Table 4. Serum LDL-cholesterol (mg /dl) for each 10-year group in Japanese

Age	all			men			women		
	N	mean	S.D.	N	mean	S.D.	N	mean	S.D.
0-9	154	104	22	70	101	22	84	106	22
10-19	162	103	24	51	101	21	111	104	25
20-29	713	97	24	240	105	26	472	93	22
30-39	751	112	29	484	119	29	267	101	25
40-49	1,179	121	30	750	124	31	429	116	29
50-59	1,243	127	30	733	125	30	510	130	30
60-69	726	129	31	387	124	30	338	135	29
70-79	246	126	28	117	120	27	129	130	28
80-89	32	123	29	10	113	27	22	127	30
Total	5,206	118	31	2,842	121	30	2,362	115	31

Table 5. Serum RLP-cholesterol (mg /dl) for each 10-year group in Japanese

Age	all			men			women		
	N	mean	S.D.	N	mean	S.D.	N	mean	S.D.
0-9	154	1.9	0.6	70	2.0	0.6	84	1.9	0.7
10-19	161	2.5	1.2	51	2.5	1.1	110	2.5	1.3
20-29	712	3.5	3.1	240	4.5	4.2	471	2.9	2.2
30-39	762	5.0	6.0	493	6.2	6.9	269	2.7	2.6
40-49	1,211	5.2	7.7	774	6.2	8.7	437	3.2	4.9
50-59	1,322	4.8	6.2	791	5.2	7.4	531	4.3	3.7
60-69	662	4.6	7.3	363	5.1	9.4	298	4.1	3.5
70-79	206	4.1	3.7	98	4.3	4.4	108	4.0	2.9
80-89	28	3.7	2.5	8	2.4	1.6	20	4.2	2.7
Total	5,218	4.5	6.2	2,888	5.4	7.6	2,328	3.4	3.5

Table 6. Fasting glucose (mg/dl) for each 10-year group in Japanese

Age	all			men			women		
	N	mean	S.D.	N	mean	S.D.	N	mean	S.D.
0-9	158	88	7	74	88	7	84	87	6
10-19	170	85	6	57	87	7	113	85	6
20-29	996	88	16	340	89	20	655	87	13
30-39	1,281	92	15	886	93	14	395	90	18
40-49	2,865	95	18	2,018	97	19	847	90	12
50-59	2,909	99	20	2,002	101	20	907	94	19
60-69	1,489	98	21	752	102	25	737	95	15
70-79	531	98	16	257	99	16	274	97	15
80-89	52	103	27	22	104	36	30	102	20
Total	10,451	95	19	6,408	98	20	4,042	92	16

Table 7. HbA1c for each 10-year group in Japanese

Age	all			men			women		
	N	mean	S.D.	N	mean	S.D.	N	mean	S.D.
0-9	155	4.7	0.2	72	4.7	0.2	83	4.7	0.2
10-19	171	4.7	0.3	58	4.7	0.3	113	4.6	0.3
20-29	1,147	4.6	0.4	374	4.6	0.6	772	4.6	0.3
30-39	1,261	4.7	0.5	871	4.7	0.5	390	4.7	0.4
40-49	2,536	4.9	0.6	1,844	4.9	0.7	692	4.8	0.5
50-59	2,676	5.1	0.7	1,879	5.1	0.7	797	5.1	0.7
60-69	1,141	5.2	0.8	614	5.3	0.9	527	5.2	0.6
70-79	443	5.3	0.7	209	5.3	0.7	234	5.4	0.8
80-89	52	5.4	0.8	22	5.4	1.0	30	5.3	0.6
Total	9,582	4.9	0.7	5,943	5.0	0.7	3,638	4.9	0.6

Table 8. Serum insulin (μU/ml) for each 10-year group in Japanese

Age	all			men			women		
	N	mean	S.D.	N	mean	S.D.	N	mean	S.D.
0-9	216	7	5	102	7	6	114	7	4
10-19	463	7	7	196	6	5	267	8	8
20-29	1,171	11	13	382	10	11	788	12	14
30-39	1,410	8	9	942	8	9	468	8	9
40-49	2,734	7	5	1,877	7	5	857	7	6
50-59	2,636	6	6	1,731	6	4	905	7	8
60-69	1,118	6	5	589	6	5	528	6	5
70-79	440	6	15	211	5	6	229	7	20
80-89	53	6	5	23	6	6	30	6	4
Total	10,241	7	8	6,053	7	6	4,186	8	10

