Associations Between Serum High-Density Lipoprotein Cholesterol or Apolipoprotein AI Levels and Common Genetic Variants of the *ABCA1* Gene in Japanese School-aged Children

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ATP-binding cassette transporter A1 (ABCA1) plays an important role in apolipoprotein AI (apoAI)-mediated cholesterol efflux from peripheral cells. The mild changes in ABCA1 activity due to genomic variation might be associated with interindividual variations in serum high-density lipoprotein cholesterol (HDL-C) and apoAI levels, or primary hypoalphalipoproteinemia in the general population. In the present study, we analyzed the relationships between 5 single nucleotide polymorphisms (SNPs) and 2 insertion/deletion polymorphisms in the 5′ flanking region and 5 missense polymorphisms of the *ABCA1* gene and serum lipid levels in healthy school-aged children. We detected significant associations between the K219R and V771M polymorphisms, and HDL-C or apoAI levels. The present data support the proposition that the K219 allele is an anti-atherogenic allele with increased cholesterol efflux activity. Similarly, the M771 allele appears to be anti-atherogenic, although the frequency of the M771 allele is low.

A LARGE NUMBER of epidemiological and clinical studies have shown that low serum high-density lipoprotein cholesterol (HDL-C) and apolipoprotein AI (apoAI) levels are important risk factors for coronary artery disease (CAD),[1-4] although serum levels of HDL-C and apoAI are strongly correlated.[4,5] The protective effect of HDL against atherosclerosis and CAD might be related to its key role in the transport of cholesterol from peripheral cells to the liver, a process called reverse cholesterol transport.[6,7]

ATP-binding cassette transporter A1 (ABCA1) is a member of the ATP-binding cassette transporter family, which plays a most important role in apoAI-mediated cholesterol efflux from peripheral cells, the first step in reverse cholesterol transport .[8,9] ABCA1 deficiency causes Tangier disease (TD), a single-gene disorder characterized by very low serum levels of HDL-C and apoAI and marked cholesterol ester deposition within tissue; TD is associated with some increased risk for CAD.[10-12] Studies have also shown that some familial hypoalphalipoproteinemia with decreased cholesterol efflux is caused by heterozygous mutations in the *ABCA1* gene.[10,13,14] However, the frequencies of TD and familial hypoalphalipoproteinemia due to dysfunction of ABCA1 are low.

Most cases of primary hypoalphalipoproteinemia are regarded as multifactorial disorders. Therefore, the mild changes in ABCA1 activity due to genomic variation might be associated with inter-individual variations in serum HDL-C and apoAI levels, or primary hypoalphalipoproteinemia in a general population. Thus far, the relationships between several polymorphisms in the *ABCA1* gene and plasma lipid levels have been analyzed in several studies; however, almost all of the subjects were CAD patients.[15-19] In the present study, we analyzed the relationships between polymorphisms in the *ABCA1* gene and serum lipid levels in healthy school-aged children.

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MATERIALS AND METHODS

Subjects

The subjects were 327 randomly selected unrelated school-aged Japanese children (165 boys, 162 girls; age range, 9 to 15 years; mean ± SD, 12.3 ± 1.9 years) without abnormalities known to affect lipid metabolism, who participated in a school survey in a rural town. Informed consent was obtained from parents of the subjects, and the study was approved by the Ethics Committee of University of Tsukuba, Japan.

Serum Lipid Measurements

After overnight fasting, blood was collected from each subject. Serum total cholesterol, triglyceride (TG), and HDL-C levels were measured using standard enzymatic methods. Serum apoAI and apolipoprotein B (apoB) levels were measured using turbidimetric immunoassays.[20] Lipid and lipoprotein values are presented as milligrams per deciliter. The mean values for serum lipid levels and body mass index (BMI) of the subjects according to gender are shown in Table 1.

DNA Analyses

Genomic DNA was isolated from peripheral blood leukocytes by the phenol extraction method. Potential variations in the 1,350 bp of the 5′ flanking region of the *ABCA1* gene were screened in 24 randomly selected subjects by denaturing high-performance liquid chromatography (DHPLC) with a WAVE DNA fragment analysis system (Transgenomic Inc, San Jose, CA). Nucleotide changes were confirmed by direct sequencing using Big Dye terminator chemistry (Applied Biosystems, Foster City, CA). Nucleotide numbering is according to Pullinger et al,[22] considering the sequences of GenBank accession no. AC012230. For the coding region of the *ABCA1* gene, we screened common missense polymorphisms from the dbSNP database (*http://www.ncbi.nlm.nih.gov/SNP/*).

