The Molecular Basis of Lecithin:Cholesterol Acyltransferase Deficiency Syndromes
A Comprehensive Study of Molecular and Biochemical Findings in 13 Unrelated Italian Families

Laura Calabresi, Livia Pisciotta, Anna Costantin, Ilaria Frigerio, Ivano Eberini, Paola Alessandrini, Marcello Arca, Gabriele Bittolo Bon, Giuliano Boscutti, Ghil Busnach, Giovanni Frascà, Loreto Gesualdo, Maddalena Gigante, Graziana Lupattelli, Anna Montali, Stefano Pizzolitto, Ivana Rabbone, Marina Rolleri, Giacomo Ruotolo, Tiziana Sampietro, Adalberto Sessa, Gaetano Vaudo, Alfredo Cantafora, Fabrizio Veglia, Sebastian Calandra, Stefano Bertolini, Guido Franceschini

Objective—To better understand the role of lecithin:cholesterol acyltransferase (LCAT) in lipoprotein metabolism through the genetic and biochemical characterization of families carrying mutations in the LCAT gene.

Methods and Results—Thirteen families carrying 17 different mutations in the LCAT gene were identified by Lipid Clinics and Departments of Nephrology throughout Italy. DNA analysis of 82 family members identified 15 carriers of 2 mutant LCAT alleles, 11 with familial LCAT deficiency (FLD) and 4 with fish-eye disease (FED). Forty-four individuals carried 1 mutant LCAT allele, and 23 had a normal genotype. Plasma unesterified cholesterol, unesterified/total cholesterol ratio, triglycerides, very-low-density lipoprotein cholesterol, and pre-β high-density lipoprotein (LDL) were elevated, and high-density lipoprotein (HDL) cholesterol, apolipoprotein A-I, apolipoprotein A-II, apolipoprotein B, LpA-I, LpA-I-A-II, cholesterol esterification rate, LCAT activity and concentration, and LDL and HDL particle size were reduced in a gene–dose-dependent manner in carriers of mutant LCAT alleles. No differences were found in the lipid/lipoprotein profile of FLD and FED cases, except for higher plasma unesterified cholesterol and unesterified/total cholesterol ratio in the former.

Conclusion—In a large series of subjects carrying mutations in the LCAT gene, the inheritance of a mutated LCAT genotype causes a gene–dose-dependent alteration in the plasma lipid/lipoprotein profile, which is remarkably similar between subjects classified as FLD or FED. (Arterioscler Thromb Vasc Biol. 2005;25:1972-1978.)

Key Words: familial lecithin:cholesterol acyltransferase deficiency ■ fish eye disease ■ high-density lipoproteins ■ lecithin:cholesterol acyltransferase ■ mutation

The lecithin:cholesterol acyltransferase (LCAT) (phosphatidylcholine:sterol-O-acyltransferase; EC 2.3.1.43) enzyme is responsible for the synthesis of cholesteryl esters (CE) in plasma.1 Through this action, LCAT plays a central role in the formation and maturation of high-density lipoproteins (HDL), and in the intravascular stage of reverse cholesterol transport, the major mechanism by which HDL modulate the development and progression of atherosclerosis. A defect in LCAT function would be expected to enhance atherosclerosis by interfering with this process.

The human LCAT gene encompasses 4.2 kilobases and is localized in the q21–22 region of chromosome 16.1 To date, 40 mutations in the human LCAT gene have been reported (HGMD; http://www.hgmd.org/index.html). Based on strict biochemical criteria, homozygotes or compound heterozygotes for these mutations are...
classified into 2 distinct syndromes, familial LCAT deficiency (FLD) (MIM# 245900) and fish-eye disease (FED) (MIM# 136120). In FLD, plasma LCAT is either absent or completely lacks catalytic activity; in FED, the mutant LCAT lacks activity on HDL lipids but esterifies cholesterol bound to apolipoprotein (apo)B-containing lipoproteins. All reported FED and FLD cases have greatly reduced plasma HDL concentrations; the prevalence of coronary heart disease (CHD) may be higher in FED than FLD cases. Scattered reports of heterozygous carriers of LCAT mutations indicate they may have either low or normal plasma HDL cholesterol (HDL-C) levels without premature CHD. To gain a better understanding of the role of LCAT in health and disease, we started collecting cases/families with mutations in the LCAT gene. We report here the genetic and biochemical characterization of 13 families, in which 15 novel and 2 already described mutations in the LCAT gene have been identified.