Table 9. Serum uric acid (mg /dl) for each 10-year group in Japanese

Age	all			men			women		
	N	mean	S.D.	N	mean	S.D.	N	mean	S.D.
0-9	0	-	-	0	-	-	0	-	-
10-19	3	6.7	0.7	3	6.7	0.7	0	-	-
20-29	410	4.7	1.4	137	6.1	1.3	273	4.0	0.8
30-39	927	5.6	1.5	714	6.0	1.3	213	4.0	0.9
40-49	2,425	5.5	1.5	1,763	6.1	1.3	662	4.1	0.9
50-59	2,459	5.5	1.4	1,762	6.0	1.3	697	4.3	0.9
60-69	1,141	5.2	1.4	618	5.8	1.3	523	4.5	1.0
70-79	296	5.1	1.5	152	5.8	1.4	144	4.4	1.1
80-89	25	4.9	1.6	8	5.0	0.9	17	4.9	1.8
Total	7,686	5.4	1.4	5,157	6.0	1.3	2,529	4.3	1.0

ORIGINAL ARTICLE

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Hypertriglyceridemia associated with amino acid variation Asn985Tyr of the *RP1* gene

Abstract Factors predisposing to the phenotypic features of hypertriglyceridemia have not been clearly defined. Here we report an association between a missense coding region polymorphism Asn985Tyr in the retinitis pigmentosa 1 gene (*RP1*) and plasma triglyceride (TG) levels in 332 adult individuals from an east-central area of Japan. Age and gender-adjusted levels of LDL-cholesterol, TG, and HDL-cholesterol were analyzed. When we separate the subjects into two genotypic groups regarding this amino acid variation, those who lack the 985-Asn allele (asparagine at residue 985) had significantly higher plasma TG levels than the others who had at least one 985-Asn allele (mean: 175.8 mg/dl vs 123.3 mg/dl; $P = 0.0006$, Mann-Whitney test). Similarly, the former subjects had significantly lower HDL-cholesterol levels than the latter (mean: 48.0 mg/dl vs 53.8 mg/dl; $P = 0.038$). Of the 280 individuals without a 985-Asn allele, approximately half of the individuals presented with hypertriglyceridemia, whereas only a quarter were hypertriglyceridemic among 52 individuals with the 985-Asn allele ($P = 0.04$). Although this SNP marker may itself be in linkage disequilibrium with other unexamined functional variants within this locus, our data suggest that genetic variation at the *RP1* locus is one of the likely candidate determinants for plasma triglyceride and HDL-cholesterol metabolisms.

Keywords Retinitis pigmentosa 1 · Plasma triglyceride (TG) · HDL cholesterol (HDL-C) · Single nucleotide polymorphism (SNP) · Modifier gene

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Introduction

Accumulating evidence derived from clinical, epidemiological and experimental studies suggests 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 diseases, 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 clearly only a part of the all determinants.

In the course of serial investigations of population genetics for hyperlipoproteinemia in a cohort of an area located in east-central Japan, we recognized a correlation between lipoprotein variations and polymorphism of the *RP1* locus. Here we focused on the analysis of the potential effect of genetic variation in a locus encoding

the retinitis pigmentosa 1 gene (RP1), investigating the correlation of the plasma lipoprotein profile with the genetic variation of this gene.

Subjects and methods

Subjects

Subjects were obtained from the participants of the cohort study of an area located in east-central Japan that was originally carried out concurrently with health check screening. The entire 22,228 participants for the health check were initially screened by distinctive criteria consisting of two issues that define individuals harboring hyperlipidemic risks (T-Chol ≥250 mg/dl, or HDL-C ≤ 35 mg/dl). From about 2,000 subjects sufficing these criteria, 332 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 h 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) in the RP1 gene

PCR amplification of the polymorphism at the RP1 locus was performed using conditions described previously (Nakazawa et al. 2001; Harada et al. 2001); primer sequences used are as follows:

Table 1 Physical and clinical profiles of the subjects. The P value was calculated by Mann-Whitney test, except for gender; χ^2 test was conducted for distribution analysis of gender. Values are expressed in mean ± SD; NS not significant

	Asn (+)	Asn (−)	P value
Number	52	280	–
Gender (M/F)	16/36	125/155	NS
Ages (years)	60.3 ± 8.4	60.6 ± 10.2	NS
BMI (kg/m²)	24.7 ± 6.5	23.3 ± 3.5	NS
TG (mg/dl)	123.3 ± 89.8	175.7 ± 127.9	0.0006
TC (mg/dl)	234.2 ± 38.8	237.6 ± 36.9	NS
HDL-C (mg/dl)	53.8 ± 18.6	48.0 ± 15.6	0.04
LDL-C (mg/dl)	145.2 ± 82.1	184.6 ± 163.8	NS

forward primer, 5'-CCTGAGGCTATTGCTCATCATTC-3'; reverse primer, 5'-TAGGCAAAGGCCACAGGAG-3'. The surrounding sequence of the amino acid-substituting SNP in the RP1 gene, i.e. polymorphic nucleotides A or T in codons 985 (Aat/Tat), 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).

