

Identification of genetic variants of lecithin cholesterol acyltransferase in individuals with high HDL-C levels

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Abstract. Among the most common lipid abnormalities, a low level of high-density lipoprotein-cholesterol (HDL-C) is one of the first risk factors identified for coronary heart disease. Lecithin cholesterol acyltransferase (LCAT) has a pivotal role in the formation and maturation of HDL-C and in reverse cholesterol transport. To identify genetic loci associated with low HDL-C in a population-based cohort in Tehran, the promoter, coding regions and exon/intron boundaries of LCAT were amplified and sequenced in consecutive individuals (n=150) who had extremely low or high HDL-C levels but no other major lipid abnormalities. A total of 14 single-nucleotide polymorphisms (SNPs) were identified, of which 10 were found to be novel; the L393L, S232T and 16:67977696 C>A polymorphisms have been previously reported in the SNP Database (as rs5923, rs4986970 and rs11860115, respectively) and the non-synonymous R47M mutation has been reported in the Catalogue of Somatic Mutations in Cancer (COSM972635). Three of the SNPs identified in the present study (position 6,531 in exon 5, position 6,696 in exon 5 and position 5,151 in exon 1) led to an amino acid substitution. The most common variants were L393L (4886C/T) in exon 6 and Q177E, a novel mutation, in exon 5, and the prevalence of the heterozygous genotype of these two SNPs was significantly higher in the low HDL-C groups. Univariate conditional logistic regression odds ratios (ORs) were nominally significant for Q177E (OR, 5.64; P=0.02; 95% confidence interval, 1.2-26.2). However, this finding was attenuated following adjustment for confounders. Further studies using a larger sample size may enhance the determination of the role of these SNPs.

Introduction

Cardiovascular diseases (CVDs) are the primary cause of mortality worldwide (1). The prevalence of cardiovascular risk factors, particularly low high-density lipoprotein-cholesterol (HDL-C) levels, was high (32%) in participants of the Tehran Lipid and Glucose Study (TLGS) (2). Several previous studies have found an inverse correlation between plasma HDL-C and the risk of CVDs (3,4). Since HDL-C levels are predominantly under genetic control, with heritability estimates of >80% (5), the detection and characterization of genetic variants associated with HDL-C concentration may provide novel insights in the field of life expectancy. A large number of genes contributing to HDL-C concentration have been identified; however, the variants detected in these genes together explain only a small amount (<10%) of the HDL-C variances (6). Therefore, more studies are required to identify genetic variants, and this may be achieved by sequence-based approaches on the identified genes (6).

While loss-of-function mutations in apolipoprotein A-I (APOA1), LCAT or ATP cassette transporter A1 (ABCA1) cause monogenic HDL-C deficiencies, single gene defects in hepatic lipase (LIPC) or cholesteryl ester transfer protein (CETP) are associated with high HDL-C levels in humans (7,8). LCAT, first described in 1962 (9), is a soluble enzyme that has a central role in the formation and maturation of HDL-C. The majority of the LCAT is synthesized by the liver and circulates in the blood, reversibly bound to lipoproteins. In the blood, LCAT preferentially binds to HDL and is then activated by apoA-I, the major protein component of HDL, and converts phosphatidylcholines and cholesterol of HDL into lysophosphatidylcholines and cholesteryl esters (10). The LCAT gene, located on chromosome 16q22.1 reverse strand, spans 4,382 bp (11) and contains six exons with ~1.5 kb of coding sequence that encodes a 416-amino acid protein (12).

Mutations in the LCAT gene have been found to cause fish-eye disease, as well as familial LCAT deficiency (13). A meta-analysis genome-wide association study (GWAS) on >100,000 individuals (14) and a Mendelian randomization study identified a single-nucleotide polymorphism (SNP) in LCAT as the strongest indicator of isolated variation in levels of HDL-C (15).

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The present study was performed to evaluate the role of genetic variation in LCAT (a biological candidate gene involved in HDL metabolism) in relation to HDL-C levels in epidemiological samples from the TLGS.