Methods

Subjects

Probands with primary hypoalphalipoproteinemia (HALP), defined by a plasma HDL-C level below the fifth percentile for the age- and sex-matched general population, were identified by Lipid Clinics and Departments of Nephrology throughout Italy. Plasma samples were analyzed for total and unesterified cholesterol; in 18 unrelated index cases, the results were suggestive of a defect in the LCAT gene. Genetic analysis revealed that 13 of 18 index cases carried at least 1 mutant LCAT allele. Relatives of the 13 probands were invited to participate in the study. All subjects gave an informed consent.

Blood samples were collected after an overnight fast. Blood and plasma aliquots were immediately frozen and stored at −80°C before shipment in dry ice for biochemical characterization and genotyping.

LCAT Gene Analysis

Please refer to supplementary data for details (please see http://atvb.ahajournals.org).

Plasma Lipids and Lipoproteins

Plasma total and unesterified cholesterol, HDL-C, and triglyceride levels were determined with standard enzymatic techniques. Plasma very-low-density lipoprotein and low-density lipoprotein (LDL) particles containing only apoA-I (LpA-I) and of particles containing both apoA-I and apoA-II (LpA-I: A-II) was determined by immunoturbidimetry and electroimmunodiffusion. The content of pre-B HDL was assessed by 2-dimensional electrophoresis and expressed as percentage of total plasma apoA-I. LDL and HDL particle size was determined by nondenaturing polyacrylamide gradient gel electrophoresis.

The esterification of cholesterol within endogenous lipoproteins (cholesterol esterification rate [CER]) or incorporated into an exogenous standardized substrate (LCAT activity) was determined as previously described. Plasma LCAT concentration was measured by an immunoenzymatic assay.

Statistical Analyses

Data are reported as means±SEM, if not otherwise stated. The principal end point was the comparison of biochemical parameters between cases, heterozygous relatives, and noncarrier family members (controls). When cases were compared, the variable used for proband selection (HDL-C) was excluded. Variables with a skewed distribution were log-transformed before analysis. The gene–dose effect was assessed by ANCOVA, taking as independent variable the number of mutant alleles (0, 1, or 2) in the genotype and testing for trend. Analyses were adjusted for age, sex, and family. Post-hoc comparisons were corrected for multiple testing by the Turkey method. Correlation coefficients were calculated and the significance of the correlation determined by the Pearson method. A 2-tailed P<0.05 was considered as significant.

Results

Identification of Mutations in the LCAT Gene

The pedigrees of the 13 probands’ families are shown in the Figure. DNA samples were obtained from peripheral blood leukocytes of the 13 probands, 62 relatives, and 7 spouses (Figure). Sequence analysis of probands’ DNA identified 17
different mutations (Table 1). All mutations were unique to a single family. Analysis using a computer program that predicts the effect of amino acid changes on protein function$^{13}$ indicated that 77% of the identified missense mutations were likely to adversely affect protein function (Table 1). Fifteen of the identified mutations are novel; the Y83X and R147W mutations have been previously reported in an FLD patient of unknown origin and in an Italian compound heterozygote for another unidentified mutation, respectively.$^{8,9}$ Overall, 15 of the 82 examined individuals carried 2 mutant LCAT alleles (9 homozygotes and 6 compound heterozygotes), 44 carried 1 mutant LCAT allele, and 23 (including 7 spouses) had a normal genotype (Figure). Analysis of 140 unrelated individuals with low, average, or high plasma HDL-C levels failed to identify any of the described mutations, indicating they are not polymorphisms.