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 of the general Japanese population. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical traits in each genotypic group did not always distribute normally, we applied a 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 invoked to detect Hardy-Weinberg equilibrium.

Results

To carry out a correlation analysis of the potential effect of the amino acid substituting SNP, Asn985Tyr, in the RP1 gene, 332 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. Because of initial screening for the health check assessment, the basal level of each value was a little higher than that of data from the general population of Japanese (Table 1). However, the average differences were less than 0.5×SD in general. When the subjects were genotypically categorized into three groups (two homozygous minor 985-Asn allele carriers, 50 heterozygous carriers and 280 homozygous 985-Tyr allele carriers), no deviation of genotype frequencies from Hardy-Weinberg equilibrium was observed ($P = 0.99$, χ^2-test).

The distribution and mean values of TG, LDL-cholesterol and HDL-cholesterol were analyzed among these groups. Although the LDL-cholesterol levels among these three groups were almost the same, TG and HDL-cholesterol levels were significantly different. Plasma TG levels of homozygous minor 985-Asn allele carriers ($n = 2$), heterozygous carriers ($n = 50$) and homozygous 985-Tyr allele carriers ($n = 280$) were 114.4 ± 51.7 mg/dl, 123.7 ± 91.3 mg/dl, and 175.7 ± 127.9 mg/dl, respectively, indicating a co-dominant TG lowering effect of minor 985-Asn allele ($r = 0.15$, $P = 0.0056$). Similarly, plasma HDL-cholesterol levels were 41.8 ± 3.2 mg/dl, 54.4 ± 18.8 mg/dl, and 48.0 ± 15.6 mg/dl, where a dominant effect was assumed. Because the subjects carrying a minor 985-Tyr allele was rare ($n = 2$), suitable categorization of the subjects was

re-considered. We thus separated the subjects into two groups, those who lack the 985-Asn allele (asparagine at residue 985) and those who bear at least one 985-Asn allele, the former subjects had significantly higher plasma TG levels than the latter (mean ± SD: 175.8 ± 127.9 mg/dl vs 123.3 ± 89.8 mg/dl; $P = 0.0006$). Similarly, the former subjects had significantly lower HDL-cholesterol levels than the latter (mean ± SD: 48.0 ± 15.6 mg/dl vs 53.8 ± 18.6 mg/dl; $P = 0.038$) (Table 1).

Since the data presented here suggested involvement of the *RP1* locus in expression of the hypertriglyceridemic phenotype in this population, we correlated the manifestation of hypertriglyceridemia with respect to presence or absence of 985-Asn or 985-Tyr alleles of *RP1*. Hypertriglyceridemia was defined as plasma triglyceride level above the reference values after age and sex adjustment (TG > 150 mg/dl). This criterion is based on a recommended pre-clinical level of hyperlipidemic individuals who have to be alerted for high risk for ischemic heart disease. It classified 150 subjects as having hypertriglyceridemia among the study population consisting of 332 individuals. Of the 280 individuals without the 985-Asn allele, 136 presented with hypertriglyceridemia (49%), whereas only 14 did so among 52 individuals with 985-Asn allele (27%) The difference of the distribution was significant when tested by Fisher's exact test ($P = 0.04$; relative risk = 0.45; 95% confidence interval = 0.25–0.79) (Fig. 1).

Discussion

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

Hypertriglyceridemia is one of the important risk factors of ischemic heart disease (Matsuzawa 1995; Norioka 2000). In the present study, we showed a tendency that hypertriglyceridemic patients lacked a minor variant allele of *RP1* gene (985-Asn). An association study revealed an elevating effect of plasma total triglyceride and a lowering effect of HDL-cholesterol by the lack of this *RP1* 985-Asn allele. The high prevalence (49%) of hypertriglyceridemia (mean ± SD: 175.7 ± 127.9 mg/dl) among the individuals with this genotype (985-Tyr/Tyr) is in contrast to the scarceness (27%) of hypertriglyceridemia (mean ± SD: 123.7 ± 91.3 mg/dl and 114.4 ± 51.7 mg/dl) among the rest of the study subjects (985-Tyr/Asn and 985-Asn/Asn, respectively). It might be interesting to test if distribution of familial combined hyperlipidemia patients among the subject groups of our cohort is different, although insufficient information of the subjects did not allowed us to analyze it at this point. Nevertheless, our results indicated

