Transmission of two novel mutations in a pedigree with familial lecithin:cholesterol acyltransferase deficiency: structure–function relationships and studies in a compound heterozygous proband

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Abstract **Two novel mutations were identified in a compound heterozygous male with lecithin:cholesterol acyltransferase (LCAT) deficiency. Exon sequence determination of the LCAT gene of the proband revealed two novel heterozygous mutations in exons one (C110T) and six (C991T) that predict non-conservative amino acid substitutions (Thr13Met and Pro307Ser, respectively). To assess the distinct functional impact of the separate mutant alleles, studies were conducted in the proband's 3-generation pedigree. The compound heterozygous proband had negligible HDL and severely reduced apolipoprotein A-I, LCAT mass, LCAT activity, and cholesterol esterification rate (CER). The proband's mother and two sisters were heterozygous for the Pro307Ser mutation and had low HDL, markedly reduced LCAT activity and CER, and the propensity for significant reductions in LCAT protein mass. The proband's father and two daughters were heterozygous for the Thr13Met mutation and also displayed low HDL, reduced LCAT activity and CER, and more modest decrements in LCAT mass. Mean LCAT specific activity was severely impaired in the compound heterozygous proband and was reduced by 50% in individuals heterozygous for either mutation, compared to wild type family members. It is also shown that the two mutations impair both catalytic activity and expression of the circulating protein.—**Argyropoulos, G., A. Jenkins, R. L. Klein, T. Lyons, B. Wagenhorst, J. St. Armand, S. M. Marcovina, J. J. Albers, P. H. Pritchard, and W. T. Garvey. **Transmission of two novel mutations in a pedigree with familial lecithin:cholesterol acyltransferase deficiency: structure–function relationships and studies in a compound heterozygous proband. *J. Lipid Res.* 1998.** 39: **1870–1876.**

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An inborn error of lipid metabolism was first described in Norway in 1966 in sisters with normochromic anemia, proteinuria, and corneal deposits of lipid (1). This disease is now known to result from loss of function of lecithin:cholesterol acyltransferase (LCAT), a key enzyme in extracellular cholesterol metabolism (2). LCAT associates with α and, to a lesser extent, with β-lipoprotein particles and catalyzes cholesterol esterification (3, 4). Specifically, cholesterol is transferred to HDL particles from peripheral cells and other lipoproteins, becomes esterified through the action of LCAT on high density lipoprotein (HDL), and is incorporated into the core of the lipoprotein (5). LCAT preferentially binds to HDL molecules that contain apolipoprotein A-I, which is the major activator of the enzyme (1, 2). Moderate LCAT enzyme deficiency is referred to as fish-eye disease (FED), characterized by corneal opacities, significant reduction of HDL, and substantial reduction of cholesterol esterification rates (6). In FED, LCAT is unable to esterify cholesterol in the HDL molecule, while it retains its activity in VLDL and LDL, hence resulting in near normal levels of plasma cholesteryl esters (7, 8). Mutations causing severe LCAT deficiency are less often characterized by the fish-eye abnormality but can be associated with anemia, proteinuria, renal impairment, and premature atherosclerosis.

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; Lp[a], lipoprotein[a]; CER, cholesterol esterification rate; FLD, familial LCAT deficiency; FED, fish-eye disease; PCR, polymerase chain reaction.

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The gene encoding LCAT protein has been cloned and its genomic organization has been determined (9). The gene maps on chromosome 16p22–p22, spans 4.2 kb, consists of six coding exons, and is predominantly expressed in the liver (9, 10). The mature LCAT protein is comprised of 416 amino acids and a leader sequence of 24 residues (11). Several mutations impairing enzyme activity have been described in the LCAT gene (12–14). Depending on the measured LCAT activity, LCAT mass, HDL concentrations, and clinical characteristics, mutations in the human LCAT gene have been described and classified (15) as familial LCAT deficiency (FLD) or as fish-eye disease (FED).

In this report, two novel, missense mutations in the LCAT gene are described in a compound heterozygous male proband with familial LCAT deficiency. Both mutations resulted in non-conservative amino acid substitutions. Furthermore, mutational screening of the nuclear family showed that the father and two daughters of the proband were heterozygous for one mutation and that the proband's mother and two sisters carried the other mutation. Construction of a three-generation pedigree provided a clear mode of Mendelian inheritance of the two mutations. The functional effect of the two heterozygous mutations (occurring singly or in the compound form) was elucidated by clinical and biochemical studies including assessment of HDL, LCAT mass, LCAT activity, and cholesterol esterification rate.

