Association of an intronic haplotype of the *LIPC* gene with hyperalphalipoproteinemia in two independent populations

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Abstract Hepatic lipase (HL) plays a major role in the regulation of plasma lipids. Several groups seeking to find association between the gene encoding HL (*LIPC*) and plasma concentrations of high-density lipoprotein cholesterol (HDLc) using various methods and populations have reported conflicting results. We have approached the problem of demonstrating a relationship between the *LIPC* locus and HDLc by means of haplotype association using four single nucleotide polymorphisms (SNPs) (rs12594375G/A, rs8023503C/T, rs4775047C/T, and rs11634134T/A) located in intron 1 of the *LIPC* gene in two independent Japanese populations consisting of 2,970 and 1,638 individuals, respectively. Significant association

between hyperalphalipoproteinemia and a specific haplotype in this intron was detected in both populations. When HDLc levels among the three haplotypic categories were analyzed [haplotype rs8023503C/rs12594375G (haplotype-1; H1) homozygotes (H1H1), haplotype rs8023503T/rs12594375A (haplotype-2; H2) homozygotes (H2H2), and heterozygotes (H1H2)], HDLc levels were lowest among H1H1 [mean $\pm$ standard error (SE) = 58.4 ± 0.4 mg/dl], highest among H2H2 (62.5 ± 0.8 mg/dl), and intermediate among H1H2 (59.2 ± 0.4 mg/dl) ($P = 0.00011$), indicating that H2 haplotype elevates plasma HDLc levels. This association was validated in the second population ($n = 1,638$) ($P = 0.00070$). The results provide convincing evidence that the *LIPC* locus influences HDL metabolism.

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Abbreviations

ANOVA	analysis of variance
CETP	cholesteryl ester transfer protein
HDLc	high-density lipoprotein cholesterol
HL	hepatic lipase
LD	linkage disequilibrium
LDLc	low-density lipoprotein cholesterol
<i>LIPC</i>	hepatic lipase gene
LPL	lipoprotein lipase
QTL	quantitative trait locus
RLP	remnant-like particle cholesterol
SNP	single nucleotide polymorphism
TC	total cholesterol

TG	triglyceride
VLDL	very-low-density lipoprotein

Introduction

Genetic mechanisms remain obscure for the majority of human hyperalphalipoproteinemias that involve elevated high-density lipoprotein cholesterol (HDLc) levels in plasma (Glueck et al. 1975, 1977). Rare Mendelian monogenic conditions do exist, however. One of them is an autosomal recessive trait familial deficiency of hepatic lipase (HL) (Hegele et al. 1991a, b). Such patients present with elevated plasma HDLc as well as triglyceride-rich beta very-low-density lipoprotein (VLDL), LDL, and HDL lipoproteins (Carlson et al. 1986). The molecular basis of HL deficiency has been demonstrated biochemically by lack of HL activity in postheparin plasma from affected cases (Carlson et al. 1986; Little et al. 1986). Hegele et al. found several mutations of a gene designated *LIPC* among patients with familial HL deficiency, establishing a genetic basis for this condition (Hegele et al. 1991a, b). Human *LIPC* is a large gene spanning more than 60 kb on human chromosome 15q21 and consists of nine exons and eight introns (Cai et al. 1989; Sparkes et al. 1987).

HL is a 477-amino-acid glycoprotein that is synthesized and secreted primarily in the liver. It belongs to a family of enzymes involved in regulation of lipoprotein metabolism in plasma. It catalyzes the hydrolysis of triglycerides of large remnant VLDL, LDL, and nascent HDL1 to form dense HDL(2) (Kinnunen 1984; Jensen et al. 1982). Elevated enzymatic activity of HL has been associated with high HDL levels (Santamarina-Fojo et al. 1998; Dugi et al. 2000; Zambon et al. 2000).

The enzyme also mediates hepatic uptake of HDL cholesteryl esters and contributes to the reverse cholesterol transport system (Lambert et al. 1999). Association between low HL activity and coronary artery disease might reflect a decrease in HL-mediated uptake of lipoproteins by the liver (Brunzell and Deeb 2001). The enzymatic activity of HL varies widely in normal individuals (five- to eight-fold), probably under the influence of genetic variation and other environmental factors (Zambon et al. 2003).