Fig. 1A–C Comparisons of plasma lipoprotein levels among genotypically determined groups according to the RP1 Asn985Tyr variation. A Adjusted plasma HDL-cholesterol levels were compared. B Adjusted plasma triglyceride levels were compared by Mann-Whitney test (*P* < 0.01). Open circles represent mean values. Error bars represent the 95% confidence interval. C Distribution analysis of hypertriglyceridemic patients among *RP1* Asn985Tyr classified-genotypes: *985-Asn(+)/(−)*. The 2×2 table was analyzed by Fisher's exact test (*P* < 0.05). Clinical phenotypes were defined by adjusted plasma total triglyceride (*TG*) levels above 150 mg/dl
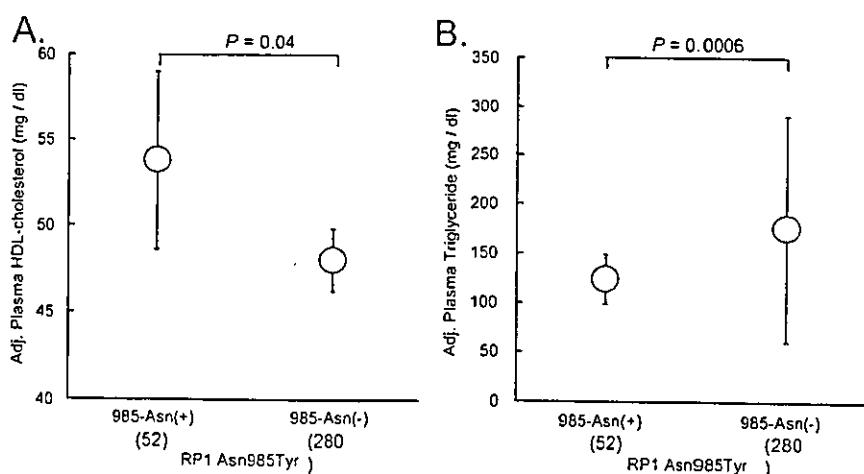
C. RP1 Asn958Tyr	985-Asn Allele		Total
	(+)	(-)	
.High TG (percentage)	14 (27%)	136 (49%)	
Normal TG (percentage)	38 (73%)	144 (51%)	
Total	52 (100%)	280 (100%)	332

that *RP1* variation might modify the lipoprotein phenotype of plasma triglyceride and HDL-cholesterol.

The suspected link between functional changes of RP1 protein and plasma triglyceride metabolism was unexpected. *RP1* was originally identified as a gene response to in vivo retinal oxygen levels in mouse model, and a causative mutation gene for a human autosomal dominant type of retinitis pigmentosa (Pierce et al. 1999; Sullivan et al. 1999). RP1 protein is a cytosolic protein that mainly exists in connecting cilia of retina photoreceptor cells, and was suggested to participate in transport of proteins or maintenance of cilial structure. Although the exact tissue distribution of the *RP1* gene product has not been clarified yet, Western blot analysis of previous study in mouse implied its existence in several types of tissues other than in retina, including skeletal muscle (Liu et al. 2002). If it affects the regulation of triglyceride metabolism in plasma, the Asn985-Tyr variation might bring about conformational changes of RP1 protein, resulting in considerable differences in metabolic function. The possibility cannot be ruled out, however, that this SNP marker may itself be in linkage disequilibrium with other unexamined functional variants localized in close proximity to Asn985Tyr. The contribution of another four non-synonymous coding SNPs in *RP1* gene that are archived in the dbSNP database may have to be tested, although a statistically significant contribution of rare SNPs is less likely. A true mechanistic basis for the associations needs to 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 at geographically distinct populations and other ethnic groups, including domestic different cohorts as well as those from other countries. Those studies will be conducted in the future.

In summary, we noted an association between amino acid variation of the retinitis pigmentosa 1 (*RP1*) gene and hypertriglyceridemia in 332 subjects from an area of the east-central region of Japan. Given our genetic results, we expect that the effects of multiple genes, both additive and interactive (Lalouel and Rohrwasser 2001; Takada et al. 2002), will eventually prove to be responsible for many cases of common, inherited, mixed dislipidemias.

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ORIGINAL ARTICLE

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

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).

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 $0.5 \times SD$ 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

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/m²)	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

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

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

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 $\pm$ 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 $\pm$ 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.
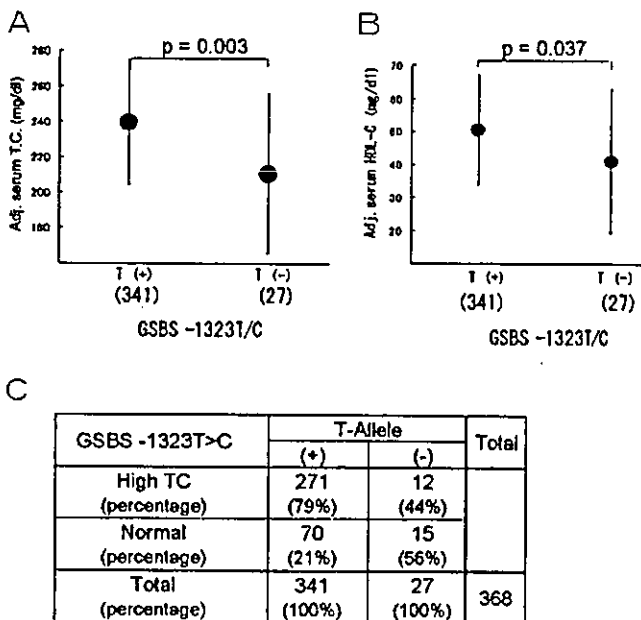
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In summary, we noted association between a putative regulatory region variation of the G-substrate (*GSBS*) gene and hypercholesterolemia in 368 subjects from an area of east-central region of Japan. Given our genetic results, we expect that the effects 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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ORIGINAL ARTICLE