METHODS

Proband and family samples

The proband, a 32-year-old Caucasian male, was referred to an ophthalmologist for investigation of a corneal arcus that the patient had noticed in 1988. Over the past year, he had developed some difficulty in accommodating between bright light and dark areas but without any change in his visual acuity. Apart from mild episodic asthma and hay fever he was well. There were no symptoms of anemia, renal disease, or macrovascular disease. He is a non-smoker, rarely consumes alcohol, and is not on regular medication. There is no past history of major illness although serological testing revealed evidence of a previous and now-inactive hepatitis B infection. The proband had a mild normocytic anemia (hematocrit 38%) and normal creatine clearance of 97 ml/min without albuminuria. His parents, siblings, wife, and children have no evidence of corneal opacification, renal impairment, or vascular disease. However, he has a positive family history of premature ischemic heart disease.

For biochemical studies, blood was drawn, after a 10–12-h fast, into vacutainers. Plasma and serum were prepared by centrifugation at 1800 rpm for 25 min. Samples obtained from the parents and siblings of the proband were drawn at regional hospitals. The study was approved by the Medical University of South Carolina Internal Review Board and informed consent was given by each subject prior to participation.

Lipid profile, lipoprotein and apolipoproteins

Lipid profile, apolipoprotein assays, renal function tests, and hematological tests were performed. Using Vitros autoanalyzers, total and HDL-cholesterol were measured by a colorimetric oxidase technique and triglycerides were measured by a glycerophosphate colorimetric technique (Johnson and Johnson, Rochester, NY). Lipids were measured according to CDC reference methods. LDL and VLDL were calculated according to the Friedewald equation. Serum BUN and creatine were also measured on a Vitros autoanalyzer using a colorimetric urease technique and a two-point rate method, respectively (Johnson and Johnson, Rochester, NY). Full blood examination and complete blood count was assisted by a Coulter Counter (Coulter Corp., Miami, FL). For lipoprotein composition analyses, VLDL, LDL, and HDL cholesterol were isolated from plasma by sequential ultracentrifugation using 60Ti and SW41 rotors (Beckman, Palo Alto, CA) (16). Apolipoproteins A-I, B, and lipoprotein[a] were measured by nepholometry using Beckman (Brea, CA) reagents (17). Agarose gel electrophoresis of plasma lipoproteins (Paragon LIPO lipoprotein electrophoresis, Beckman, Brea, CA) was performed according to the manufacturer's instructions. The distribution of free and esterified cholesterol in isolated lipoproteins and in plasma was determined using gas chromatography, as previously described (18).

Measurements of LCAT mass, LCAT activity, LCAT specific activity, and cholesterol esterification rate (CER)

The concentration of LCAT was measured by a double antibody radio-immunoassay as previously described (19, 20). LCAT activity was measured as the ability of plasma to esterify [^{3}H]cholesterol in an exogenously provided analogue of HDL, as previously described (21). The calculation of the activity assumes that during the incubation, the [^{3}H]cholesterol does not equilibrate with the endogenous cholesterol pool of the sample. CER is a measure of the rate of observed cholesteryl ester synthesis as a function of both the amount and activity of LCAT in plasma together with the endogenous plasma lipoprotein substrate composition (22). The specific activity was measured as the ratio of LCAT activity and LCAT mass.

Genomic DNA isolation, amplification and sequence determination of LCAT exonic sequences

Genomic DNA was isolated from whole blood using a standardized DNA isolation kit (Gentra Systems, Puregene, Minneapolis, MN). The polymerase chain reaction (PCR) was used to amplify (23) all six exons of the LCAT gene. PCR primers were designed according to the sequence and genomic structure of the LCAT gene (GenBank Accession number: X04981), using the Primer3 program {Steve Rozen, Helen J. Skaletsky (1996, 1997), Primer3. Code available at: http://www-genome.wi.mit.edu/genome_software/other/primer3.html}. In addition, previously reported PCR primers (24) were also used. The following primers were used to amplify and sequence all exonic sequences of the gene:

Exon 1:
E1F: CAC TGG CCA GGC CGT CCC TC
E1R: GGG GCT TAT GCA GGG CAG AAG
(PCR product: 357 bp)

Exons 2, 3, 4 and 5:
E2F: ACT GCA GCA TCT GGG GTG ACG
E5R: GAG CAG GAG CCG CAA TGA AGG
(PCR product: 1008 bp)

Additional sequencing primers:
LCAT4: TGG CTG TAC CTG GTG TTA TC
LCAT5: GCC CCG CAG GGT TGT CTA CA
LCAT6: CTC ACC GGG CTC CAG CCG CC
1896: CCA CCC TAG CCC CAA CAC G
2185: TGG TGC AGA ACC TGG TCA A
NEX5F: GGC CAG CAG GAG GAG

Exon 6:
LCAT8: TCC ACA GGT GAC AAC CAG
LCAT11: CAG GGC TTA CGG TAG CAA A
(PCR product: 610 bp)

Additional sequencing primers:
LCAT9: GCC CAC GCC GTA AAG ACA GT
4601: ACA GGC CGT GAC TTC CAA CGC

PCR reactions were carried out in 25-μl volumes in 0.02-ml-thin-wall tubes in a GenAmp 9600 temperature cycler (Perkin Elmer, Foster City, CA). For each sample, 50–100 ng of genomic DNA was mixed with the above primers (25 pmol of each primer), the four dNTPs (200 μmol each), $MgCl_2$ (1.5 mm), 0.15 units of *Taq* DNA polymerase (GIBCO-BRL, MD), and 1× PCR buffer, as provided by the manufacturer (GIBCO-BRL, MD). After an initial denaturation step (94°C, 3 min), PCR was carried out for 35 cycles, each cycle consisting of three steps of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec. An extension step at 72°C for 5 min was performed upon completion of the 35 cycles.

DNA sequence analysis

To examine for mutations in the LCAT gene, PCR products were purified from gel slices using the Qiaquick gel extraction kit (Qiagen Inc., Chatsworth, CA) and the DNA sequence was determined, bidirectionally. DNA sequencing of the proband was, initially, performed manually, using the Amplicycle sequencing kit (Perkin Elmer, Foster City, CA). Subsequent sequencing of family members was performed using an automated ABI Prism 373 sequencer (Perkin Elmer, Foster City, CA).

RESULTS

Clinical and biochemical characteristics

Repeat LIPO gel analysis (data not shown) of the proband indicated absence of HDL and the patient became a candidate for further biochemical and genetic testing for fish-eye disease (FED) or familial LCAT deficiency (FLD). Members of the immediate family of the proband also participated in the study and circulating lipids, lipoproteins, LCAT activity, LCAT mass, and cholesterol esterification rate were measured in all participating individuals (**Table 1**). HDL levels were <5 mg/dl in the proband, substantially reduced in the mother and the father, and below the normal reference range in daughter 1 and sister 1 (Table 1). HDL levels in the remaining family members were within the reference range, although in some cases in the low range of normal. As expected, the serum apolipoprotein A-I level of the proband was less than 50% of the minimum of the reference range (Table 1) and was depressed, albeit less dramatically, in the father, mother, sister 2, and daughter 1 (Table 1). It was also noted, that the lipoprotein[a] level of the proband, sister 2, and the father were within the expected range (Table 1), while all other family members had higher levels of Lp[a].

Genetic analyses

All six exons of the proband's LCAT gene were amplified by PCR and sequenced bidirectionally. A heterozygous mutation in exon one (**Fig. 1**), at position 110 of the cDNA (counting from "A" of the translation initiator "ATG") was revealed with one allele having a thymidine in place of the wild-type, cytosine (C110T). The predicted translation of the mutant allele resulted in a missense, non-conservative, amino acid substitution of the threonine for a methionine (Thr13Met). An additional heterozygous mutation in the LCAT gene was identified in exon 6 (Fig. 1). This mutation was in position 991 of the cDNA with one allele having a thymidine instead of the wild-type cytosine (C991T) and resulting in a missense, non-conservative, amino acid substitution of the proline by a serine (Pro307Ser). Exons two, three, four, and five of the LCAT gene of the proband were also amplified and sequenced, but no other sequence variations were identified. The proband was thus classified to be compound heterozygous for the two mutations in exons one and six. Both mutations were identified by parallel determination