Previous attempts to correlate variations of *LIPC* with HDLc levels in general populations have shown conflicting results. In particular, recent evidence argues both for and against association between a single nucleotide polymorphism (SNP) in the promoter region, −514C/T, and plasma HDLc levels. Some studies have shown a significant association with plasma HDL (Jansen et al. 1997; Couture et al. 2000; Guerra et al. 1997; Hubáček et al. 2001; Jansen

et al. 1999; Murtoimäki et al. 1997; Ordovas et al. 2002; Yamakawa-Kobayashi et al. 2002; Zambon et al. 2003; Chen et al. 2003; Ji et al. 2002; Ko et al. 2004), but other studies (Vega et al. 1998; Tahvanainen et al. 1998; Grundy et al. 1999; Carr et al. 2002; Fang and Liu 2002; Hegele et al. 1999; Juo et al. 2001; Hong et al. 2000; St-Pierre et al. 2003) have failed to demonstrate it. The disparate data might reflect differences in ethnicity, gender, and/or heterogeneity of the test populations in clinical settings, as well as the fact that most of these studies involved only small population samples. Earlier studies in Japanese groups suffered from selection bias; for example, subjects were chosen from among patients undergoing hemodialysis or postmenopausal hormone replacement therapy.

We approached the problem of defining a possible relationship between the *LIPC* locus and HDLc levels in plasma by typing multiple SNPs located in intron 1 of *LIPC* and investigating haplotype association. Here we describe association between a haplotype in intron 1 of *LIPC* and hyperalphalipoproteinemia in two independent Japanese populations for applying in medical practice.

Materials and methods

Study populations

This investigation was part of an ongoing Molecular Epidemiological Study utilizing the Regional Characteristics of the Twenty-first Century Centers of Excellence (COE) Program in Japan. The first regional population was Takahata (T), a local area of northeastern Japan with a resident population of 15,222 adults over the age of 40 years (men 7,109, women 8,113), as described previously in detail (Konta et al. 2006). In 2004 or 2005, 1,343 men and 1,626 women (total 2,970 residents in regional population T) took part in the program, agreed to join the study, and went through the medical checkup. In this regional population, mean age and body mass index (BMI) with standard error (SE) were 63.0 ± 0.2 years and 23.5 ± 0.1 kg/m², respectively. Mean values and SD of HDLc in regional population T were 59.1 ± 0.3 mg/dl (Table 1). The second regional population, Funagata (F), consisted of 1,638 individuals in another local area of northeastern Japan; all agreed to join the study. A Caucasian regional population studied elsewhere for ethnic comparison from the state of Utah, USA (U), consisted of a random sample of 488 men and 541 women (total 1,029 individuals).

All works were approved by the institutional ethical committee, and all participants gave written informed consent.

Measurement of plasma lipoproteins

The protocols for lipid and lipoprotein measurement were based on Centers for Disease Control and Prevention (CDC)-approved procedures referred from ARUP National Reference Laboratory, as described previously (Wu et al. 1989). In brief, plasma cholesterol and triglyceride concentrations were assayed enzymatically, and concentrations of HDLc were determined by MgCl_2 -dextran precipitation methods.

Selection and genotyping of single nucleotide polymorphisms (SNPs)

We extracted four SNPs present in intron 1 of the *LIPC* gene according to the dbSNP database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/SNP/>) (Fig. 1). Genotyping of these four SNPs

Table 1 Clinical characteristics of 2,970 individuals in Takahata, Japan (cohort T)

Characteristics	Statistics
Age, year	63.0 (0.2) ^a
Body mass index (kg/m^2)	23.5 (0.1) ^a
TC (mg/dl)	200.7 (0.6) ^a
HDLc (mg/dl)	59.1 (0.3) ^a
LDLc (mg/dl)	124.1 (0.5) ^a
TG (mg/dl)	104.6 (1.0) ^a
RLP (mg/dl)	6.6 (0.1) ^a
Systolic blood pressure (mmHg)	134.2 (0.3) ^a
Diastolic blood pressure (mmHg)	79.3 (0.2) ^a
Drinking	1,231/2,970
Smoking	965/2,970

TC total cholesterol, HDLc high-density lipoprotein cholesterol, LDLc low-density lipoprotein cholesterol, TG triglycerides, RLP remnant-like particle cholesterol

^a Values are the mean $\pm$ [standard error (SE)]

(rs12594375, rs8023503, rs4775047, and rs11634134) was performed by Invader assay (Third Wave Technologies, Madison, WI, USA) according to the manufacturer's protocol (Mein et al. 2000; Lyamichev et al. 1999). The manufacturer provided designed probe sets along with the required reagents. Some genotypes were determined by TaqMan Assay (Applied Biosystems) according to the manufacturer's protocol using the ABI prism 7900HT (Livak 1999).