Materials and methods

Study population. In this cross-sectional study, 150 individuals, aged between 15 and 75 years, with HDL-C levels <5th percentile adjusted for age and gender (84 individuals) and those with levels of HDL-C >95th percentile adjusted for age and gender (66 individuals), without any other lipid abnormalities, were selected among TLGS participants. The TLGS is a prospective population-based longitudinal cohort study with >15,000 participants designed to determine the risk factors for major noncommunicable disorders in Tehran (16).

To increase the likelihood of genetic causes, only individuals who had the same trait in all four TLGS phases consistently and had at least one family member with the same trait were included. Obese individuals [body mass index (BMI) ≥ 30 kg/m²] and those receiving drugs affecting HDL-C levels were excluded from the study. A standardized questionnaire was used to collect data on demographics and medication usage for lipid disorders and hypertension treatment.

The study was conducted at the Research Institute for Endocrine Sciences in Shahid Beheshti University of Medical Sciences (Tehran, Iran) following approval by the Institutional Ethics Committee. Each participant signed a written informed consent form.

Biochemical assessment. Details of collection, preparation and lipid determination methods, together with details regarding quality control have been previously published (17). Briefly, a venous blood sample was obtained from each subject, and fasting blood glucose (FBS), total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and HDL-C were measured using a Selectra 2 Auto-Analyzer (Vital Scientific, Spankeren, The Netherlands) the same day. TC and TG were measured using the cholesterol enzymatic colorimetric assay kit (Pars Azmoon Inc., Tehran, Iran). Serum HDL-C levels were measured following precipitation of apo B-containing lipoproteins with dextran-magnesium sulfate (18). LDL-C was calculated in serum samples with TG <400 mg/dl using the Friedwald formula (19).

Gene resequencing. In order to analyze the genetic polymorphisms of LCAT, genomic DNA was isolated from the blood of the participants using the standard proteinase K salting-out method (20). Sequences of human LCAT coding regions (GenBank NM_000229.1) were acquired from the University of California, Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu/>) (21). Six DNA fragments were amplified, covering 857 bp upstream of exon 1, all six exons of LCAT and the exon-intron boundaries. Primers were designed using web-based Primer3 software (22) and National Center for Biotechnology Information (NCBI) primer blast programs (23). Primers were verified using electronic PCR on the UCSC Genome Browser to ensure one unique genomic hit, and were also inspected for known SNPs using the SNP

Database (dbSNP) (24) and Ensembl (25). Polymerase chain reaction (PCR) conditions and primer sequences are provided in Table I.

Each PCR amplification was performed with 100 ng genomic DNA in a 30- μ l final volume reaction, containing 40 pmol each primer, 0.2 mmol/l each deoxynucleotide triphosphate, 1.5 mmol/l MgCl₂, 10 mmol/l Tris (pH 8.4) and 0.25 units Taq polymerase (Fermentase Co., Burlington, ON, Canada). Hybridization was conducted in a DNA Thermal Cycler (Corbett Life Sciences, Sydney, Australia), in which, following pre-denaturation for 5 min at 96°C, there were 32 cycles of 96°C for 50 sec, an annealing step at the temperature given for the primer pair (Table I) for 35 sec and an elongation step at 72°C for 50 sec, followed by a final extension at 72°C for 5 min. The PCR products were run on 1.5% agarose gel and the gel was then treated with 1 mg/dl ethidium bromide solution for 10 min. The DNA fragments were subsequently visualized using the gel documentation system OptiGo (Isogen Life Science, De Meern, Netherlands).

Purification and sequencing of the PCR product. The PCR product was purified using the Qiagen kit (Germantown, MD, USA) in order to eliminate excess nucleotides, dimers and nonspecific bands, and the PCR product was then processed through direct sequencing using an Applied Biosystems 3730/3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence alignment analysis was performed using the Basic Local Alignment Search Tool (BLAST) (26), ClustalW (<http://www.ebi.ac.uk/clustalw/>) and chromas programs.

