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Novel P143L polymorphism of the LCAT gene is associated with dyslipidemia in Chinese patients who have coronary atherosclerotic heart disease

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Abstract

Coronary atherosclerotic heart disease (CAD) is a multifactorial disorder resulting from numerous gene–gene and gene–environment interactions. Lecithin:cholesterol acyltransferase (LCAT), a key enzyme in reverse cholesterol transport and the metabolism of high-density lipoprotein (HDL), is thought to be a candidate gene related to dyslipidemia and CAD. Variations in the LCAT gene were investigated in 190 CAD patients and 209 age- and gender-matched controls by denaturing high-performance liquid chromatography, and confirmed by sequencing and RFLP assay. In CAD patients, a novel single-nucleotide polymorphism (P143L) in exon 4 of the LCAT gene was discovered in nine males and two females (frequency of 5.79%), which was found in none of 209 controls. The genotype and allele distribution of P143L is significantly ($P < 0.04$) higher in the low HDL-C subgroup than in the normal HDL-C subgroup in both male patients and all CAD patients. P143L was also found to be significantly ($P < 0.01$) associated with the low HDL-C phenotype in both male patients and all CAD patients, with odds-ratios of 7.003 (95% CI 2.243–21.859) and 5.754 (95% CI 1.893–13.785), respectively. Thus, the P143L polymorphism may play a role in causing decreased HDL-C levels, leading to increased risk of dyslipidemia and CAD in Chinese.

Keywords: LCAT gene; P143L polymorphism; DHPLC; Dyslipidemia; Coronary atherosclerotic heart disease

Coronary atherosclerotic heart disease (CAD) is one of the main leading causes of death worldwide. It is a complex disease resulting from numerous gene–gene and gene–environment interactions. Epidemiological studies over the past 50 years have revealed that—among the multiple risk factors—the relative abundance of the various plasma lipoproteins is of primary importance in the pathogenesis of CAD. Moreover, it has been established that dyslipidemia, including elevated levels of atherogenic lipoproteins and/or reduced levels of high-density lipoprotein (HDL), is a prerequisite for most forms of CAD [1,2].

Lecithin:cholesterol acyltransferase (LCAT), which is responsible for the synthesis of most of the cholesteryl esters in plasma by catalyzing the transacylation of the sn-2 fatty acid of lecithin to the free 3′-hydroxyl group of cholesterol in HDLs, plays a key role in cholesterol and HDL metabolism. It also facilitates a pivotal step in reverse cholesterol transport (RCT), a physiologic process by which peripheral cell-derived cholesterol

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is transported through plasma to the liver for catabolism [3–5]. In humans, the plasma HDL-cholesterol (HDL-C) concentration has been found to be correlated positively with plasma LCAT concentration [6]. In addition, the role of LCAT in facilitating RCT, modulating lipoprotein metabolism and atherosclerosis has been demonstrated in vivo. Therefore, LCAT is thought to be one of the candidate genes associated with dyslipidemia and CAD, as well as a putative new target for gene therapy to prevent CAD [7].

The human LCAT gene is located on chromosome 16q22.1. The mRNA (NM000229) of this gene is 1354 bp in length, and the gene spans 4.6 kb and is divided into six exons (NT010478). Substantial levels of the LCAT mRNA—in the human, rabbit, and mouse—are present in liver, with much lower levels in the brain [5].

Although the DNA sequence of LCAT is highly conserved among mammalian species, 38 mutations in the gene, leading to complete or partial losses of enzymatic function, have been reported (HGMD; http://uwc-mml1s.uwcm.ac.uk/uwcm/mg/search/119359.html). The LCAT protein seems to be very vulnerable in that all these mutations impair its function, although to varying degrees, and lead to familial LCAT deficiency (FLD) syndrome or fish eye disease (FED). Most of these mutations, however, are rare, with frequencies of less than 1 in 10,000. In addition, it is noteworthy that homozygotes or compound heterozygotes in families with FLD or FED exhibit a virtual absence, or marked deficiency, of mature plasma HDLs [8].