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Hypercholesterolemia associated with splice-junction variation of inter-α-trypsin inhibitor heavy chain 4 (*ITIH4*) gene

Abstract Factors predisposing to the phenotypic features of higher total cholesterol (T-Cho) have not been clearly defined. Here we report an association between a C/T single nucleotide polymorphism at IVS17 + 8 in the inter-alpha-trypsin inhibitor heavy chain 4 gene (*ITIH4*) and plasma total cholesterol levels in 351 adult individuals from an east-central area of Japan. Age and gender-adjusted levels of plasma T-Cho, LDL-cholesterol, triglyceride, and HDL-cholesterol were analyzed. When we separate the subjects into two genotypic groups regarding this single nucleotide polymorphism (SNP), those who lack the T-allele had significantly higher plasma T-Cho levels than the others who bear T-allele (mean 252.3 mg/dl versus 241.7 mg/dl; $p = 0.009$). Of the 309 individuals without the T-allele, approximately 90% presented with hypercholesterolemia, whereas only 10% were hypercholesterolemic among 42 individuals with the T-allele ($p < 0.0001$). These data suggest that genetic variation at *ITIH4* locus is one of the likely candidate determinants for plasma cholesterol metabolisms.

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 diseases 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 the all determinants, obviously.

ITIH4 is a glycoprotein mainly expressed in the liver tissue (Nishimura H et al. 1995). It belongs to the inter-α-trypsin inhibitor family of serine protease inhibitors (ITIH1, 2, 3, and 4), which have diverse functions as antiapoptotic and matrix-stabilizing molecules important throughout the development. In addition, *ITIH4* gene locates in chromosomal region 3p21.1-p22 were recently described as one of the candidate quantitative trait locus (QTL) for dyslipidemias (Yuan et al. 2000). Expression in the liver, the role as a protease inhibitor,

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and the location in the candidate QTL region constituted rationales for selecting this gene as a candidate to study the correlation between genetic variation and lipoprotein profile.

In the course of serial investigations of population genetics for hyperlipoproteinemia in a cohort of an area located in east-central Japan, we recognized a correlation between the plasma total cholesterol (T-Cho) levels and a variation of the inter-alpha-trypsin inhibitor heavy chain 4 (*ITIH4*) gene. Here we focused on the analysis of the potential effect of genetic variation in *ITIH4*, investigating the correlation of plasma lipoprotein profile with the genetic variation of this gene.

Subjects and methods

Subjects

Subjects were obtained from participants of a cohort study that was originally carried out concurrently with health-check screening in an area in east-central Japan, as described in detail previously (Fujita et al. 2003). In brief, 22,228 participants were initially screened, and hypercholesterolemic individuals whose LDL-cholesterol (LDL-C) levels were higher than 140 mg/dl (LDL-C ≥140 mg/dl) were selected. To the present study, 351 individuals were recruited who gave their written informed consent prior to the 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,

Table 1 Physical and clinical profiles of the subjects. *NS* not significant, *M/F* male/female, *BMI* body mass index, *TC* total cholesterol, *LDL-C* LDL-cholesterol, *HDL-C* HDL-cholesterol, *TG* triglyceride

	T-allele (+)	T-allele (−)	p value
Number	42	309	−
Gender (M/F)	15/27	140/169	NS
Ages (years)	60.0 ± 8.6	61.4 ± 9.1	NS
BMI (kg/m²)	23.8 ± 3.2	23.8 ± 3.8	NS
TC (mg/dl)	241.7 ± 23.8	252.3 ± 23.5	0.009
LDL-C (mg/dl)	168.8 ± 22.0	169.7 ± 20.3	NS
HDL-C (mg/dl)	46.9 ± 10.9	51.5 ± 12.6	0.014
TG (mg/dl)	146.7 ± 77.1	160.4 ± 89.6	NS

The p value was calculated by Mann-Whitney test, except for gender; x2 test was conducted for distribution analysis of gender Values are expressed in mean ± SD

such as pituitary disease, hypothyroidism or hyperthyroidism, diabetes mellitus, liver disease, and renal disease. None was receiving antihyperlipidemic therapy. The selected participants visited lipid clinics for detailed examination of their lipoprotein profile and other clinical parameters. Blood was collected after 12–16 h of fasting. Genomic DNA was extracted, as previously described (Yoshida et al. 2002).