Statistical analysis. The allele frequency and consistency with the Hardy-Weinberg equilibrium were calculated using the PowerMarker software version 3.25 (27). The SPSS Version 15 software package (SPSS, Inc., Chicago, IL, USA) and STATA version 9.1 (StataCorp LP, College Station, TX, USA) were used to analyze the data. Categorical variable distribution was analyzed using the χ^2 test. Multivariate logistic regressions were used to control the effects of potential confounders, including age, BMI, smoking and gender. For all tests, $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of the study population. The mean ages of individuals in the low and high HDL-C groups were 41 ± 13 and 37 ± 16 years, respectively. Table II summarizes the demographic and biochemical parameters, as well as the clinical characteristics of the studied population. The BMI, FBS, TC, TG and LDL-C in the low HDL-C group were significantly different from those in the high HDL-C group.

Identification of SNPs in the LCAT gene. The region (~1,500 bp) of the LCAT gene encoding 440 amino acids was determined by sequencing and NCBI BLAST. A total of 14 SNPs were identified through the sequencing of 300 chromosomes (Fig. 1). The type and position of the SNPs, as well as their frequency of occurrence, are listed in Table III. Six of the nucleotide substitutions were found to be in exons, whilst six SNPs occurred in introns and two were located in the

Table I. Polymerase chain reaction primers used for the analysis of the lecithin cholesterol acyltransferase gene.

Primer name	Primer sequence (5' to 3')	Annealing (°C)	Product size (bp)
Promoter	F: TGTTGCCTCCTTGACTTGAG R: GGGAAGAGCACATTGAGGAG ^a	53	957
Exon 1	F: CCTTTCCGGCAATCTCTG ^a R: TCACAGTGTGGTGGGAGAAG	56	481
Exons 2 and 3	F: CCAGACTGGGTGTTTGCTC R: TGTGTGCAGGTACCCTGTG	59	731
Exon 4	F: AGCAAGCTGGCAGGTTTGTGTCA ^a R: AAGACAGGCTTCCCATAGGCAG	63	362
Exon 5	F: AACAAATGGCTACGTGCGGGACGA ^a R: AGTGGTAGATAGCACCCCTAGAG	62	485
Exon 6	F: TGAGCCTACACTCAGCAGGTTGTG ^a R: CCCATCTTGCCTCACTGCACACA	69	781

^aPrimers used for sequencing. F, forward; R, reverse.

Table II. Characteristics of the study population.

Variables	Subjects with high HDL-C	Subjects with low HDL-C	P-value
Gender, M/F (%/%)	59.1/40.9	75/25	NS
Age (years)	37±16	41±13	NS
TC (mg/dl)	194±42	168±47	0.001
LDL-C (mg/dl)	104±35	90±28	0.012
TG (mg/dl)	81±42	251±179	<0.001
BMI (kg/m ²)	23±3	26±3	<0.001
FBS (mg/dl)	93±9	108±34	0.001
SBP (mmHg)	113±19	114±17	NS
DBP (mmHg)	73±11	77±9	0.016

Demographics and lipid levels among subjects with high and low HDL-C levels are shown. HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; BMI, body mass index; FBS, fasting blood glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; NS, not significant.

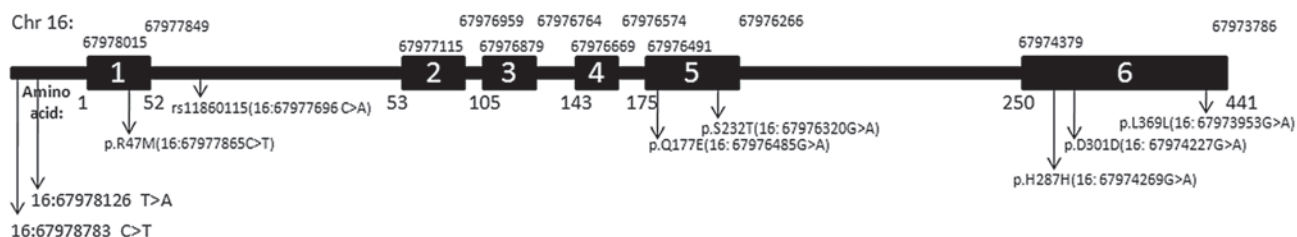


Figure 1. Structural diagram of the lecithin cholesterol acyltransferase gene showing the position of important single-nucleotide polymorphisms identified. Black boxes indicate exons 1-6. Nucleotide numbering reflects chromosome (NC_000016.9) and amino acid numbering is according to GenBank: AAB34898.1.