To date, only three coding single-nucleotide polymorphisms (cSNP) of the LCAT gene have been reported. They are 608C > T, 911T > C, and 1188C > T. The frequencies of each are less than 10% [9,10]. There have been no extensive population studies of these polymorphisms, however, especially on CAD populations, and no data about Chinese population have ever been reported.

Recently, we characterized SNP frequencies in a series of genes relevant to lipid metabolism in patients with CAD [11,12]. Here, to further our understanding of the possible role played by SNPs in dyslipidemia and CAD, we screened the SNPs of the LCAT gene in 190 Chinese patients with CAD and 209 controls from West China by high-throughput denaturing high-performance liquid chromatography (DHPLC). A novel cSNP, P143L, was discovered, and a gender-specific association with dyslipidemia was observed.

Materials and methods

Subjects in this study. For this study, 190 unrelated CAD patients, 134 males, and 56 females, aged from 48 to 77 with a mean age of 59.66 ± 8.7, were enrolled from the West China Hospital, Sichuan University. The diagnosis was confirmed by coronary angiography using the Judkins technique. Individual having at least one stenosis >60% in any of the major branches of a coronary artery (left anterior descending, left circumflex, right coronary artery) was qualified as a "CAD patient." None of the patients took hypolipidemic drugs prior to coronary angiography and lipid measurements. In addition, 209 unrelated age- and gender-matched subjects free of any clinical or biochemical signs of CAD were selected via health examinations at the same hospital as controls. Informed consents were obtained from all of the 399 subjects involved in this study. Measurement of lipids and lipoproteins of the patients and controls was performed as previously described [11].

PCR amplification. Genomic DNA was extracted from leukocytes of peripheral blood samples by the "salting-out" procedure and then stored at 4 °C for use. Five sets of primer pairs were designed, according to the sequence of the LCAT cDNA (NM000229) and genomic DNA (NT010478) to amplify its six exons (Table 1). The PCR profile was 94 °C for 2 min followed by 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 30 s, 30–36 cycles with a final extension at 72 °C for 5 min.

Variation screening of the LCAT gene by DHPLC. DHPLC screening for single-nucleotide variations of all six exons in 190 CAD patients and 209 controls was performed on an automated HPLC instrument (Hewlett Packard, Palo Alto, CA), as described [11,12]. Briefly, the support for the stationary phase was made of the specially prepared wide-pore silica with a dense layer of an aliphatic organosilane packed into a 50×4.6-mm i.d. dsDNA-analysis column (Hewlett Packard). The mobile phase was 0.1 M triethylammonium acetate (PE BioSystems, Foster City, CA) buffer at pH 7.0 containing 0.1 mM ethylenediaminetetraacetic acid. PCR products were eluted with a linear acetonitrile gradient. The temperatures required for successful resolution of heteroduplex molecules were determined by the DHPLC algorithm (http://insertion.stanford.edu/melt.html), and adjusted empirically by running it at different temperatures until the best resolution between homo- and heteroduplexes was obtained. Temperatures used for the five amplified fragments were 66, 61, 62, 62, and 63 °C, respectively. The appearance of additional peaks or shoulders during the chromatography was regarded as indicative of a single-base mismatch in the heteroduplex DNA fragments. Such samples were then subjected to two additional turns of PCR-DHPLC to exclude the possibility of false positive heterozygosity resulting from PCR.

DNA sequencing and restriction fragment length polymorphism assay.The confirmed heteroduplex DNA fragments obtained by DHPLC were cloned into the pGEM-T Easy Vector (Promega).

Table 1
Primers for PCR amplification of the six exons of the LCAT gene and lengths of the products amplified

Region amplified	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size of products (bp)
Exon 1	GGCAGTAGGCACCAGG	GCTTACCGAGGATGACG	182
Exon 2–4[a]	ATCCAGAGTCCAGAGTGAGG	CCGCAGAGACACTCACC	592
Exon 5	GCCAGCAGGAGGAGTACTAC	TGGACCTAAGTGTTCGAGG	253
Exon 6A	TGTCCCACCTTGCTCC	CCGTGTCATCACCATCC	392
Exon 6B	ATGAGGATGGTGATGACACG	GGGCTTACGGTAGCAAAGG	256

[a] This fragment includes introns 2 and 3.