TABLE 1. Summary of all lipids, apolipoproteins, LIPO gel data, free cholesterol, cholesteryl ester, LCAT mass. LCAT activity, cholesterol esterification rate, and mutational changes for the entire pedigree presented in this study

Parameter	Reference Range	**Proband 32 yr Thr 13Met Pro307Ser**	Mother 55 yr Pro307Ser	Sister 1 33 yr Pro307Ser	Sister 2 27 yr Pro307Ser	Father 58 yr Thr13Met	Daughter 1 9 yr Thr13Met	Daughter 2 7 yr Thr13Met	Brother 24 yr Wild type	Wife 32 yr Wild type
Total cholesterol, mg/dl	100–200	**108**	230	157	176	187	129	133	158	123
Triglyceride, mg/dl	<250	**136**	243	58	86	272	60	61	96	69
LDL-C, mg/dl	70–130	**90**	159	112	120	117	90	86	103	69
HDL-C, mg/dl	29–89	**<5**	22	33	39	16	27	35	36	40
VLDL, mg/dl	3–40	**27**	49	12	17	54	12	12	19	14
Lp[a], mg/dl	<25	**<10**	34	44	<10	10	108	118	54	114
ApoA-I, mg/dl	101–199	**52**	102	111	82	87	102	125	121	138
ApoB, mg/dl	49–103	**78**	185	85	127	150	69	71	97	52
LIPO Gel		**No HDL**	IIb[a]	N	N	IIb[a]	N	N	N	N
FC/CE	0.33–0.43	**1.26**	0.47	0.41	0.45	0.43	0.4	0.39	0.37	0.4
LCAT mass, μg/ml	6.14 ± 0.98	**2.02**	6.32	4	4.05	5.21	5.16	6.36	5.73	4.82
LCAT activity, nmol/ml/h	24.6 ± 3.6	**2.1**	12.4	11.5	12	NA	12.4	13.5	24.7	21.1
CER, nmol/ml/h	123 ± 32.1	**12.8**	100.7	90	69.4	NA	109.7	91.3	119.4	105.7

C, cholesterol; Lp[a], lipoprotein[a]; apo, apolipoprotein; FC, free cholesterol; CE, cholesteryl ester; N, normal; CER, cholesterol esterification rate; NA, not available.
[a]Fredrickson classification of hyperlipidemia.

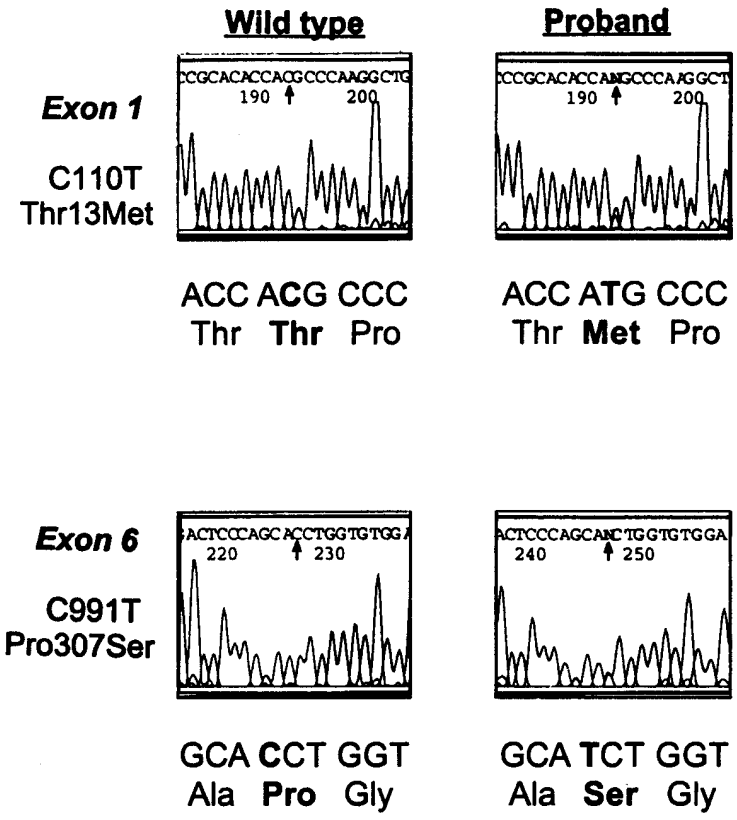

Fig. 1. Electropherograms of sequence determination of exon one (upper panel) and exon six (lower panel) of the LCAT gene of the proband. The wild-type (left-hand side panels) and the heterozygous mutations (right-hand side panels) are indicated by the arrow in the corresponding electropherograms.