The genotypes for each polymorphism were determined with polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis, using the primers and restriction enzymes described in Table 2. For the −675 to −674insGTTTGTTTT or ins-

Table 1. Clinical Characteristics and Serum Lipid Levels of the Subjects

	Total Subjects	Boys	Girls	*P*
No.	327	165	162	
Age (yr)	12.3 ± 1.9	12.4 ± 1.8	12.3 ± 1.8	.50
Total cholesterol (mg/dL)	168.5 ± 25.3	164.9 ± 26.0	172.1 ± 24.0	.0097
TG (mg/dL)*	74.6 ± 32.8	71.6 ± 33.6	77.6 ± 31.7	.052
HDL-C (mg/dL)	54.3 ± 10.5	53.7 ± 10.7	55.0 ± 10.3	.26
LDL-C (mg/dL)	99.3 ± 21.8	96.9 ± 21.8	101.6 ± 21.7	.052
apoAI (mg/dL)	131.7 ± 17.6	130.0 ± 18.1	133.4 ± 17.0	.081
apoB (mg/dL)	68.6 ± 13.6	67.1 ± 14.9	70.5 ± 12.0	.024
BMI (kg/m^2)	19.5 ± 3.4	19.3 ± 3.3	19.6 ± 3.6	.44

NOTE. Values are shown as mean ± SD. LDL-C levels were estimated based on Friedewald's formula.[21] *P* values were calculated by *t* test.
*Statistical test for TG levels was calculated on log-transformed values.

GTTTT polymorphisms, the genotypes were determined from the length of PCR-amplified DNA fragments.

Statistical Analyses

Differences in serum lipid levels due to the genotypes of each polymorphism were analyzed by multiple linear regression analyses incorporating age, sex, and BMI as covariates. Statistical analyses were performed with the JMP software package (SAS Institute, Cary, NC). A *P* value of less than .05 was considered statistically significant. No adjustment for multiple testing was made as each polymorphism and serum lipid levels are not fully independent of one another.

RESULTS

Polymorphisms of the 5′ Flanking Region

We screened for polymorphisms in the 1,350 bp of the 5′ flanking region of the *ABCA1* gene and detected a total of 5 single nucleotide polymorphisms (SNPs) and 2 insertion/deletion polymorphisms. Five of these polymorphism (−937 to −936delAT, −477C→T, −320G→C, −191G→C, and −17G→C) were identical to those reported previously.[17,18,23] The −707G→A polymorphism and a complex insertion/deletion polymorphism with 3 alleles are newly detected polymorphisms. The complex polymorphism has 3 alleles, a 9-bp (GTTTGTTTT) insertion or a 5-bp (GTTTT) insertion, or no insertion at the site between nt −675 and nt −674. Almost all pairs of these 7 polymorphisms were in tight linkage disequilibrium with each other (data not shown).

Table 3 shows the mean values for serum HDL-C, TG, and apoAI levels, according to the genotypes of these 7 polymorphisms. Significant associations between these polymorphisms in the 5′ flanking region and serum HDL-C, TG, and apoAI levels were not observed.

Missense Polymorphisms in the Coding Region

To date, in the coding region of the *ABCA1* gene, 9 SNPs inducing amino acid changes have been reported in the dbSNP database; we have ascertained that at least 5 of these missense mutations (K219R, V771M, V825I, M883I, and R1587K) are polymorphic in Japanese populations. Tight linkage disequilibliums were observed between the V771M polymorphism and the M883I polymorphism, and the V825I polymorphism and the M883I polymorphism (data not shown). These data suggest ethnic differences in the allele frequencies of these SNPs. The

Table 2. PCR Primers, Annealing Temperatures, Sizes of Amplified Fragments, and Restriction Enzymes Used for Genotyping of Each Polymorphism

