Molecular defects underlying Wolman disease appear to be more heterogeneous than those resulting in cholesteryl ester storage disease

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Abstract **Human lysosomal acid lipase/cholesteryl ester hydrolase (hLAL) is essential for the intralysosomal metabolism of cholesteryl esters and triglycerides taken up by receptor-mediated endocytosis of lipoprotein particles. The key role of the enzyme in intracellular lipid homeostasis is illustrated by two lysosomal storage diseases inherited as autosomal recessive traits. Wolman disease, associated with deficient hLAL activity, leads to massive intracellular substrate accumulation and is always fatal in early infancy. Cholesteryl ester storage disease (CESD), in contrast, is characterized by very low levels of enzymic activity sufficient to allow survival of the affected patients into adulthood. In order to elucidate the underlying molecular defects in Wolman disease, we have characterized the hLAL gene in two female Wolman patients of German and Turkish origin by SSCP and DNA sequence analysis. Our results demonstrate that the German proband was compound heterozygous for an 8-bp deletion in exon 3 and a 2-bp deletion in exon 4 of the hLAL gene. These frameshift mutations lead to protein truncation at amino acid positions 24 and 116 and to complete loss of hydrolytic activity. The Turkish proband, in contrast, was homozygous for a G_{1064}→T substitution in exon 10 of the hLAL gene which converts the completely conserved glycine (GGG) residue at position 321 of the mature enzyme to tryptophan (TGG). In vitro expression of the hLAL(Gly_{321}→Trp) cDNA construct revealed that the amino acid replacement results in a more than 99% reduction of neutral lipid hydrolysis. The mutations provide new insights into the molecular basis of Wolman disease which is apparently more heterogeneous at the genetic level than cholesteryl ester storage disease.**—Lohse, P., S. Maas, P. Lohse, A. C. Sewell, O. P. van Diggelen, and D. Seidel. **Molecular defects underlying Wolman disease appear to be more heterogeneous than those resulting in cholesteryl ester storage disease.** ***J. Lipid Res.*** **1999.** 40: **221–228.**

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Human lysosomal acid lipase/cholesteryl ester hydrolase (hLAL; EC 3.1.1.13) is synthesized by all nucleated cells and plays a central role in intracellular neutral lipid metabolism (for review see refs. 1–3). The 378-amino acid protein catalyzes the intralysosomal hydrolysis of cholesteryl esters and triglycerides contained within endocytosed lipoproteins. Cholesterol, di- and monoglycerides, and free fatty acids are subsequently transported to the cytoplasm for re-esterification or storage, or they are used for energy provision and biosynthetic processes such as membrane biogenesis, steroid hormone production, and bile acid synthesis.

The increase in size of the cytoplasmic free cholesterol pool triggers three important regulatory steps. First, activity of the key enzyme of intracellular cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is suppressed. Second, the synthesis of low density lipoprotein (apoB,E) receptors is down-regulated, leading to a lower influx of cholesteryl ester-carrying lipoproteins into the lysosomal compartment. Third, activation of acyl-CoA:cholesterol acyltransferase (ACAT) stimulates the intracellular formation of cholesteryl esters.

An inherited deficiency or low activity of hLAL results in the intralysosomal storage of the lipid substrates, producing the fatal Wolman disease of the infant or the more benign cholesteryl ester storage disease (CESD) of the adult. It also causes the deregulation of the negative and positive feedback mechanism which normally ensure intracellular cholesterol homeostasis.

Cloning of the hLAL cDNA in 1991 by Anderson and Sando (4) led to the isolation of the gene (5–7) which is responsible for Wolman disease and CESD. It consists of ten exons dispersed over a 38.8-kb region on chromosome 10q23.2–q23.3 (8, 9). Subsequent analysis of Wolman patients revealed seven causal mutations thus far. One patient (cell line FeD) was compound heterozygous for a Leu_{179}→Pro substitution and a T-insertion after residue 634

Abbreviations: CESD, cholesteryl ester storage disease; hLAL, human lysosomal acid lipase.

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of the cDNA (5). In two other children affected with this disorder, homozygosity for a G→A mutation at position +1 of the conserved intron 8 splice donor site has been observed. This mutation results in the skipping of exon 8 and in the loss of 24 amino acids from the mature enzyme (10). Three additional probands of Japanese, African, and Italian origin were homozygotes for the stop codon mutations Tyr22X (11), Gln277X (12), and Tyr303X (13), respectively. A patient from a sixth kindred was homozygous for the insertion of an A at codon 117 (351insA), leading to premature termination of protein translation at position 124 (14). In the rat model of Wolman disease, a 4.5-kb deletion of genomic DNA and a 60-bp substitution produced a premature stop codon at amino acid position 368, resulting in the synthesis of a truncated protein missing the carboxyl terminal 29 residues (15).