For each fragment, at least 12 clones obtained by blue/white screening were selected for plasmid preparation, and six clones with the desired inserts were sequenced using the Thermo Sequenase Cycle Sequencing Kit (Amersham–Pharmacia) and an ALFexpress Automatic DNA Sequencer (Pharmacia). The variations obtained from sequencing were then confirmed by restriction fragment length polymorphism assay (RFLP) in which the DNA was digested with *Mnl*I.

Statistical analysis. The phenotypic data of the dyslipidemic vs. other CAD patients were adjusted for age and gender, and were statistically analyzed using Student's t test. χ^2 analyses were performed to compare overall allelic and genotype frequency of the polymorphic site in male and female patients separately, and in the normal HDL-C (NHDL) and low HDL-C (LHDL) subgroups, divided according to the representative lipoprotein index (HDL-C level). The adjusted odds-ratios (ORs) for the CAD trait and for the NHDL and LHDL groups were calculated using logistic regression analysis.

Results

Phenotype of the patients

Table 2 lists the demographics and lipid profiles (phenotypes) of the 399 subjects studied. Values for the plasma cholesterol, plasma triglycerides, and LDL-cholesterol were considerably higher in both male and female CAD patients than those in controls. HDL-C was considerably lower in male and female CAD patients, compared with that in controls.

Abnormal postprandial lipid and lipoprotein levels are generally thought to be best determined after an overnight fast. Of the 190 CAD patients investigated (Table 3), 89 subjects fulfilled the commonly accepted clinical normal criteria (NCEP-ATPIII, National Cholesterol Education Program Adult Treatment Panel III) for the LHDL subgroup (HDL-C < 1.03 mmol/L). Another 101 patients belonged to the NHDL subgroup (HDL-C $\geqslant 1.03$ mmol/L). As shown in Table 3, plasma cholesterol and LDL-cholesterol levels in the LHDL subgroup for males and females were significantly higher. The reverse was seen for the HDL-C levels in the LHDL subgroup. Adjusted for the body mass index, the lipid data for each group remained unaltered.

Genotype of the 380 chromosomes of CAD patients

As the result of screening for possible DNA variations in all six exons, a novel SNP P143L (+511C > T) was discovered in exon 4 (Fig. 1). This C > T transition causes a non-synonymous mutation and creates a new *Mnl*I restriction site that can be easily confirmed by an RFLP assay (Fig. 2). Using the RFLP assay as a check in both the CAD patients and controls, we screened again for possible heterozygotes and homozygotes that might have been missed by DHPLC analysis; this RFLP screen would also exclude any false positive signals

Table 2
Demographics and lipid profiles of CAD patients and controls

Trait	Patients		Control		P[b]	
	Male ($n = 134$)	Female ($n = 56$)	Male ($n = 124$)	Female ($n = 85$)	M vs. M	F vs. F
Age	59.4 ± 9.0[a]	60.2 ± 8.09	56.2 ± 10.11	55.6 ± 8.55	NS[c]	NS[b]
BMI	24.1 ± 0.92	23.8 ± 1.01	23.9 ± 1.18	24.1 ± 1.34	NS	NS
Plasma cholesterol	5.31 ± 0.93	5.69 ± 1.07	4.50 ± 0.79	4.33 ± 0.24	<0.01	<0.01
Plasma triglycerides	1.91 ± 1.00	2.17 ± 1.13	1.16 ± 0.49	1.20 ± 0.58	<0.01	<0.01
LDL-cholesterol	3.17 ± 0.61	3.29 ± 0.75	2.55 ± 0.60	2.37 ± 0.35	<0.01	<0.01
HDL-C	1.07 ± 0.29	1.19 ± 0.26	1.79 ± 0.66	1.84 ± 0.42	<0.01	<0.01

[a] All levels are given in mmol/L except body mass index (BMI) which is given in kg/m^2 and age given in years.
[b] Statistical analysis was performed using Student's t test.
[c] NS, no significant difference ($P > 0.05$).