	Forward Primer	Reverse Primer	Annealing Temp (°C)	Size of Amplified Fragment (bp)	Restriction Enzyme
5′-flanking region					
−937 to −936delAT	F: CCTCTTCTACGGGTCTGTCCTG	R: CAGCCTCCCTGTGATAAAAACA	64	321, 323	*Nla*III
−707G→A	F: TGGAGGTCTGGAGTGGCTACAT	R: CACAGAACAATATGGACCAGGCC	60	156	*Stu*I
−675 to −674ins GTTTGTTTT or insGTTTT	F: CTGGTCCATATTGTTCTGTGTT	R: CATAAATTGAGAGGAAGGAGGC	58	76, 81, 85	—
−477C→T	F: CGCCTATCAAAAATCAAAGTCC	R: TCCGCGGTCTGCGTCCCCTTCC	60	371	*Aci*I
−320G→C	F: AAAAAATTGCGGAAAGTA	R: GCCGCAGACTCTCTAGTCCA	56	86	*Rsa*I
−191G→C	F: CAAATTCCACTGGTGCCCTTGG	R: TGTCTTAGGGTCCGCGGTCTGGGT	60	140	*Ava*II
−17G→C	F: ACCCCCACCCCACCCACCTCCC	R: GTGCTCTCCCTCCTCCCCGCCGC	62	158	*Bst*UI
Coding region					
K219R	F: TGACAAGTCTGTGCAATGGA	R: GGCTTAAACTCAGCCACACC	58	379	*Sty*I
V771M	F: AGTGCTTGGGATTGTTGAGG	R: CACTGAAGAAAGGCCAGAGG	58	300	*Bsa*AI
V8251	F: CTGCTGTCTCCTGTGGCTTT	R: GCTGCCTGTCCTTGGACTAT	58	349	*Bsm* AI
M883I	F: ACCCTGGTTCCAACCAGAAGAGGAT	R: TTTAGAAAGGCAGGAGACATCG	58	125	*Eco*RV
R1587K	F: AGATTTATGACAGGACTGGACATCA	R: CTGCCAACTTTACCATGAGTTG	55	129	*Hpy*188I

NOTE. Mismatch nucleotides in primers are underlined.

Table 3. Relations Between Genotypes of Polymorphisms in the 5′-Flanking Region and the Serum Levels of HDL-C, TG, and apoAI

	Genotype	n	HDL-C (mg/dl)	*P*	TG (mg/dL)*	*P*	apoAI (mg/dL)	*P*
−937 to −936delAT	ins/ins	170	53.8 ± 10.4	.91	73.0 ± 32.0	.65	130.5 ± 17.1	.69
	ins/del	139	54.2 ± 11.0		78.1 ± 36.6		132.4 ± 18.8	
	del/del	18	53.6 ± 6.6		75.3 ± 18.7		130.8 ± 12.0	
−707G→A	GG	183	54.8 ± 10.4	.75	73.5 ± 32.6	.87	132.7 ± 17.3	.42
	GA	125	52.8 ± 11.1		77.9 ± 35.1		129.2 ± 18.5	
	AA	19	55.4 ± 9.2		75.4 ± 32.1		133.3 ± 15.2	
−675 to −674insGTTTGTTTT or insGTTTT	del/del	111	53.6 ± 10.9	.48	77.5 ± 32.9	.42	130.4 ± 17.4	.43
	ins9bp/del	101	53.5 ± 10.7		76.4 ± 37.3		131.7 ± 18.3	
	ins9bp/ins9bp	22	56.1 ± 10.7		82.5 ± 29.8		136.2 ± 21.1	
	ins9bp/ins5bp	25	55.6 ± 11.4		66.9 ± 34.5		132.2 ± 16.7	
	ins5bp/del	61	53.8 ± 9.6		68.9 ± 29.3		129.9 ± 16.5	
	ins5bp/ins5bp	7	58.9 ± 12.3		72.7 ± 24.8		139.7 ± 17.5	
−477C→T	CC	116	54.1 ± 10.9	.35	77.9 ± 34.0	.44	131.6 ± 17.4	.38
	CT	146	53.3 ± 10.0		74.8 ± 36.0		130.2 ± 16.7	
	TT	65	55.6 ± 11.5		71.2 ± 26.0		133.9 ± 20.3	
−320G→C	GG	125	54.6 ± 10.5	.13	76.4 ± 33.7	.77	132.2 ± 16.8	.33
	GC	152	53.3 ± 10.3		74.8 ± 35.3		130.8 ± 17.0	
	CC	50	56.6 ± 11.0		74.1 ± 28.1		135.0 ± 19.0	
−191G→C	GG	116	54.1 ± 10.8	.098	77.0 ± 33.8	.58	131.2 ± 17.6	.21
	GC	151	53.2 ± 10.3		74.7 ± 35.2		130.3 ± 17.0	
	CC	60	56.2 ± 11.0		73.1 ± 28.8		134.8 ± 19.5	
−17G→C	GG	155	54.1 ± 10.9	.60	74.9 ± 32.5	.92	131.3 ± 18.7	.79
	GC	93	54.4 ± 11.0		75.9 ± 33.9		132.1 ± 17.4	
	CC	79	53.5 ± 9.8		75.1 ± 35.6		131.1 ± 16.3	