In the present report, we describe the molecular basis of Wolman disease in two patients of Turkish and German origin. The lysosomal storage disorder presented in both cases in early infancy with hepatosplenomegaly, bilateral adrenal calcifications, abdominal distension, vomiting, steatorrhea, and failure to thrive. The infants died at ages 2.5 and 3 months from fulminant hepatic failure and haemorrhagic diathesis and from acute heart failure, respectively.

MATERIALS AND METHODS

Study subjects

Patient T. S. was a newborn Caucasian female, whose German parents are healthy and non-consanguineous. Patient Y. O. was the second child of healthy Turkish parents who are second cousins.

Cell culture

Skin fibroblasts of the probands were grown in DMEM supplemented with 2 mm l-glutamine, 1 g glucose/l, and 10% fetal calf serum (Sigma-Aldrich) in a humidified incubator (Heraeus Instruments) with 5% CO_2.

Isolation of genomic DNA and total RNA

High molecular weight chromosomal DNA and total RNA were isolated from the fibroblast cultures, using genomic tips and the RNeasy total RNA purification system (QIAGEN Inc.).

Oligonucleotides

Synthetic oligonucleotide primers based on the hLAL gene sequence were prepared on a DNA synthesizer (Perkin-Elmer Applied Biosystems, Inc., model 381A) using the phosphoramidite method. Each primer contained an incorporated restriction enzyme site for BamH I, EcoR I, or Hind III (**Table 1**) for subcloning into sequencing vector pBluescript II KS (Stratagene). Oligonucleotides were desalted on NAP-5 columns (Amersham Pharmacia Biotech) and used for PCR amplification without further purification.

Reverse transcription of total RNA

In the case of proband T. S., partial complementary copies of hLAL mRNA were obtained by first strand cDNA synthesis of 1–5 μg of total fibroblast RNA in a 20-μl reaction volume with Superscript II RNase H^- reverse transcriptase (GIBCO BRL Life Technologies) using oligonucleotide primer AL-27 (5′-CACATAATA GAATTCTTCTTGGCC-3′) in exon 5 of the hLAL gene.

cDNA and DNA amplification by the polymerase chain reaction

One-tenth of the reverse transcription reaction products and 1 μg of genomic DNA were amplified by the PCR technique (16) using an automated DNA Thermal Cycler (Hybaid Ltd., model OmniGene) and 20 μm each of two hLAL-specific primers (Table 1). Amplifications were performed in 100-μl reaction volumes containing 50 mm KCl, 10 mm Tris-HCl, pH 9, 1.5 mm $MgCl_2$, and 200 μm each dATP, dCTP, dGTP, and dTTP with 5 units of Taq DNA polymerase (Amersham Pharmacia Biotech). The cycle profile usually included denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s for 35 to 40 cycles.

Single-strand conformation polymorphism (SSCP) analysis

One set of double-stranded PCR products each of the two patients was heat-denatured and subjected to non-denaturing poly-

TABLE 1. Synthetic oligonucleotide primers used for hLAL exon amplification

Primer	Position	Sequence	Restriction Site
AL-100	5′-flanking region	CCTACGGCG***A***AT***T***CTCTATGGGGT	EcoR I
AL-109	intron 1 (5′)	TTGCTGAAGGCAC***A***AGCTTCCACG	Hind III
AL-94	intron 1 (3′)	CTTTTTGTGG***A***AGC***T***TTAATTTACC	Hind III
AL-87	intron 2 (5′)	CTGGATCGGGGAA***T***T***C***GATGCATT	EcoR I
AL-70	intron 2 (3′)	GCTTTCTTAAAGC***T***TGGAGAACATAG	Hind III
AL-75	intron 3 (5′)	CACAAAACCCAGAAGAATTCTGGT	EcoR I
AL-74	intron 3 (3′)	GTTTTGAAGCTTGGTGCTACTGCC	Hind III
AL-61	intron 4 (5′)	TTCTGCTGGA***TC***CCTGTTGTCTGC	BamH I
AL-62	intron 4 (3′)	TCAATGAAAG***CT***TTCCTTTCTTGG	Hind III
AL-41	intron 5 (5′)	CAACTTC***G***GATC***C***TATTTACATAC	BamH I
AL-68	intron 5 (3′)	CAGATGATGGAATTCCTGTTTTCT	EcoR I
AL-69	intron 6 (5′)	GAAGGCA***A***AG***C***TTATCCCTCCCCT	Hind III
AL-40	intron 6 (3′)	GTACTTGTCT***AA***GCTTCTGAGGTG	Hind III
AL-83	intron 7 (5′)	AGACTCTTTAGA***A***TTCTGATGAGG	EcoR I
AL-84	intron 7 (3′)	TATCAATGC***A***A***G***CTTAATGCTGTT	Hind III
AL-89	intron 8 (5′)	TCTGGGGAAGAA***TT***CCAGATCAGA	EcoR I
AL-80	intron 8 (3′)	GTTATCTCTAA***GC***TTGGTGTCAGA	Hind III
AL-47	intron 9 (5′)	GCCTGGCCAGAAT***T***CTGATAGGCC	EcoR I
AL-42	intron 9 (3′)	CTAGACAA***AG***C***T***TAATGGAGATTG	Hind III
AL-1	3′-nontranslated region (5′)	GACATAATCATTGA***A***TT***C***GTGGTACAC	EcoR I