Table 3
Serum lipid and lipoprotein levels (means ± SD) in CAD patients with the LHDL or NHDL phenotype[a]

Trait	Patients with LHDL		Patient with NHDL		P	
	Male ($n = 65$)	Female ($n = 24$)	Male ($n = 69$)	Female ($n = 32$)	M vs. M	F vs. F
Age	59.6 ± 8.91	60.2 ± 9.84	59.1 ± 9.15	60.2 ± 7.49	NS[b]	NS[b]
BMI	24.1 ± 0.79	23.6 ± 0.91	24.2 ± 1.08	23.8 ± 1.03	NS	NS
Plasma cholesterol	3.83 ± 0.78	3.85 ± 1.11	4.74 ± 0.94	4.99 ± 0.84	<0.01	<0.01
Plasma triglycerides	1.97 ± 0.90	1.89 ± 0.74	1.92 ± 0.83	2.34 ± 0.58	NS	NS
LDL-cholesterol	1.95 ± 0.66	1.77 ± 0.55	2.39 ± 0.72	2.48 ± 0.65	<0.01	<0.01
HDL-C	0.86 ± 0.17	0.88 ± 0.15	1.31 ± 0.33	1.32 ± 0.22	<0.01	<0.01

[a] The LHDL phenotype is characterized by HDL-C <1.03 mmol/L, as the commonly accepted standard (NCEP-ATPIII, National Cholesterol Education Program Adult Treatment Panel III). All lipid levels are given in mmol/L; the body mass index (BMI) is given in kg/m^2 and the age is given in years. Statistical analysis was performed by using Student's t test.
[b] NS, no significant difference ($P > 0.05$).

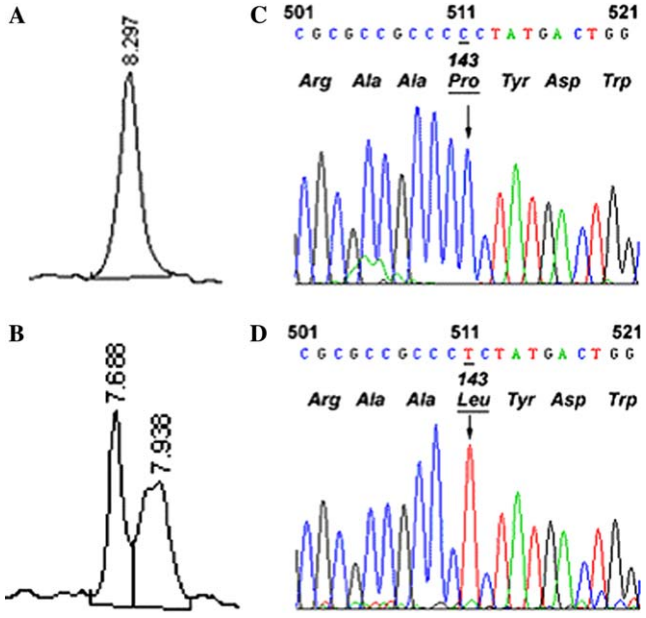

Fig. 1. DHPLC screening for, and DNA sequencing of, the P143L polymorphism in the LCAT gene. (A,B) Chromatographic patterns of the wide-type homozygote and heterozygote, respectively. (C,D) Corresponding sequencing results. The nucleotides and amino acids are numbered according to NM000229 and NP000220. The variations in them are underlined and indicated by the arrows.

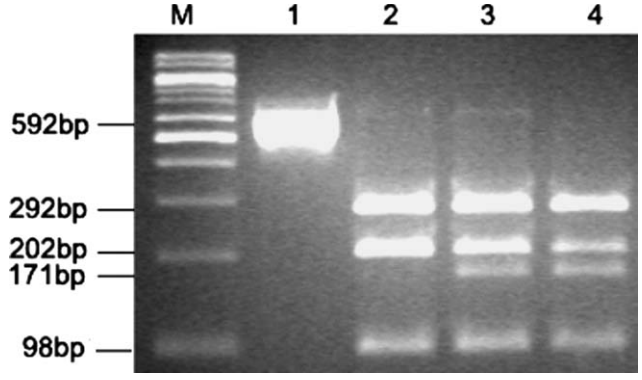

Fig. 2. RFLP assay of the polymorphism P143L (+511C > T) using the restriction endonuclease *Mnl*I. Lane 1, the expected 592-bp PCR-amplified product; lane 2, wild-type homozygote presenting with four bands: 292, 202, 98 bp, and an invisible 10 bp; and lanes 3–4, P143L heterozygotes, showing an additional 171-bp band. M, 100-bp DNA ladder.

