that the effects of multiple genes, both additive and interactive (Lalouel et al. 2001; Takada et al. 2002), will eventually prove to be responsible for many cases of common, inherited, mixed dislipidemias.

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