NOTE. Values are shown as mean ± SD. *P* values were calculated by multiple linear regression analyses incorporating sex, age, and BMI as covariates.

*Statistical tests for TG levels were calculated on log-transformed values.

minor allele frequencies of each SNP in the Japanese are higher than those in Caucasians. In particular, for the K219R and M883I polymorphisms, the minor allele in Caucasians is the major allele in the Japanese.

The relationships between serum HDL-C, TG, or apoAI levels and the genotypes of these 5 missense polymorphisms are shown in Table 4. Significant associations between the K219R polymorphism and serum HDL-C or apoAI levels, and between the V771M polymorphism and apoAI levels, were observed in multiple linear regression analysis with sex, age, and BMI as covariates. However, the gene dosage effects of each allele were not observed in these associations. We have classified the subjects into 2 groups, namely, carriers of the minor allele and noncarriers for these 2 polymorphisms. The mean serum HDL-C and apoAI levels of the carriers with the R219 allele (52.8 ± 10.1 mg/dL and 129.5 ± 16.9 mg/dL,

Table 4. Relations Between Genotypes of Missense Polymorphisms and the Serum Levels of HDL-C, TG, and apoAI

	Genotype	n	HDL-C (mg/dL)	*P*	TG (mg/dL)*	*P*	apoAI (mg/dL)	*P*
K219R	KK	97	56.9 ± 11.3	.016	74.3 ± 36.6	.43	136.0 ± 18.8	.012
	KR	160	52.2 ± 10.3		77.2 ± 32.3		128.7 ± 17.3	
	RR	70	54.3 ± 9.7		71.8 ± 31.9		131.2 ± 15.7	
V771M	VV	265	53.4 ± 10.5	.13	74.4 ± 32.3	.76	130.1 ± 17.5	.035
	VM	59	56.8 ± 10.5		79.3 ± 39.2		137.5 ± 17.3	
	MM	3	57.7 ± 18.2		71.0 ± 14.0		133.3 ± 23.5	
V825I	VV	134	53.2 ± 10.9	.53	76.3 ± 35.3	.77	130.7 ± 18.3	.54
	VI	156	54.9 ± 10.3		74.8 ± 31.6		132.5 ± 17.2	
	II	37	53.8 ± 11.2		73.1 ± 35.4		129.5 ± 17.4	
M8831	MM	120	53.9 ± 11.4	.73	75.3 ± 35.1	.24	131.2 ± 18.0	.90
	MI	162	53.8 ± 10.2		76.5 ± 32.3		131.1 ± 18.1	
	II	45	55.3 ± 10.6		70.6 ± 33.7		133.0 ± 15.7	
R1587K	RR	114	54.5 ± 10.7	.52	75.2 ± 34.8	.063	131.7 ± 16.9	.49
	RK	154	54.0 ± 10.3		78.1 ± 34.3		131.9 ± 17.5	
	KK	59	53.4 ± 11.7		67.7 ± 27.7		129.5 ± 19.7	

NOTE. Values are shown as mean ± SD. *P* values were calculated by multiple linear regression analyses incorporating sex, age, and BMI as covariates.