Haplotyping, linkage disequilibrium, and statistical analysis

Quantitative associations between genotypes and clinical parameters were analyzed by analysis of variance (ANOVA) with regression analysis as a post hoc test using SPSS version 15.0.1J (SPSS, Chicago, IL, USA). The three genotypic categories of each SNP were indicated, e.g., C/C, C/T, and T/T for rs8041059, with values corresponding to the number of chromosomes possessing a minor allele of the respective SNP. Association was examined among subjects in two Japanese regional populations. Significant association was defined when the given *P* value of the ANOVA *F* test was 1% ($P < 0.01$). Chi-square tests were used to ascertain Hardy–Weinberg equilibrium among genotypes ($P > 0.05$) using the R2.4.1 package, genetics (<http://www.r-project.org/>). Linkage disequilibrium (LD) for all possible two-way combinations of the SNPs was tested with *D'* by using Haploview Ver.3.3 (Barrett et al. 2005). Haplotype frequencies were calculated by the haplo.stats program, which implements the methods of Schaid et al. (2001). Haplotypes were inferred, and haplotype frequencies were estimated using the modified expectation-maximization (EM) method of haplotype inference included in the haplo.stats program (Schaid et al. 2001). Haplotype-based associations with clinical traits were performed using the haplo.stats program, and output global-score statistics and haplotype-specific scores were derived from generalized linear models.

Fig. 1 Map of the q22.1 region of human chromosome 15, which contains the *LIPC* gene. The exon–intron structure of the gene is shown; (1)–(4) are single nucleotide polymorphisms present in intron 1

Table 2 Summary of genotyping data for the four *LIPC* polymorphisms examined in this study

NCBI ref. ID ^a	Chr. ^a	Location ^a	Major allele ^a	Minor allele ^a	Allele frequency ^b (heterozygosity %)
rs12594375	15	Intron 1	G	A	0.63/0.37 (30.5)
rs8023503	15	Intron 1	C	T	0.62/0.38 (30.6)
rs4775047	15	Intron 1	C	T	0.58/0.42 (31.5)
rs11634134	15	Intron 1	T	A	0.52/0.48 (34.1)

Chr chromosome

^a According to the SNP database of the National Center for Biotechnology Information (home page: <http://www.ncbi.nlm.nih.gov/>) (Build = 36.2)

^b 2,970 individuals were genotyped

Fig. 2 Linkage disequilibrium (LD) analysis of four single nucleotide polymorphisms in *LIPC*, as measured by D' (a) and r^2 (b)

Results

Almost complete linkage disequilibrium between SNPs rs8023503C/T and rs12594375G/A in intron 1 of *LIPC*

A panel of 2,970 Japanese adult participants was first analyzed in detail for genotypes of four SNPs present in intron 1 of *LIPC*: rs12594375G/A, rs8023503C/T, rs4775047C/T, and rs11634134T/A. Each of these sites had a minor allele frequency >0.1 in this test population at a call rate >99% (Table 2). No deviation of genotype frequencies from Hardy–Weinberg equilibrium occurred among our subjects in any of the polymorphisms. We analyzed LD between the four SNPs among all 2,970 subjects using Thompson's method (D' and r^2). We observed an obvious consistency of genotypes between two

SNPs, rs8023503C/T and rs12594375G/A, by detecting almost complete LD ($D' = 0.97$ and $r^2 = 0.91$) (Fig. 2a, b).