### Differential Diagnosis

Seven of the 13 probands had undetectable CER and LCAT activity and were diagnosed as FLD cases (Table 1). Three probands had detectable CER and undetectable LCAT activity, and were classified as FED cases. The remaining 3 probands had detectable CER and LCAT activity and could not be classified as either FLD or FED (Table 1).

### Clinical Findings

Seven of the 13 probands were recruited by Lipid Clinics, and 6 by Departments of Nephrology. The anthropometric and clinical findings in the 15 carriers of 2 mutant LCAT alleles are shown in Table 2. None had cardiovascular disease; 5 were hypertensive and none had abnormalities of glucose metabolism. The most frequent clinical findings were bilateral corneal opacity (15/15) since early adolescence and normochromic anemia of varying severity (12/15). Nine cases (8 FLD and 1 FED) had proteinuria; 6 FLD cases had end-stage renal failure, requiring hemodialysis treatment, and 3 of them underwent an orthotopic kidney transplantation. Thirty-four of the 44 heterozygotes were apparently healthy. Five heterozygotes had high blood pressure and elevated blood lipids, and 2 were overweight and had diabetes mellitus type 2; 2 had a stroke and 1 had proteinuria caused by IgA nephropathy.

### Biochemical Findings

Plasma samples from 66 of the 82 examined subjects were available for biochemical evaluation; these included samples from 15 carriers of 2 mutant LCAT alleles, 38 heterozygotes, and 13 noncarrier family members.

The plasma lipid/lipoprotein and LCAT levels in the 15 carriers of 2 mutant LCAT alleles are shown in Table 3. Plasma total and LDL cholesterol and triglyceride levels showed a wide interindividual variability, even among cases carrying the same mutation(s) in the LCAT gene (Table 3). No clear relationship between plasma lipid levels and differential diagnosis was found. All had remarkably low plasma HDL-C
but individual levels were quite variable among families; no significant correlation was found between HDL-C and either plasma LDL-cholesterol or triglycerides. Except for proband 2, who is homozygous for a truncating mutation at position H11002 and completely lacks plasma enzyme, all cases had detectable but remarkably low plasma LCAT concentrations (Table 3); a highly significant positive correlation ($R = 0.814$, $P = 0.0002$) was found between plasma LCAT and HDL-C concentrations.

In a first analysis, the average plasma lipid, lipoprotein, and cholesterol esterification values were calculated for carriers of 2 and 1 mutant LCAT alleles, and for noncarrier family members (controls); comparisons were then made using covariance analysis, with age, sex, and family as covariates (Tables 4 and 5). Carriers of 2 mutant LCAT alleles tended to have lower plasma total and LDL cholesterol levels and smaller LDL particles than controls, but the differences did not achieve statistical significance. Unesterified cholesterol, the unesterified/total cholesterol ratio, very-low-density lipoprotein cholesterol, and triglycerides were significantly elevated, whereas HDL-C, apoA-I, apoA-II, and apoB were significantly reduced compared with controls. The HDL-C/apoA-I ratio ($0.24 \pm 0.04$) was significantly lower, and the

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<th>TC, mg/dL</th>
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<th>LDL-C, mg/dL</th>
<th>HDL-C, mg/dL</th>
<th>UC/TC, ratio</th>
<th>LCAT, $\mu$g/mL</th>
<th>Diagnosis</th>
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<td>180</td>
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<td>1.00</td>
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Reference values: $< 200 < 150 < 130 > 40 < 0.28 3.1–6.7$

HDL-C indicates high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; UC/TC, unesterified/total cholesterol.
apoA-I/A-II ratio (5.57 ± 0.64) was significantly greater than in controls (0.43 ± 0.04 and 3.80 ± 0.20, respectively). Plasma CER and LCAT concentration were significantly lower in controls; LCAT activity was undetectable. The average plasma concentration of both LpA-I and LpA-I:A-II particles in controls; LCAT activity was undetectable. The average HDL3 size was smaller than that of control HDL3. When carriers of 2 mutant LCAT alleles were divided in homozygotes and LCAT gene–dose-dependent effect on a number of biochemical parameters, including the plasma unesterified/total cholesterol ratio, LCAT activity and concentration, HDL cholesterol and apolipoproteins, and HDL subpopulations.