of the DNA sequence of a control individual (with normal HDL levels and no visible corneal opacification) and performance of sequence alignments. The GenBank entry of the LCAT gene with Accession Number: X04981 was also used to validate the consensus sequence obtained.

Genomic DNA from all family participants was also isolated and exons one and six were amplified and sequenced to examine for presence of the two mutations. The wife and the brother of the proband were found to have wild type alleles only. Transmission of the mutant alleles is shown by the pedigree in **Fig. 2**. The mother and the two sisters of the proband were found to be heterozygous for the same mutation as the proband in exon six (C991T, Pro307Ser). On the other hand, the father and the two daughters of the proband were found to be heterozygous for the mutation in exon one (C110T, Thre13Met). It is apparent that the exon one mutation was transmitted to the proband and subsequently to the two daughters from the heterozygous father of the proband. The exon six mutation was transmitted to the proband and to the two sisters from the heterozygous mother (Fig. 2).

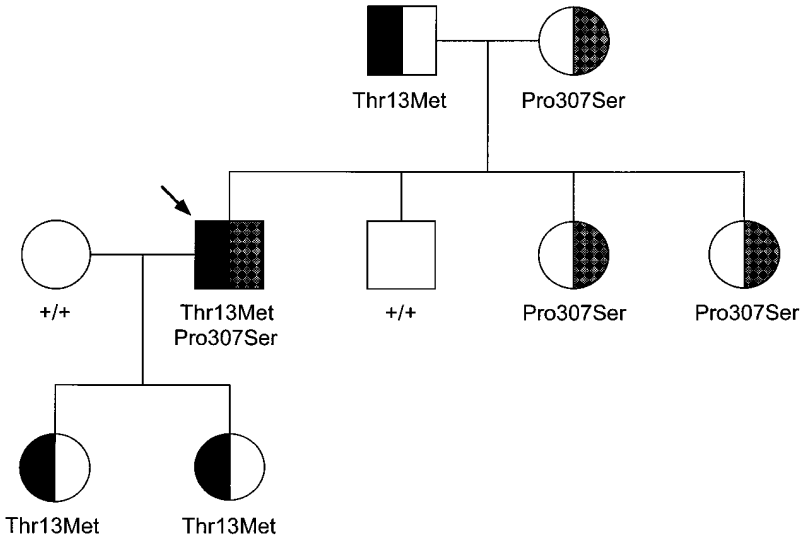

Fig. 2. Pedigree presentation of the family studied. Heterozygous individuals, for either mutation, are presented with half-filled shaded areas. The compound heterozygous proband is presented with both halves filled. The wife and brother of the proband had wild-type alleles only (no shaded area).

Structure–function relationships

The ratio of serum-free cholesterol (FC) to cholesteryl ester (CE) of the proband was 3.3 times higher than that of the expected mean, while the FC/CE ratio was within the expected range for all other family members (Table 1). Accordingly, the cholesterol esterification rate (CER) of the proband was significantly reduced, at a level of 10% of the expected minimum (Table 1), while the CER of all other family members was within the normal range. The LCAT mass of the proband was measured to be 33.3% of the expected value and the LCAT mass of the two sisters (Pro307Ser) was found to be at 65% the level of the expected value. The LCAT mass of the other family members was estimated to be within the expected range (Table 1). As expected, the LCAT activity of the proband was notably lower (8.5%) than expected. Likewise, the LCAT activity in the mother, the two sisters (Pro307Ser), and the two daughters (Thr13Met) was found to be lower than the expected value (at 50% level). The LCAT activity in the brother and the wife was normal (Table 1). The LCAT activity and CER of the father of the proband were not estimated due to insufficient sample volume.