Calculating the frequencies of the constructed haplotypes predicted two major haplotypes segregating among the test population. The most frequently observed haplotype, rs8023503C/rs12594375G, designated here as haplotype-1 (H1), constituted 66.9% of the predicted haplotypes (Table 3). The other haplotype, rs8023503T/rs12594375A, designated here as haplotype-2 (H2), represented 31.3% of the haplotypes. Because the predicted frequencies of two other haplotypes, rs8023503C/rs12594375A and rs8023503T/rs12594375G, were rare (about 2%), we regarded them as negligible for this analysis.

Association of haplotype 2 with elevated HDLc levels

Association of the determined genotypes and haplotypes to plasma levels of HDLc was analyzed among all 2,970 Japanese participants. ANOVA and linear regression tests identified significant association for each of two SNPs: rs12594375G/A ($P = 0.00003$) and rs8023503 ($P = 0.00010$) (Table 4). Similar analysis of the two frequent haplotypes versus HDLc levels in the same individuals revealed a significant association ($P = 0.00011$). Comparison of HDLc levels among the three haplotypic categories—haplotype-1 homozygotes (H1H1), heterozygotes (H1H2), and haplotype-2 homozygotes (H2H2)—revealed the lowest HDLc levels among H1H1 individuals (mean $\pm$ SE = 58.4 ± 0.4 mg/dl), highest for H2H2 (62.5 ± 0.8 mg/dl), and intermediate for H1H2 (59.2 ± 0.4 mg/dl). Possession of haplotype-2 appeared to elevate plasma HDLc.

Table 3 Association of high-density lipoprotein cholesterol with major *LIPC* haplotypes

Haplotypes	rs12594375	rs8023503	Frequency	Positive correlation	<i>P</i> value
H1	G	C	0.66906	−3.76611	0.00017
H2	A	T	0.31264	4.13929	0.00003

Haplotypes with frequencies exceeding 5% are listed

When subjects were separated into two groups, those who bore at least one haplotype-1 (H1H1 and H1H2) and those who lacked haplotype-1 (H2H2 homozygotes), the former group registered significantly lower plasma HDLc levels than the latter (mean $\pm$ SE = 58.8 ± 0.3 mg/dl vs. 62.5 ± 0.8 mg/dl, $P = 0.00005$) (Fig. 3).

Validation of the association of haplotype-2 with high HDLc in a separate Japanese population

To examine reproducibility of our findings, we chose to analyze genotypes of rs8023503 against HDLc for validation of association in regional population F. ANOVA and

Table 4 Analysis of single nucleotide polymorphisms versus various cholesterol parameters in plasma

No.	NCBI reference ID	P value				
		TC	TG	RLP	LDLc	HDLc
1	rs12594375	NS	NS	NS	NS	0.00003
2	rs8023503	NS	NS	NS	NS	0.00010
3	rs4775047	NS	NS	NS	NS	NS
4	rs11634134	NS	NS	NS	NS	NS

TC total cholesterol, TG triglycerides, RLP remnant-like particle cholesterol, LDLc low-density lipoprotein cholesterol, HDLc high-density lipoprotein cholesterol, NS not significant ($P > 0.01$)

Fig. 3 Plasma high-density lipoprotein cholesterol levels (mg/dl) in regional population T (Takahata) (2,970 Japanese adults); P values were calculated by analysis of variance. Histogram bars represent mean values; T -bars above them indicate standard error. The number of individuals analyzed for each haplotype is shown in parentheses under the respective columns

logistic regression analysis in these subjects again revealed a significant association between the *LIPC* allele and HDLc levels; i.e., mean plasma concentrations of HDLc were higher among 173 homozygotic T-allele carriers (mean $\pm$ SE = 60.9 ± 1.1 mg/dl) and 735 heterozygotes (60.1 ± 0.5 mg/dl) than among 725 homozygotic C-allele carriers (57.5 ± 0.5 mg/dl) ($P = 0.00070$) (Fig. 4).

Association of *LIPC* alleles with HDLc levels in the Caucasian study population was not possible because no variation of the rs8023503 SNP was detectable in the regional population U.

Multivariate analysis of HDLc levels against SNPs in intron 1 of *LIPC* and other parameters

To examine association with HDLc levels of other factors, we investigated multiple logistic regression analysis including two SNPs (rs12594375 and rs8023503), gender, age, BMI, systolic blood pressure, diastolic blood pressure, and lifestyle (drinking and smoking status) as confounding factor showed that each of these factors was associated with HDLc. There was interaction between HDLc and rs12594375 ($P < 0.05$), but it was not shown in rs8023503 ($P > 0.05$). Other factors—BMI, drinking (drinker and nondrinker), and smoking (smoker and nonsmoker), also were associated with HDLc ($P < 0.05$, respectively).