In a second analysis, the effect of the number of copies of mutated alleles (gene–dose effect) on plasma lipid/lipoprotein and cholesterol esterification parameters was investigated by ANCOVA, with the number of mutant alleles (0, 1, or 2) as independent variable and testing for trend (Tables 4 and 5). A mutation in the LCAT gene had a significant gene–dose-dependent effect on a number of biochemical parameters, including the plasma unesterified/total cholesterol ratio, LCAT activity and concentration, HDL cholesterol and apolipoproteins, and HDL subpopulations.

In a third analysis, a comparison was made between FLD and FED cases; no significant differences were found in the lipid/lipoprotein profile except for higher plasma unesterified cholesterol levels and unesterified/total cholesterol ratio in the former (153.2 ± 21.3 versus 68.7 ± 23.9 mg/dL and 0.94 ± 0.03 versus 0.62 ± 0.03, respectively).

### Discussion

In the present study, 13 families were identified carrying 17 different mutations in the LCAT gene. Forty mutations in the
The present study demonstrates that: (1) the inheritance of a mutated LCAT genotype causes a remarkable and gene–dose-dependent alteration in the plasma lipid/lipoprotein profile; and (2) the lipid/lipoprotein profile is indistinguishable between subjects classified as FLD or FED.

Plasma total and LDL cholesterol levels in carriers of 2 mutant LCAT alleles showed a wide interindividual variability, which may be caused by environmental, metabolic, or genetic factors. Elevated plasma triglycerides were a frequent finding among cases, and LCAT mutations had a gene–dose-dependent effect on plasma triglycerides and very-low-density lipoprotein cholesterol, which argues for a metabolic relationship between defective cholesterol esterification and hypertriglyceridemia. A decreased postheparin lipoprotein lipase activity has been detected in LCAT-deficient mice and in some FLD cases, suggesting that defective lipolysis may contribute to the elevated plasma triglycerides.

All carriers of 2 mutant LCAT alleles had remarkably low plasma HDL-C, apoA-I, and apoA-II levels; no significant difference was found between FLD and FED cases, confirming that the inheritance of a completely, or partially defective LCAT causes HALP. A remarkable variability in the severity of the HALP was, however, found among cases with different LCAT genotypes, as exemplified by the 6-fold variation in plasma HDL-C (Table 2). Such variability is clearly unrelated with the inherited defect in LCAT function, because FLD and FED cases had overlapping plasma HDL-C levels. The severe HALP in the carriers of 2 mutant LCAT alleles is associated with multiple alterations in HDL structure and particle distribution, with a selective depletion of LpA-I:A-II particles, a predominance of small, pre-β-migrating HDL1, and a complete lack of HDL2. Such changes likely reflect the accumulation in plasma of CE-poor, apoA-I-containing, discoidal HDL, which cannot mature into spherical HDL because of the lack of LCAT activity. These findings are consistent with an accelerated catabolism of LpA-I:A-II particles as a common metabolic cause of HALP in FLD and FED. With the exception of the homozygous carrier of the X-14 mutation, all FLD and FED cases had remarkably low plasma LCAT protein concentrations. The striking positive correlation between plasma LCAT and HDL-C levels suggests that HDL may function as a vehicle for LCAT in plasma, stabilizing the enzyme and preventing its catabolism. Consistent with this hypothesis is the repeated observation of partial LCAT deficiency in individuals with primary HALP caused by mutations in the apoA-I gene.