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 (HDL-C) were determined by the MgCl₂-dextran precipitation method. LDL-C concentration was calculated by subtracting HDL-C level from the fraction of both LDL-C and HDL-C, as described elsewhere (Ishii et al. 2002).

Selection criteria of single nucleotide polymorphisms (SNPs)

We focused on all SNPs that either accompanies amino acid substitution, SNPs adjacent to the exon/intron junction, and SNPs that locate in the putative promoter region in the entire *ITIH4* gene. The complete list of such SNPs in Table 2 describes nucleotide variation, amino acid variation, location, reference database (JSNP and dbSNP), allele frequency, and heterozygosity in the population. We examined SNPs whose percent-heterozygosity exceeded 10% to obtain substantial statistic analytical power. Thus, out of the six SNPs listed in Table 2, IVS17+8C/T and IVS20+8A/G were selected for the present study.

Genotyping for SNP in the *ITIH4* gene

PCR amplification of the flanking sequence for the polymorphism IVS17+8C/T and IVS20+8A/G at the *ITIH4* locus was performed using a condition described previously (Iida et al. 2002). The surrounding sequence of the SNP as well as the primer sequences and the experimental condition were obtained from the published reports (Haga et al. 2002). SNP genotyping was performed by Invader assay (Third Wave Technologies. Madison, WI, USA) (Saito et al. 2002) using PCR products of the flanking sequence and the probes designed and synthesized by the supplier (Ohnishi et al. 2001).

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 of the general Japanese population. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical

Table 2 Summary of the selected polymorphisms in the ITIH4 gene. *SNP* single nucleotide polymorphism

No.	SNP	nt.	Location	JSNP-ID[a]	dbSNP[b]	Allele frequency (heterozygosity)[c]
1	L669Q	T/A	Exon17	IMS-JST073530	rs2276814	0.997:0.003 (0.7%)
2	IVS17+8C/T	C/T	Intron17	IMS-JST182668	rs3821831	0.933:0.067 (12.5%)
3	P698T	C/A	Exon18	−	rs4687657	ND (ND)
4	I714M	C/G	Exon18	IMS-JST073528	rs2256734	0.997:0.003 (0.7%)
5	IVS20+8A/G	A/G	Intron20	IMS-JST073526	rs2245538	0.566:0.434 (49.1%)
6	L791P	T/C	Exon21	−	rs2535621	ND (ND)

[a] Number from Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/index_ja.html)
[b] Number from dbSNP database of NCBI (http://www.ncbi.nlm.nih.gov/SNP/)
[c] Data from dbSNP database or from JSNP database

traits in each genotypic group did not always distribute normally, we applied a nonparametric 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 (Fujiwara et al. 2002). Fisher's exact test was used to compare differences in the prevalence of hypercholesterolemia among the population. Chi-square tests were invoked to detect Hardy-Weinberg equilibrium.

Results

To carry out a correlation analysis of the potential effect of SNPs, IVS17+8C/T and IVS20+8A/G, 351 individuals were selected from participants for a cohort of an area initially screened by criteria that defined individuals harboring hyperlipidemic risks. Because of initial screening for the health-check assessment, basal level of each value was a little higher than that of data from the general Japanese population. However, the average differences were less than 0.5×SD in general. When the subjects were genotypically categorized into three groups for each SNP (for instance, three homozygous minor T-allele carriers, 39 heterozygous carriers and 309 homozygous C-allele carriers, for IVS17+8C/T), no deviation of genotype frequencies from Hardy-Weinberg equilibrium was observed (p = 0.38, x^2-test).

Distribution and mean values of T-Cho, TG, LDL-C and HDL-C were analyzed among the genotypically divided groups by each SNP, and significant difference in T-Cho and HDL-C was detected among the three groups divided by IVS17+8C/T genotypes. Plasma T-Cho levels of homozygous minor T-allele carriers (n = 3), heterozygous carriers (n = 39), and homozygous C-allele carriers (n = 309) were 200.4±39.7 mg/dl, 244.9±19.5 mg/dl, and 252.3±23.5 mg/dl respectively, indicating a codominant T-Cho lowering effect of the minor T-allele (r = 0.18, p = 0.0006). Similarly, plasma HDL-C levels were 31.1±6.9 mg/dl, 48.1±10.2 mg/dl, and 51.5±12.6 mg/dl, respectively, indicating a codominant HDL-C lowering effect of the minor T-allele (r = 0.15, p = 0.006). No statistically significant difference was detected in the plasma lipoprotein levels among the groups categorized by IVS20+8A/G, suggesting presence of a break of haplotype blocks within the gene (data not shown).