promoter. The nucleotide variations in the 5' regulatory region occurred in one gene copy. These substitutions included a C instead of T at position 4,233 (g.4233T>A) and G instead of A at position 4,890 (g.4890C>T) (positions given according to GenBank entry NG_009778.1).

Three of the SNPs in the protein coding regions were non-synonymous, whilst the others were synonymous. One SNP in exon 1 was observed in only one individual. No polymorphisms were found in exon 2. Three of the SNPs were in exon 6, two of which were synonymous and one of which was

Table III. Summary of variations identified in lecithin cholesterol acyltransferase.

Variants	HGVS names/SNP ID	Location	Amino acid position	Amino acid change	MAF	Predicted effect (PolyPhen)	Score
Chr: 16:67978783	g.4233T>A	Promoter	NA	NA	0.004		
Chr: 16:67978126	g.4890C>T	Promoter	NA	NA	0.004		
Chr: 16:67977696 ^a	g.5320T>G rs11860115	Intron	NA	NA	0.007		
Chr: 16:67977865 ^a	g.5151G>A COSM972635 (R47M)	Exon 1	47	Arg-Met	0.004	Possibly damaging	0.739
Chr: 16:67976686	g.6330G>A	Intron	NA	NA	0.004		
Chr: 16:67976682	g.6334G>A	Intron	NA	NA	0.007		
Chr: 16:67976555	g.6461G>A	Intron	NA	NA	0.004		
Chr: 16:67976261	g.6755G>A	Intron	NA	NA	0.004		
Chr: 16:67973791	g.9225A>G	Intron	NA	NA	0.004		
Chr: 16:67976485	g.6531C>G or c.529C>G (Q177E)	Exon 5	177	Gln-Glu	0.046	Possibly damaging	0.953
Chr: 16:67976320 ^a	g.6696T>A or c.694T>A rs4986970 (S232T)	Exon 5	232	Ser-Thr	0.004	Probably damaging	0.977
Chr: 16:67973953 ^a	g.9063C>T or c.1177C>T rs5923 (L369L)	Exon 6	393	Leu-Leu	0.050		
Chr: 16:67974227	g.8789C>T (D301D)	Exon 6	301	Asp-Asp	0.004		
Chr: 16:67974269	g.8747C>T (H287H)	Exon 6	287	His-His	0.004		

^aThese sequence variations have been identified previously. HGVS, Human Genome Variation Society; SNP, single-nucleotide polymorphism; MAF, minor allele frequency; Arg, arginine; Met, methionine; Gln, glutamine; Ser, serine; Thr, threonine; Leu, leucine; Asp, aspartic acid; His, histidine; PolyPhen, Polymorphism Phenotyping.

Table IV. Allele frequencies of lecithin cholesterol acyltransferase variants.

Number	Variant	Allele frequency (%)		P-value ^a
		High HDL group	Low HDL group	
1	Chr: 16:67978783 C>T	0.9	0.0	0.41
2	Chr: 16:67978126 G>A	0.0	0.6	0.52
3	Chr: 16:67977696 A>C	0.0	1.2	0.37
4	Chr: 16:67977865 C>T	0.0	0.6	0.52
5	Chr: 16:67976687 G>T	1.5	0.0	0.44
6	Chr: 16:67976682 C>T	0.0	1.2	0.37
7	Chr: 16:67976555 C>T	0.9	0.0	0.41
8	Chr: 16:67976261 C>T	0.0	0.6	0.52
9	Chr: 16:67973791 T>C	0.0	0.6	0.52
10	Chr: 16:67976485 G>C	1.5	7.5	0.09
11	Chr: 16:67976320 A>T	0.9	0.0	0.41
12	Chr: 16:67973953 G>A	2.6	8.2	0.16
13	Chr: 16:67974227 G>A	0.9	0.0	0.41
14	Chr: 16:67974269 G>C	0.9	0.0	0.41

^aFisher's exact test. HDL, high-density lipoprotein.

non-synonymous. Whilst all nucleotide substitutions in the 5' sequences were transition mutations, two of the substitutions within the coding sequences and two of the substitutions within the intron sequences were transversions.