The availability of a relatively large number of carriers of 2 and 1 mutant LCAT alleles allowed us to identify a significant LCAT gene–dose-dependent effect on cholesterol esterification measurements, as well as on a number of HDL-related parameters. These findings underline the importance of proper LCAT function for efficient plasma cholesterol esterification process and appropriate HDL maturation/metabolism. The inheritance of a single mutant LCAT allele leads to a biochemical phenotype intermediate between those of carriers of 2 or zero copies of mutant alleles, thus indicating that the biochemical abnormalities are expressed as codominant traits in families carrying mutations in the LCAT gene. The heterozygous carriers of mutant LCAT alleles had lower average plasma HDL cholesterol and apolipoproteins, apoA-I–containing lipoprotein particles, LCAT activity and concentration, and higher pre-β HDL content than controls.

According to the present knowledge, the abnormalities in the HDL profile of carriers of either 2 or 1 mutant LCAT alleles are all indicative of a high CHD risk. No evidence of increased CHD in LCAT-deficient families was instead found in the present study. The association between inheritance of a functional defect in LCAT and CHD risk remains debated, based on contradictory findings in both humans and animals. Large follow-up studies in carriers of LCAT mutations are needed to clarify this issue.

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References

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**LCAT gene analysis**

Genomic DNA was extracted using a standard procedure. The *LCAT* gene was amplified using the following primers: Exon 1, 5'-GGC TCC CTG AAG CTT TGC CCC TTT-3' (f) and 5'-TGG CGT GGT GAA TTC GGG GCC TGG-3' (r); Exons 2+3, 5'-CAC GGG GGG AAG CTT CTT GAG TCC AGA-3' (f) and 5'-TGT GGG CCA GAA TTC AGG CCT GGA-3' (r); Exon 4, 5'-AGC AAG CTG GCA GGT TTG TGT CA-3' (f) and 5'-AAG ACA GGC TTC CCA TAG GCA G-3' (r); Exon 5, 5'-AAC AAT GGC TTC TTG TGT TGT CA-3' (f) and 5'-AGT GGT AGA TAG CAC CCC TAG AG-3' (r); Exon 6, 5'-TGA GCC TAC ACT CAG CAG GTT GTG-3' (f) and 5'-CCC ATC TTG CCT CAC TGC ACA CA-3' (r). Briefly, the amplification mix (50 µl) included 350 ng of genomic DNA, 1 U of Taq DNA polymerase (Expand™ High Fidelity PCR System, Roche) in 1 x PCR buffer, 0.2 mM of each dNTP, 20 pmol of each primer, 1.5 mM MgCl₂ and 10% DMSO. The amplification conditions were: Exons 1 and 2+3: 94 °C for 3 min; 35 cycles at 94° C for 15 sec, 56° C for 30 sec, 72° C for 1 min; 72° C for 7 min; other Exons: 94 °C for 3 min; 30 cycles at 94° C for 15 sec, 58° C for 30 sec, 72° C for 2 min; 72° C for 7 min. The amplification products were purified by QIAquick kit (Qiagen, Milan, Italy), quantified on 1% agarose gel and sequenced by automatic sequencer CEQ2000 DNA Analysis System (Beckman Coulter, Fullerton, CA). Reference GenBank numbers for the LCAT genomic DNA and cDNA were X04981 and NM_000229, respectively.