We repeatedly evaluated the genotypic effect by categorization of the subjects into two groups, those who lack the T-allele (C/C) and those who bear at least one T-allele (C/T and T/T), since the homozygous subjects carrying the minor allele was rare (n = 3). By this categorization, the former subjects had significantly higher plasma T-Cho levels than the latter (mean±SD 252.3±23.5 mg/dl versus 241.7±23.8 mg/dl; p = 0.009) (Fig. 1A). Similarly, the former subjects had significantly higher HDL-C levels than the latter (mean±SD 51.5±12.6 mg/dl versus 46.9±10.9 mg/dl; p = 0.014).

Since the data presented here suggested an involvement of *ITIH4* locus in the expression of the hypercholesterolemic phenotype in these populations,
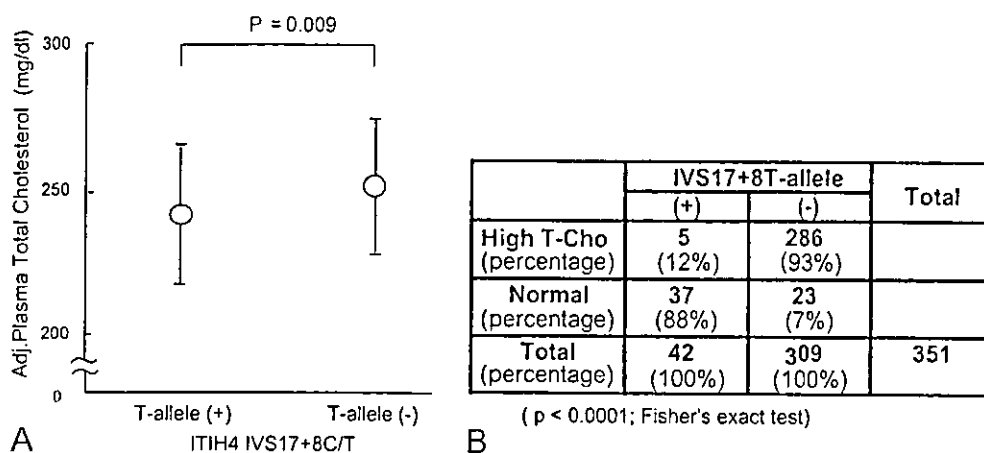
we correlated the manifestation of the hypercholesterolemia with respect to presence or absence of T-alleles of *ITIH4* IVS17+8C/T. Hypercholesterolemia was defined as plasma cholesterol level above the reference values after age and gender adjustment (T-Cho >220 mg/dl). This criterion is based on a recommended preclinical level of hyperlipidemic individuals who have to be alerted for high risk for ischemic heart disease. It classified 291 subjects as having hypercholesterolemia among the study population consisting of 351 individuals. Of the 309 individuals lacking the T-allele, 286 presented with hypercholesterolemia (93%) whereas only five did so among 42 individuals with T-allele (12%). Distribution difference was significant when tested by Fisher's exact test (p <0.0001; Relative risk = 2.44; 95% CI: 1.80–3.30) (Fig. 1B). As to plasma HDL-C, the distribution analysis (low HDL <40 mg/dl versus high HDL ≥ 40 mg/dl) was not significantly deviated.

Correlation analysis of a population of 108 normolipidemic subjects from the same geographical area that we carried out preliminarily did not reach statistical significance (data not shown), suggesting that the ITIH4 may exert its modifying effect only in the hypercholesterolemic metabolic state. A subsequent, large-scale cohort study is obviously necessary to clarify whether or not the ITIH4 effect is also present in normolipidemic populations.

Discussion

Hypercholesterolemia is one of the important risk factors of ischemic heart disease (Matsuzawa 1995; Norioka 2000). In the present study, we showed a correlation between hypercholesterolemia and homozygosity for the C-allele of IVS17+8 C/T SNP in the *ITIH4* gene. An association study revealed an elevating effect of plasma T-Cho of the homozygosity for the C-allele. The high prevalence (93%) of hypercholesterolemia (mean±SD 252.3±23.5 mg/dl) among the individuals with this genotype (C/C) is in contrast to the scarceness (12%) of hypercholesterolemia (mean±SD 244.9±19.5 mg/dl and 200.4±39.7 mg/dl) among the rest of the study subjects (C/T and T/T). Our results indicated that *ITIH4* variation might modify the lipoprotein phenotype of plasma lipoprotein metabolism.