A review of the dbSNP found that, to date (May 2013), 161 SNPs in the human LCAT gene have been submitted (24). Additionally, the Ensembl Genome Browser (25) has indexed 1,029 variants in the human LCAT gene with 1,136 downstream gene variants and 1,872 upstream gene variants (http://www.ensembl.org/Homo_sapiens/Gene/Variation_Gene/Table?g=ENSG00000213398;r=16:67973653-67978034). In addition, >80 causative mutations in LCAT have been described (28) in the Human Gene Mutation Database (HGMD; <http://www.hgmd.cf.ac.uk/ac/>) (29).

Ten of the identified SNPs were found to be novel. The L393L, S232T and 16:67977696 C>A polymorphisms have been previously reported in the dbSNP Human Build 137 (as rs5923, rs4986970 and rs11860115, respectively), and the non-synonymous R47M mutation (COSM972635) was observed in Ensembl (release 18, 2013) and has been reported in the Catalogue of Somatic Mutations in Cancer (<http://cancer.sanger.ac.uk/cosmic/mutation/overview?id=972635>) (30).

Six of the 14 polymorphisms were found only in the high-HDL-C group, whilst six were only in the low-HDL-C group. Three of the 150 individuals carried two of the SNPs of LCAT. The most common variant was L393L in exon 6, found in 15 of 300 alleles, and the Q177E in exon 5, found in 14 of 300 alleles. Allele frequencies of low and high HDL-C groups are shown in Table IV. No significant differences were detected between the study groups. Evaluating genotype frequencies in low versus high HDL-C groups using Fisher's exact test demonstrated a significant association for the synonymous L393L (P=0.04) and the non-synonymous Q177E (P=0.02) SNPs. Univariate conditional logistic regression odds ratios

(ORs) were nominally significant for Q177E models only [OR, 5.64; P=0.02; 95% confidence interval (CI), 1.2-26.2]. Multivariate logistic models with adjustment for age, gender, smoking, FBS and BMI attenuated the effect (OR, 2.58; P=0.47; 95% CI, 0.16-39.5).

Three of the identified SNPs (position 6,531 in exon 5, position 6,696 in exon 5 and position 5,151 in exon 1) gave rise to amino acid substitutions. Computational tools, for example Sorting Intolerant From Tolerant (31) and Polymorphism Phenotyping (PolyPhen) (32) are able to predict 90% of damaging SNPs. Table III shows analysis using PolyPhen, which revealed that two of the missense SNPs were possibly damaging, and one of them is probably damaging (available at <http://genetics.bwh.harvard.edu/pph2/index.shtml>).

Discussion

In the present study, enzymatic amplification and direct sequencing of the LCAT gene from individuals with high and low HDL-C were performed in order to investigate the association between LCAT and HDL-C levels in a population-based study in Tehran. Since LCAT is a biological candidate for HDL-C levels, the common and/or rare variants of LCAT are hypothesized to contribute to variation in HDL-C levels between individuals.

To date, candidate genes, genome-wide linkage and GWASs have identified several genetic variations that may account for plasma HDL-C levels (8). GWASs independently identified numerous genetic loci for HDL-C (33-35). For example, eight GWASs analyzing HDL-C levels have confirmed the following genes that were recognized from functional studies: LCAT, CETP, LIPC, lipoprotein lipase, ABCA1, endothelial lipase and the gene cluster APOA1C3A4A5 (6). A meta-analysis GWAS, including >100,000 individuals, identified an SNP in

the LCAT gene as the strongest marker of isolated variation in HDL-C levels (14). This indicates that variants in the LCAT gene associated with levels of HDL-C may be useful for a formal causality test of HDL-C levels for determining the risk of CVDs (15).