Fig. 4 Association of one *LIPC* polymorphism with high-density lipoprotein cholesterol levels (mg/dl) in regional population F (Funagata); P values were calculated by analysis of variance. Histogram bars represent mean values; T -bars above them indicate standard error. The number of individuals analyzed for each genotype is shown in parentheses under each column

Discussion

In this study, we found that significant association between HDLc and a specific haplotype in intron 1 of the *LIPC* gene was detected in both populations. HDL system has received attention in recent years because of its close relationship to the incidence of coronary heart disease. A number of epidemiological investigations have shown an inverse relationship between the incidence of coronary heart disease and high levels of HDLc in plasma (Castelli et al. 1977; Nikkila 1976; Miller and Miller 1975).

Several attempts have been made to examine a potential relationship between HDLc and variation in the *LIPC* gene, focusing on an SNP in the 5' flanking region, –514C/T (Jansen et al. 1997). Jansen et al. first reported association of this promoter SNP with HDLc levels in 1997; subsequent studies confirmed the significance of this association (Jansen et al. 1997; Couture et al. 2000; Guerra et al. 1997; Hubáček et al. 2001; Jansen et al. 1999; Murtomäki et al. 1997; Ordovas et al. 2002; Yamakawa-Kobayashi et al. 2002; Zambon et al. 2003; Chen et al. 2003; Ji et al. 2002; Ko et al. 2004; Arai et al. 2005), and rejected (Vega et al. 1998; Tahvanainen et al. 1998; Grundy et al. 1999; Carr et al. 2002; Fang and Liu 2002; Hegele et al. 1999; Juo et al. 2001; Hong et al. 2000; St-Pierre et al. 2003). Although genetic heterogeneity and other confounding factors, such as ethnic differences, are clearly possibilities, the contradictory results might reflect the limitations of analysis using only the –514C/T variation. The exclusive use of this single SNP in every previous study overlooked other regions of the *LIPC* gene that might have shown associations with HDLc.

In the work reported here, we studied haplotype association by analyzing four SNPs present in intron 1 of *LIPC* in two independent Japanese adult populations (regional population T and regional population F). By constructing haplotypes and analyzing them for association with plasma lipoprotein levels, we demonstrated a significant association of elevated HDLc levels with one of the major haplotypes constructed from two intronic SNPs that were in almost complete LD. Plasma HDLc levels were higher among haplotype-2 homozygotes than among heterozygotes or haplotype-1 homozygotes. These data implied that variation within the first intron of *LIPC* might have affected lipoprotein metabolism in these individuals, eventually inducing variation in plasma HDLc levels. Elevated HDLc could be a result of accelerated turnover to mature HDL(2) particles after uptake of HDL particles by the liver, or a reduced liver uptake of HDL. Future analysis of haplotype variation among various ethnicities, ages, and disease states would be required to prove the hypothesis.

Several interpretations are possible for the association with HDLc that we observed for the two SNPs that were

in strong LD in our Japanese population. First, one site or the other might play a predominant mechanistic role. Second, both nucleotide changes could be required to impart the functional change that underlies the observed association (i.e., a codominant effect of the two polymorphisms). Third, these markers might themselves be in LD with as yet unmeasured and functional variants that are the true mechanistic basis for the association. These possibilities should be distinguished by functional studies in the future. In addition, multivariate analysis showed that intronic variations of the *LIPC* gene and other risk factors (BMI, smoking, and drinking) were associated with HDLc level.

In conclusion, we identified a haplotype association between intronic variations of the *LIPC* gene and plasma HDLc levels in a Japanese population consisting of 2,970 adults and validated the finding in a second Japanese population. The implied involvement of these genetic variations in HDL metabolism may explain, at least in part, the wide variations in plasma HDLc levels noted among individuals and may contribute to establishment of designs for medical checkup, suitable treatments, and preventive strategies to address low HDL as a risk factor for coronary artery disease.

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