resulting from cloning and sequencing. Based on this comprehensive analysis by DHPLC, DNA sequencing, and the RFLP assay, all the SNPs and the genotypes were obtained. As a result, 11 P143L heterozygotes but no homozygotes were found in the 190 CAD patients; in the 209 controls, not a single heterozygote or homozygote of this SNP was found (Table 4). In addition, the R399C mutation in exon 6, which has been previously reported, was found in two patients; because this was regarded as a rare polymorphic variant, we did not carry out any statistical analysis on this polymorphism.

Table 4
Genotype and allele distribution of SNP P143L in the LCAT gene and its association with dyslipidemia in 190 Chinese patients with CAD

LHDL patients					NHDL patients						*P* value[c]				
Male		Female		Total		Male		Female		Total		Genotype-wise		Allele-wise	
Wt[a]	Het	Wt	Het	Wt	Het	Wt	Het	Wt	Het	Wt	Het	M vs. M	L vs. N[b]	M vs. M	L vs. N
57	8	22	2	79	10	68	1	32	0	100	1	0.030	0.003	0.033	0.004

[a] Abbreviations are identical to those in previous tables except Wt and Het represent the individual numbers of wild type and P143L heterozygotes, respectively.
[b] L vs. N represents the LHDL vs. NHDL subgroup.
[c] Statistical analysis was performed using χ^2 test.

The P143L polymorphism in CAD patients with or without dyslipidemia

Because gender is likely to have a significant effect on heart disease, the possible association of P143L in the LCAT gene with CAD was analyzed separately in males and in females. As shown in Table 4, the genotype and allele distribution of P143L between the NHDL and LHDL subgroups is significantly different, both in males and in the CAD patient group, with ORs [95% confidence intervals] of 7.003 [2.243–21.859] and 5.754 [1.893–13.785], respectively. No further statistical analysis was done for female patients, because only two P143L alleles were found in the 24 LHDL subjects and none in the 32 NHDL subjects. Considering all CAD patients and controls, it is worthy of note that the cSNP P143L was found only in CAD patients and not in controls, and that this difference was statistically significant. Furthermore, the P143L polymorphism was found to be in accordance with Hardy–Weinberg expectations for the CAD group (data not shown).

Discussion

LCAT plays an important role in lipoprotein metabolism, especially in the process termed reverse cholesterol transport. The enzyme is synthesized in the liver and circulates in blood plasma as a complex with components of HDL. Excess cholesterol from peripheral cells is transferred to HDL particles, esterified through the action of LCAT on plasma HDL, and incorporated into the core of the lipoprotein. Thus, LCAT promotes not only flux of more cholesterol from tissues into HDL, but also promotes the maturation of HDL. The cholesterol ester is thereby transported to the liver for catabolism. Since most cholesterol esters present in plasma are produced by LCAT, lack of LCAT activity would be expected to lead to accumulation of free cholesterol in the tissues and decrease of mature HDL (HDL-C) [13]. In humans, plasma HDL-C concentrations have been found to be positively correlated with plasma LCAT concentrations [6].

Numerous epidemiological studies have shown a negative correlation of HDL-C level with the incidence and severity of CAD. Moreover, decreased HDL-C levels have been used as a marker for dyslipidemia and an increased risk factor of CAD, especially in populations of subjects more than 50 years of age [14]. Decreases in normal HDL-C could lower an individual's resistance against atherosclerosis. In the human, about half of the patients reported with low LCAT activity display low HDL-C levels and premature CAD. Studies on transgenic animal models have also demonstrated the important physiological role of LCAT in modulating HDL metabolism and in preventing diet-induced CAD in the intact animal; such studies show the protective effect of LCAT on CAD and LCAT as a potential target for therapeutic manipulation [15–17].