The genotyping platforms typically used in GWASs include mostly common variants [minor allele frequency (MAF)>0.05] that are unlikely to tag most of the rare variants in the genome or to identify the true causative variant due to the small effect sizes of common variants. One of the strategies used to identify these rare variants is the resequencing of extreme phenotypes, for which individuals at both extremes of the phenotype (e.g. highest and lowest five percentiles) are selected for further sequencing. These individuals may be part of a population-based cohort or a case-control study (36). Variants with major phenotypic effects are likely to be found only at one extreme, while alleles found in both the high and low HDL-C groups are most likely neutral with respect to HDL-C levels (37). Resequencing, unlike GWASs, requires a decision as to which gene to target. Resequencing is usually used to analyze the regions within a gene rather than the region between genes, and within exons rather than introns, and it is able to detect not only SNPs, but also structural variations such as copy-number variations (deletions and insertions) and copy-neutral variants (inversions and translocations) (36). Furthermore, resequencing requires smaller sample sizes, and previous studies have obtained positive results with initial sample sizes of ≤ 200 (100 from each phenotypic extreme) (38). These positive findings may then be followed by studies on populations enriched for the variants, for example families of individuals who carry the risk alleles.

Cohen *et al* (37) resequenced three candidate genes (ABCA1, APOA1 and LCAT) in 256 individuals constituting the upper and lower 5% of the distribution of plasma HDL-C levels in a population-based study. The number of synonymous variants was similar in high and low HDL-C groups. Non-synonymous sequence variants were significantly more common (16 vs. 2%) in individuals with low HDL-C compared with those with high HDL-C (six LCAT non-synonymous sequence variations were unique to the low-HDL-C group) (37). Therefore, it was concluded in the study that multiple rare, non-synonymous sequence variants of the genes that are involved in HDL metabolism may influence HDL-C levels. The results of the study supported the 'rare variant-common disease' hypothesis (39).

In the present study, a similar approach was used as Cohen *et al* (37); however, the entire gene and flanking regions were sequenced so that the regulatory variants were also identified. A total of 150 individuals with HDL-C levels in the extremes were sequenced, and 14 variants were identified. Three of the variants were non-synonymous sequence variants, of which one was significantly more common in individuals with low HDL-C compared with those with high HDL-C. The other two were present only in the high HDL-C group. Three synonymous mutations were found in exon 6; only one of these mutations had an MAF of 5%, and this mutation was more common in the low HDL-C group. The majority of identified variants (71.4%) were absent from the dbSNP Human Build 137; however, some of them (21.4%) were present in the 1,000 Genomes Project (<http://www.1000genomes.org>) and

one SNP (COSM972635) was present in Ensembl (24). A synonymous H287H mutation in the coding region of exon 6 of the LCAT gene was observed in an individual with HDL-C levels of 75 mg/dl. CD952176 is the accession number of the coding sequence variant from the HGMD-PUBLIC dataset December 2012 (release 2012.4) that is present at 16:67974268. A previous study (40) demonstrated that point mutations (deletion or frameshift mutations) at this position result in LCAT deficiency.

Tietjen *et al* (41) resequenced 178 unrelated probands with HDL-C <10th percentile and showed that mutations were most frequent in LCAT, followed by ABCA1 and APOA1; together, these explain >40% of familial hypoalphalipoproteinemia. Furthermore, they observed a clear elevation in coronary artery disease risk among individuals with the mutations and a large reduction in HDL-C (41). Holleboom *et al* (28) found high mutation frequencies of LCAT in individuals with low levels of HDL-C in the Netherlands, and 50% of the LCAT mutations were a p.T147I mutation (28).

Due to time constraints and technical issues in the present study, the identified variants were not genotyped. When all variants are genotyped in the entire population, it may be possible to determine the extent to which LCAT variants influence HDL-C levels in the TLGS population.

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