*Statistical tests for TG levels were calculated on log-transformed values.

respectively) were significantly lower than those of the homozygotes of the K219 allele (56.9 ± 11.3 mg/dL and 136.0 ± 18.8 mg/dL, respectively) ($P = .0086$, $P = .0045$, respectively). The mean serum HDL-C and apoAI levels of the carriers with the M771 allele (56.8 ± 10.8 mg/dL and 137.3 ± 17.4 mg/dL, respectively) were significantly higher than those of the homozygotes of the V771 allele (53.4 ± 10.5 mg/dL and 130.1 ± 17.5 mg/dL, respectively) ($P = .042$, $P = .011$ respectively). These data suggest that the R219 allele is associated with decreased HDL-C and apoAI levels, whereas the M771 allele is associated with increased HDL-C and apoAI levels. However, no significant associations were observed between the remaining 3 missense polymorphisms (V825I, M883I, and R1587K) and serum HDL-C, TG, and apoAI levels (Table 4).

DISCUSSION

The ABCA1 protein plays a most important role in the first step of the reverse cholesterol transport system.[8,9] To date, several common variations in the *ABCA1* gene have been identified. In the 5′ flanking region, weak associations exist between the −477C→T polymorphism and levels of HDL-C or apoAI, and significant correlation is seen between this polymorphism and the severity of coronary atherosclerosis.[17] In addition, significant associations have been observed between the −17G→C and the −191G→C polymorphisms and susceptibility of atherosclerosis, even though neither polymorphism affects plasma lipid levels.[18] In the coding region, weak associations between the K219R polymorphism and plasma HDL-C or TG levels and strong association between this polymorphism and the severity of atherosclerosis have been demonstrated.[16] A recent study has shown that the K219 allele has a protective effect against CAD in patients with hyperlipidemia without modification of plasma HDL-C levels.[24,25]

Polymorphisms of the *ABCA1* gene appear to be more strongly associated with susceptibility of atherosclerosis than with serum lipid levels.[16-18,24,25] Despite near ubiquitous expression of *ABCA1*, the accumulation of cholesterol observed in Tangier disease or *ABCA1* knockout mice appears to be restricted primarily to macrophages.[26,27] *ABCA1* in macrophages is expected to play an important role in mediating the efflux of cholesterol and phospholipids to apolipoprotein, a process necessary for HDL formation. Studies using mouse models have indicated that selective inactivation of *ABCA1* in macrophages increases atherosclerosis and foam cell accumulation, but such properties of *ABCA1* are independent of plasma HDL-C levels.[28-30] Thus, the possibility exists that the mild change in *ABCA1* activity due to such genomic variations primarily affects macrophages. Therefore, the effects of *ABCA1* polymorphism might be more easily detected in atherosclerosis than in the change in HDL-C levels.

We analyzed the relationships between five SNPs and 2 insertion/deletion polymorphisms in the 5′ flanking region and 5 missense polymorphisms of the *ABCA1* gene and serum lipid levels in healthy school-aged Japanese children. We detected significant associations between the K219R and V771M polymorphisms, and HDL-C or apoAI levels. The determination of plasma lipid levels is controlled by multiple complex pathways and influenced by many environmental factors, such as dietary habits, exercise, smoking, and alcohol intake. In the present study, the subjects were healthy school-aged children, aged 9 to 15 years. Few Japanese children at this age are habitual drinker or smokers, and the effects of such environmental factors including smoking and alcohol intake are probably lower in school-aged children than in adults. However, almost all of them are in puberty, a time when the serum sex hormone levels change dramatically, and these changes affect serum lipid levels.[31,32] Unfortunately, we were unable to determine serum sex hormone levels in our subjects. We examined the relationship between each polymorphism and lipid levels after adjustment for sex, age, and BMI by multiple linear regression analysis. However, it remains necessary to ascertain the relationships between lipid levels and these polymorphisms in the *ABCA1* gene in a differently aged population.

The K219 allele is thought to be an anti-atherogenic allele that increases cholesterol efflux activity.[16] Our data support this proposal as the serum HDL-C and apoAI levels of homozygotes of the K219 allele are higher than those of the carriers with the R219 allele. In addition, the M771 allele also seems to be an anti-atherogenic allele, although the gene frequency of the M771 allele is low.

Further epidemiological and genetic studies are required to understand the relationships between hypoalphalipoproteinemia in the general population or CAD patients and these common variants of the *ABCA1* gene. In addition, *ABCA1* has been expected to be a potential target for development of new pharmacological agents that may raise serum HDL-C levels. Therefore, it will also be interesting to examine the relationship between responses to such drugs and the genotypes of the *ABCA1* gene.

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