The enzymatic function of LCAT is vulnerable to small changes in the protein. To date, 38 highly dispersed mutations of the LCAT gene have been described (HGMD; http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/119359.html). Extensive evidence suggests that these rare mutations cause the clinical phenotype of either familial lecithin:cholesterol acyltransferase deficiency, characterized by the total loss of LCAT activity) or fish eye disease (FED, characterized by the partial loss of LCAT activity). Although for the time being the exact site of these mutations does not allow us to predict the associated biochemical or clinical phenotype, homozygous or compound heterozygous mutations in the LCAT gene, such as G230R in Finns, represent a relatively common genetic cause of diminishing HDL-C levels [18]. In addition, the P10Q mutation found in Netherlands or the T123I mutation in Germany appears to lead to premature CAD, resulting in the lack of normal maturation of HDL due to LCAT dysfunction [19–21]. However, it is still controversial as to whether LCAT deficiency is associated with premature CAD, due to the small patient population size that was studied and the limited data about the effect of LCAT gene mutations on HDL-C concentrations and risk of CAD at the population level. Thus, to advance our knowledge of the LCAT gene, population-based studies on LCAT polymorphisms or mutations are still needed.

In the present study we have discovered a novel cSNP, +511C > T (P143L), in the LCAT gene with a frequency of 5.79% in 190 Chinese CAD patients. This polymorphism was not found in 209 controls and therefore seems to have a significant association with the CAD trait in heterozygotes carrying this allele. Thus, the frequency of P143L heterozygotes was significantly elevated in the low HDL-C (LHDL) group, compared with that in the normal HDL-C (NHDL) group, among the 190 CAD patients; the P143L heterozygote frequency was also significantly elevated in male patients with LHDL. This significant association between P143L and the LHDL phenotype (Table 4) suggests that the P143L polymorphism might play a role in causing lowered HDL-C levels, and hence in the development of dyslipidemia and CAD, in these patients. Only two female heterozygous individuals were found in the present study, due to the smaller size of the female patient population. Moreover, no homozygotes were found in this study. The possibility of finding a homozygote would be very low in a population of 190 patients. The polymorphism frequency was 0.0579, meaning the likelihood of finding a +511T/T homozygote would be $(0.0579)^2 = 0.0033$, or 3 in 1000 patients; not finding one homozygote in 190 patients has a P value by χ^2 analysis of >0.05. Whether a P143L homozygous subject exists,

or whether this polymorphism is compatible with life, remains to be determined.

This P143L polymorphism is located in exon 4, which is 96 bp in length and encodes 31 amino acids (from residue 120 to 150). Although the exact function of this region is currently unknown, 10 of 38 common mutations in the LCAT gene have been found in this limited area. These include eight non-synonymous mutations—T123I, N131D, R135Q, R135W, R140H, A141G, Y144C, and R147W—-one 2-bp (TG) deletion at 138 and one 3-bp (GGC) insertion at 141. Among these mutations, only the R135Q and Y144C carriers presented with the FED phenotype, and the others presented with the FLD phenotype. The exon-4 region therefore seems to be a hot area for disease mutations. As suggested by Kuivenhoven et al. [8], amino acids 123–156 of LCAT are important for interaction of the enzyme with lipoproteins, by regulating its affinity with the lipoprotein substrate. This point of view is further supported by site-directed mutagenesis. For instance, whereas R147W and T123I alter the lipid binding or transesterification domains, they do not disrupt the functional domain mediating LCAT phospholipase activity [22]. Whether the P143L polymorphism reported here might be critical to substrate binding is not yet known. It is well known, however, that proline is an essential amino acid residue involved in formation of a β-turn, an important aspect of secondary structure; hence, the replacement of proline by leucine at position 143 may change the β-turn and lead to impairment of LCAT function. Further studies are needed to confirm this hypothesis.

In conclusion, our present study suggests that the novel cSNP, +511C > T (P143L), may contribute to increased risk of dyslipidemia and CAD. Obviously, additional independent population studies of this variant in several ethnic groups are required to confirm its association with dyslipidemia and CAD, and to elucidate the exact function of this mutation. In addition, only the three-dimensional structure of LCAT is firmly established, the role of different mutations in the LCAT gene during the development of dyslipidemia and atherosclerosis could be fully understood.

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