To better illustrate the functional effect of the two mutations, the LCAT specific activity was calculated as the ratio of LCAT activity and LCAT mass. The mean LCAT specific activity was compared among individuals heterozygous for one or the other mutation, the individuals with no mutations, and the compound heterozygous proband (**Fig. 3**). The mean LCAT specific activity of the family members with either the Thr13Met (2.26 ± 0.14 nmol/h·μg) or the Pro307Ser (2.6 ± 0.32 nmol/h·μg) mutation was significantly lower ($P = 0.005$ and $P = 0.025$, respectively) than that of wild-type family members (4.34 ± 0.035 nmol/h·μg). LCAT specific activity of the compound heterozygous proband (1.04 nmol/h·μg) was less than half of the mean value in family members with either mutation and four times lower than that in the wild-type brother and wife.

DISCUSSION

The lecithin:cholesterol acyltransferase gene consists of six translated exons and encodes a plasma glycoprotein that plays a major role in lipid metabolism and, in particular, cholesterol esterification in HDL particles (10, 25). In the present study, two novel heterozygous mutations were identified in a proband with LCAT deficiency (C110T in exon one and C991T in exon six). Both mutations resulted in non-conservative amino acid substitutions (Thr13Met and Pro307Ser) and led to a profoundly low level of plasma HDL as well as severe defects in cholesterol esterification rate and LCAT specific activity. Mutational analyses of the LCAT gene of the parents of the proband revealed that the Thr13Met mutation was transmitted from the father and the Pro307Ser mutation was transmitted from the mother. The maternal mutation was also transmitted to the two sisters of the proband, whereas the paternal mutation was transmitted, via the proband, to the two daughters.

HDL levels of the compound heterozygous proband were extremely low (<5 mg/dl), whilst HDL levels of family members were widely varied, irrespective of heritability of either LCAT genotype. While LCAT levels are characteristically reduced in patients with FLD or FED (15), family members heterozygous for either LCAT mutation had normal HDL levels and no clinical evidence for either FLD or FED. However, HDL levels could also be affected by other factors such as age, hormone status, diabetes status, body mass index, diet, alcohol intake, and exercise (26–28). Cholesterol esterification rates, LCAT mass, and LCAT specific activity were also assessed in order to establish a precise relationship between structure and function of the LCAT gene in this pedigree. Mean LCAT specific activity in family members with either the Thr13Met or Pro307Ser missense heterozygous mutation was significantly reduced ($P = 0.005$ and $P = 0.025$, respectively) when compared with the LCAT activity of wild-type family members. This is consistent with the dramatic reduction

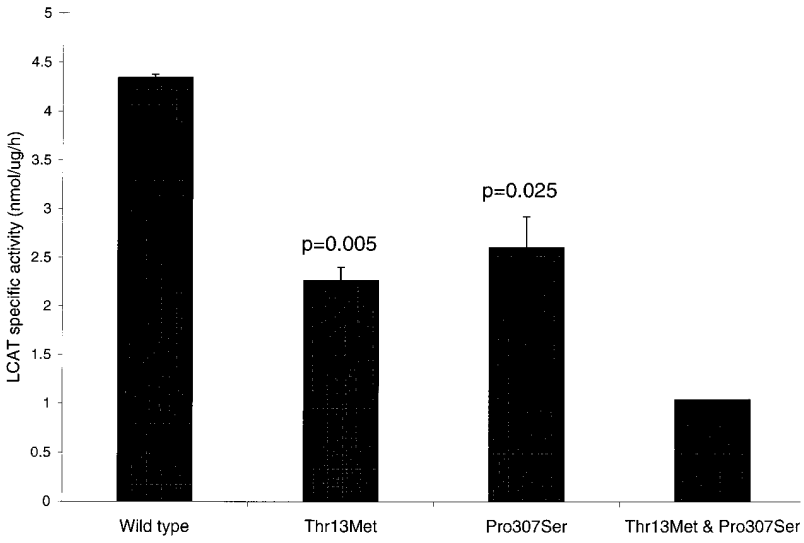

Fig. 3. Effect of the two heterozygous mutations on the corresponding LCAT specific activity. The LCAT specific activity was estimated as the ratio of LCAT activity and LCAT mass and averaged (mean ± SEM) for each category [wild-type: 4.35 ± 0.035 nmol/h·μg; Thr13Met: 2.26 ± 0.14 nmol/h·μg; Pro307Ser: 2.6 ± 0.32 nmol/h·μg; (Thr13Met and Pro307Ser): 1.04 nmol/h·μg). The Student's *t*-test was used to determine levels of significance. Only one value for the LCAT specific activity of the compound heterozygous mutation (Thre13Met and Pro307Ser) was available and, therefore, no *P* value was calculated.