**Screening tests for LCAT gene mutations**

*Screening for c.31delG (frameshift>X-14) in exon 1*

The screening for the mutation was performed by heteroduplex analysis. The DNA fragment which included the nucleotide deletion (232 bp) was amplified using the following primers: 5'-CAG ATA AGG ACA GCC CAG TGC CGC T-3' (forward) and 5'-CAT CAG GGG CTT GGT GGG GGC TTA-3' (reverse); the conditions were: 94°C for 5 min; 5 cycles at 94°C for 30 sec, 62°C for 30 sec, 72°C for 1 min; 30 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min; final extension at 72°C for 7 min. EDTA 0.25mM was added to the amplification product that, to drive to completion the heteroduplex formation, was submitted to incubation at 95° C for 3 min and then slow cooled to room temperature in about 40 min. The electrophoresis was carried out on 7.5% polyacrylamide gel for 40 min at 180 V. The wild DNA homoduplex gave a 232 bp band and the heteroduplex a 260 bp band.

*Screening for c.35 C>T transition (T-13>M) in exon 1*
Exon 1 and flanking intronic sequences were amplified using the primers mentioned in sequencing method. The amplification product (343 bp) was digested with Mae III and the fragments were separated on 2.5% agarose gel electrophoresis. The mutation abolished a Mae III restriction site. The wild DNA gave three fragments of 184, 97 and 62 bp and the mutant DNA two fragments of 281 and 62 bp.

**Screening for the 27 nucleotides deletion (g.131-157del; frameshift>X26) in exon 1**
The deletion was easily detectable by the amplification of exon 1 and flanking intronic sequences using the primers mentioned in sequencing method. The electrophoresis on 2% agarose gel of the amplified products showed a 343 bp band for the wild allele and a 316 bp band for the mutant allele.

**Screening for the 5 nucleotides deletion (c.141-145del; frameshift>X76) in exon 1**
The screening for the mutation was performed by heteroduplex analysis. The exon 1 and flanking intronic sequences were amplified using the primers mentioned in sequencing method. EDTA 0.25mM was added to the amplification product that, to drive to completion the heteroduplex formation, was submitted to incubation at 95° C for 3 min and then slow cooled to room temperature in about 40 min. The electrophoresis was carried out on 10% polyacrylamide gel for 40 min at 180 V. The wild DNA homoduplex gave a 340 bp band and the heteroduplex a 510 bp band.

**Screening for c.209 (g.942) T>A transversion (V46>E) in exon 2**
The mutation eliminated a restriction site for the enzyme Hph I. Genomic DNA was amplified with a forward primer in intron 1 (5'-TGG GGG TTG AGG GTA TGG GAG GTG-3') and a reverse primer in exon 2 (5'-GAC TTC TTC ACC ATC TGG CTG GTA-3'). The amplified product (199 bp) was digested with Hph and the fragments were separated on 2% agarose gel. The wild gene gave origin to three fragments (114, 67 and 18 bp) and the mutant gene to two fragments (132 and 67 bp).

**Screening for c.321 (g.1133) C>A transversion (Y83>X) in exon 3**
This mutation, which abolished a restriction site for the enzyme Acc I, was screened using a method previously described.[2887]

**Screening for c.343 (g.1155) T>C transition (S91>P) in exon 3**
The fragment including the mutation was amplified by the following primers: 5'-AAG ACA GAG GAC TTC TTC ACC A-3' (forward); 5'-TCC ACA GAG TAG GTC TTG CCA-3' (reverse). The amplification product (254 bp) was digested with Bmy I and the resulting fragments analysed by capillary electrophoresis on microchips (Agilent DNA 500 LabChip kit) using the Bioanalyzer mod. 2100 (Agilent Technologies, Waldbronn, Germany). The
wild DNA gave fragments of 102, 84 and 68 bp; the mutant DNA fragments of 102, 84 and 63 bp.

**Screening for c.490 (g.1396) C>T transition (R140>C) in exon 4**
The fragment including the mutation was amplified by the following primers: 5'-GCA TGG CCC AAG CTG CTC CCG GTG AC T-3' (forward); 5'-TGG ACC TAA GAA TTC GAG GCC TTC T-3' (reverse). The amplification product (471 bp) was digested with Acc II and the fragments were separated on 2% agarose gel. The wild allele was undigested (471 bp) and the mutant allele gave origin to two fragments (368 and 103 bp).