ITIH4 belongs to the inter-α-trypsin inhibitor family of serine protease inhibitors, which have diverse functions as an antiapoptotic and matrix-stabilizing molecule important throughout the development. Four genes, designated *ITIH1, 2, 3* and *4*, are involved in the synthesis of inter-α-trypsin inhibitor family members. All four heavy chain precursors and the resulting mature heavy chains harbor a so-called von Willebrand type-A (vWA) domain (Chan P et al. 1995, Bork P et al. 1991). Only the heavy chain 4 precursor polypeptide harbors a plasma Kallikrein-released bradykinin-like domain on its C-terminal sequence (Nishimura H et al. 1995) and a domain with some similarity to the ATP-dependent

	IVS17+8T-allele		Total
	(+)	(-)	
High T-Cho (percentage)	5 (12%)	286 (93%)	
Normal (percentage)	37 (88%)	23 (7%)	
Total (percentage)	42 (100%)	309 (100%)	351

(p < 0.0001; Fisher's exact test)

Fig. 1A, B Comparison of plasma total cholesterol (T-Cho) levels among genotypically determined groups according to the *ITIH4* variation. A Adjusted plasma T-Cho levels were compared between genotypic groups with or without the ITIH4 IVS17+8 T-allele (*p*=0.009). Open circles represent mean values. Error bars represent the standard deviation (SD). B Distribution analysis of hypercholesterolemic patients among ITIH4 IVS17+8C/T classified-genotypes; T-allele (+)/(−). The 2×2 table was analyzed by Fisher's exact test (*p*<0.0001). High T-Cho phenotypes were defined by adjusted plasma T-Cho levels above the 220 mg/dl

proteases (Saguchi K et al. 1995). Although the suspected link between functional changes of ITIH4 protein and plasma T-Cho metabolism was not a commonly expected one, it may have unidentified functions in regulation of cholesterol synthesis and catabolism in the liver.

The IVS17+8C/T SNP locates close to the splice junction for the donor site of exon 17 of the *ITIH4* gene. Due to this polymorphic sequence near the splice junction, the *ITIH4* gene may have differential splicing that results in production of distinct ITIH4 isoforms in some organs. This possibility may need to be investigated by functional studies in the future. In addition, possibilities that this SNP marker may itself be in linkage disequilibrium with other unexamined functional variants localized in its close proximity or those in nearby genes have to be tested. HapMap initiative is now in progress among the international consortium. Haplotypic knowledge of the human genome will help clarify the contribution of the *ITIH4* gene in lipid profile through detailed haplotype association study in the near future. It would also be important to verify the association in geographically distinct populations and other ethnic groups in the future (Shastry 2002).

In summary, we noted an association between the variation of the inter-α-trypsin inhibitor heavy chain 4 (*ITIH4*) gene and hypercholesterolemia in 351 subjects from an area in the 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 dyslipidemias.

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Original Articles

Cutoff Point Separating Affected and Unaffected Familial Hypercholester-olemic Patients Validated by LDL-receptor Gene Mutants

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Familial hypercholesterolemia (FH) results from low-density lipoprotein (LDL) receptor gene mutations. Heterozygotes have twice normal LDL-cholesterol concentrations in early childhood, and experience early myocardial infarction. We demonstrated bimodal cholesterol frequency distributions, independently confirming existence of an identifiable hypercholesterolemic subpopulation. We assayed blood lipids in 181 FH patients genetically diagnosed and 100 unaffected relatives. Receiver operating characteristics curves were constructed. Total cholesterol and LDL-cholesterol concentrations showed bimodality. A total cholesterol cutoff of 225 mg/dl produced results agreeing with DNA testing (specificity, 98.5%; sensitivity, 99.4%). An LDL-cholesterol cutoff of 161–163 mg/dl produced 98.5% specificity and 98.3% sensitivity. Areas under curves were 0.9826 ± 0.0058 for total cholesterol, and 0.9852 ± 0.0043 for LDL-cholesterol. In conclusion, we define total cholesterol and LDL-cholesterol levels of 225 and 160 mg/dl, respectively, as cutoff points of normal subjects and FH patients. J Atheroscler Thromb, 2005; 12: 35–40.

Keywords: Familial hypercholesterolemia, LDL-cholesterol, Cutoff point, Receiver operating characteristics analysis

Introduction

Hypercholesterolemia is a major coronary risk factor and many epidemiologic studies have linked high serum cholesterol to higher incidence of coronary heart disease (CHD) (1). In the second report from the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP II) a cutoff point defining a high

blood cholesterol (240 mg/dl) marked a steep rise in risk for CHD. This value corresponded to approximately the 80th percentile in the third adult US population National Health and Nutrition Examination Survey (NHANES III) (2). Yet a precise definition of hypercholesterolemia is difficult to establish. Often an abnormally high laboratory variable is considered to be the value defining the upper 5% of the population (the 95th percentile). Frequency distributions of serum cholesterol concentrations have been reported to be continuous and unimodal in the general population, with no clear point of separation between individuals with normal and abnormally high values. Serum cholesterol concentrations depend upon both genetic and environmental factors, and the mean ± standard deviation (SD) in "healthy subjects" has been used to define the normal range. However, use of this definition is unreasonable, since CHD is a major cause