(25%) of the LCAT specific activity observed in the proband with the two heterozygous mutations.

It was also observed that Lp[a] levels in the proband were undetectable, although other family members had relatively high Lp[a] levels. Lp[a] is an LDL-like particle with variations in content and composition of its core lipids (29). Apo[a] seems to be the major determinant of Lp[a] size although variations in LDL components may also affect size heterogeneity (29). Low Lp[a] levels may be the result of inability to assemble the lipoprotein with LDL of abnormal composition that has resulted from LCAT deficiency (30) consistent with the undetectable Lp[a] levels in the proband. Normal or high Lp[a] levels in the heterozygotes suggest that a single mutant allele may not have a major impact. Alternatively, it cannot be ruled out that structural mutations / polymorphisms in the Lp[a] gene may also be present in the proband, possibly transmitted from the father (also with relatively low Lp[a] levels), and may affect Lp[a] concentration.

Over thirty different mutations have been identified in the LCAT gene in patients with LCAT deficiency. In most cases reported, reduced HDL concentration was the most striking common feature even though only a handful of these cases have been fully characterized in complete family pedigrees. These mutations have been categorized into five classes depending on the catalytic activity of the protein product of the LCAT gene, the LCAT mass, and the presence of HDL (31, 32). Two further major stratifications of the disease constitute familial LCAT deficiency (FLD) and the milder fish-eye disease (FED). The almost absent LCAT activity and lack of endogenous esterification, but a secreted and stable protein and absence of HDL, would suggest that the proband, with two heterozygous missense mutations, has LCAT deficiency type II. In vitro transfection experiments with mutant LCAT cDNAs have also showed that reductions in LCAT specific activity and CERs are immediate and direct biochemical measures of genetic and functional defects that are the result of missense mutations (15, 31). Previous studies have reported heterozygous mutations in the LCAT gene of subjects with FLD (33), dramatic reduction in LCAT activity (33), and a compound heterozygote with cholesterol levels (34) identical to the compound heterozygote reported here. Furthermore, mutagenesis of highly conserved histidines in LCAT has led to the identification of a histidine residue (His377) that obliterates LCAT activity (35). Moreover, mutations in the amino acid segment 154–171 of the LCAT protein have been associated with decreased interactions with lipids by decreasing the mean hydrophobicity of the hydrophobic face of the amphipathic helix of the molecule (36).

Each of the two mutations in this family also affected functional motifs in the LCAT protein. Specifically, the Thr13Met mutation renders loss of a protein kinase C phosphorylation site (37), whereas, the Pro307Ser mutation introduces two additional motifs; one casein kinase II (CK2) phosphorylation site and one N-myristoylation site (38, 39). Furthermore, secondary structure analyses of the mutant LCAT proteins were performed using the Baylor College SSP program (40) for the prediction of α-helix and β-strand segments of proteins. It was observed that the Pro307Ser mutation reduces the potential for β-strand formations in a predicted β-strand segment of the LCAT protein. The architecture of a three-dimensional model for LCAT was recently proposed and detailed descriptions of α-helices and β-strands for the C-terminus of the protein molecule have been presented (41). The lid structure in LCAT is proposed to be at the N-terminus of the molecule between residues 50 and 74 but little is known for upstream structures, near the Thr13Met mutations, as residues 1–50 of LCAT show weak homologies with other lipases (41). It has been suggested that residues 211–314 between strands six and seven of LCAT are involved in the enzyme substrate recognition (41). The Pro307Ser mutation described here is, therefore, within the substrate recognition segment and it is likely that the mutation alters the ability of the protein to bind to its substrate. The three-generation family described here provides a clear mode of Mendelian inheritance of the two novel mutations in the LCAT gene. It is also shown that the two nonconservative amino acid substitutions in exons one and six of the LCAT gene impair both catalytic activity and expression of the circulating protein.

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