**Screening for c.493 (g.1399) G>A transition (A141>T) in exon 4**
The fragment including the mutation was amplified by the following primers: 5'-TGG TGC AGA ACC TGG TCA ACA A-3' (forward in exon 4); 5'-TGC GAG CTT GCG GTA GTA CTG-3' (reverse in exon 5). The amplification product (196 bp) was digested with Fsp I and the fragments were separated on 2% agarose gel electrophoresis. The wild DNA gave an undigested fragment of 196 bp and the mutant DNA two fragments of 147 and 49 bp.

**Screening for c.614 (g.1603) G>A transition (S181>N) in exon 5**
The mutation was screened by amplification of exon 5 with a forward mutagenic primer 5'-ATC CTG TCT TCC TCA TTG GCC TCA-3' and a reverse canonical primer 5'-TTG ACC TAA GTG TTC GAG GCC TTC-3'. The mutation abolished a restriction site for the enzyme Dde I. After enzymatic digestion and 2% agarose gel electrophoresis we obtained two bands of 165 and 21 bp for wild DNA and one band of 186 bp for mutant DNA.

**Screening for c.803 (g.3676) G>A transition (R244>H) in exon 6**
The mutation was screened by amplification of exon 6 using the primers mentioned in sequencing method. The mutation abolished a restriction site for the enzyme Cfo I. After enzymatic digestion and 1% agarose gel electrophoresis we obtained two bands of 696 and 85 bp for wild DNA and one band of 781 bp for mutant DNA.

**Screening for c.892 (g.3765) A>G transition (T274>A) in exon 6**
This mutation was screened by an allele-specific amplification (PASA) method. The DNA fragment encompassing the mutation was amplified using a canonical forward primer 5'-TCC CTG TCC CAC CTT GCT CCA TAT-3' and a reverse mutagenic primer 5'-AAG CGT TGG AAG TCA CGG CCT TG G-3'. The conditions were: 94° C for 7 min; 30 cycles at 94° C for 30 sec, 58° C for 30 sec, 72° C for 1 min; final extension at 72° C for 8 min. The mutagenic primer did not allow the amplification of the normal DNA, whereas the mutant DNA gave a fragment of 197 bp detectable by 10% polyacrylamide gel electrophoresis.
Screening for c.893 (g.3766) C>T transition (T274>I) in exon 6
The mutation was screened by a PASA method similar to that above described. The DNA was amplified using a canonical forward primer 5'-TCC CTG TCC CAC CTT GCT CCA TAT-3' and a reverse mutagenic primer 5'-AGA AGC GTT GGA AGT CAC GGC CTG-3'. The conditions were: 94° C for 7 min; 30 cycles at 94° C for 30 sec, 60° C for 30 sec, 73° C for 1 min; final extension at 72° C for 8 min. The mutagenic primer did not allow the amplification of the normal DNA, whereas the mutant DNA gave a fragment of 199 bp.

Screening for c.997 (g.3870) G>A transition (V309>M) in exon 6
The mutation was screened by amplification of the 5’ half of exon 5 with a forward canonical primer 5'-TGT CCC ACC TTG CTC CAT AT-3' and a reverse mutagenic primer 5'-GCT GTA AAG ACA GTA TAC TTC GCG-3'. The mutation introduced a second restriction site for the enzyme Rsa I. After enzymatic digestion and 2% agarose gel electrophoresis we obtained two bands of 224 and 74 bp for wild DNA and three bands of 224, 50 and 24 bp for mutant DNA.

Screening for c.1187 (g.4060) T>G transversion (L372>R) in exon 6
The mutation was screened by amplification of exon 6 using the primers mentioned in sequencing method. The mutation introduced a restriction site for the enzyme Msp I. After enzymatic digestion and 1% agarose gel electrophoresis we obtained one band of 781 bp for wild DNA and two bands of 514 and 267 bp for mutant DNA.