All sequences are given in the 5′ to 3′ orientation. Nucleotides that were altered in order to create an artificial restriction enzyme site are shown in italics and boldface.

acrylamide gel electrophoresis (17, 18), using precast Clean Gels (Amersham Pharmacia Biotech) as well as 0.5× mutation detection enhancement (MDE) gels (HydroLink; AT Biochem, Malvern, PA) with and without 10% glycerol.

DNA sequence analysis

PCR-amplified DNA fragments were digested with the restriction enzymes BamH I, EcoR I, and Hind III (New England BioLabs) under the conditions recommended by the manufacturer, isolated by 2% low melting point agarose (GIBCO BRL Life Technologies) gel electrophoresis, and ligated into the pBluescript II KS phagemid. DNA sequencing was performed with the dideoxynucleotide chain termination method (19) using T7 DNA polymerase (Sequenase; Amersham Pharmacia Biotech).

Automated sequencing of PCR products

PCR fragments were purified from agarose gels with the Geneclean kit (Bio 101) and sequenced with 3′ dye-labeled dideoxynucleotide triphosphates (dye terminators) using AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Inc.). Sequence analysis was performed on an Applied Biosystems 377A DNA sequencer.

Restriction fragment length polymorphism analysis

hLAL exon 3 was amplified from genomic DNA with primers AL-70 (5′-GCTTTCTTAAAGCTTGGAGAACATAG-3′) located at the 3′-end of intron 2 and AL-75 (5′-CACAAAACCCAGAAGAAT TCTGGT-3′) at the 5′-end of intron 3. Amplification products of exon 4 were obtained with oligonucleotide AL-74 (5′-GTTTTG AAGCTTGGTGCTACTGCC-3′) and the mutation detection primer AL-119 (5′-CTGAAAGCCCAGAATTCATCCTGAGAAA CTTCG-3′) containing three nucleotides of an artificial Taq I site (5′-T/CGA-3′) at its 3′ end. Amplification of exon 10 was performed with the primer pair AL-42 (5′-CTAGACAAAGCTTAAT GGAGATTG-3′) in intron 9 and AL-1 (5′-GACATAATCATTGAA TTCGTGGTACAC-3′) located at the 5′ end of the 3′-nontranslated region.

Site-directed mutagenesis by the overlap extension PCR method, in vitro expression of hLAL cDNA constructs, and the hLAL enzyme assay were performed as described in detail elsewhere (20, 21).

RESULTS

Case history of patient T. S.

The proband was a Caucasian female who first presented at one month of age with bloody stools, abdominal distention, and 23% vacuolized lymphocytes. One month later, she was hospitalized due to persistent and forceful vomiting as well as failure to thrive. Hepatosplenomegaly and massive distention of the abdomen were noticed on presentation. Hemoglobin was decreased to 9.1 g per 100 ml and the platelet count was 140,000/ml. Transaminase activities were elevated (SGPT 46 U/l; SGOT 95 U/l). Results of hemostasis assays were also pathological (PT 46%; APTT 46 sec; TT 21 sec; AT III 35%). Sonography of the abdomen revealed massive calcification of both adrenal glands. Low-grade fever as well as elevations of C-reactive protein (3.2 mg/100 ml) and total leukocyte count (9400/ml) made it probable that she suffered from an infection. However, antibiotic therapy did not improve her

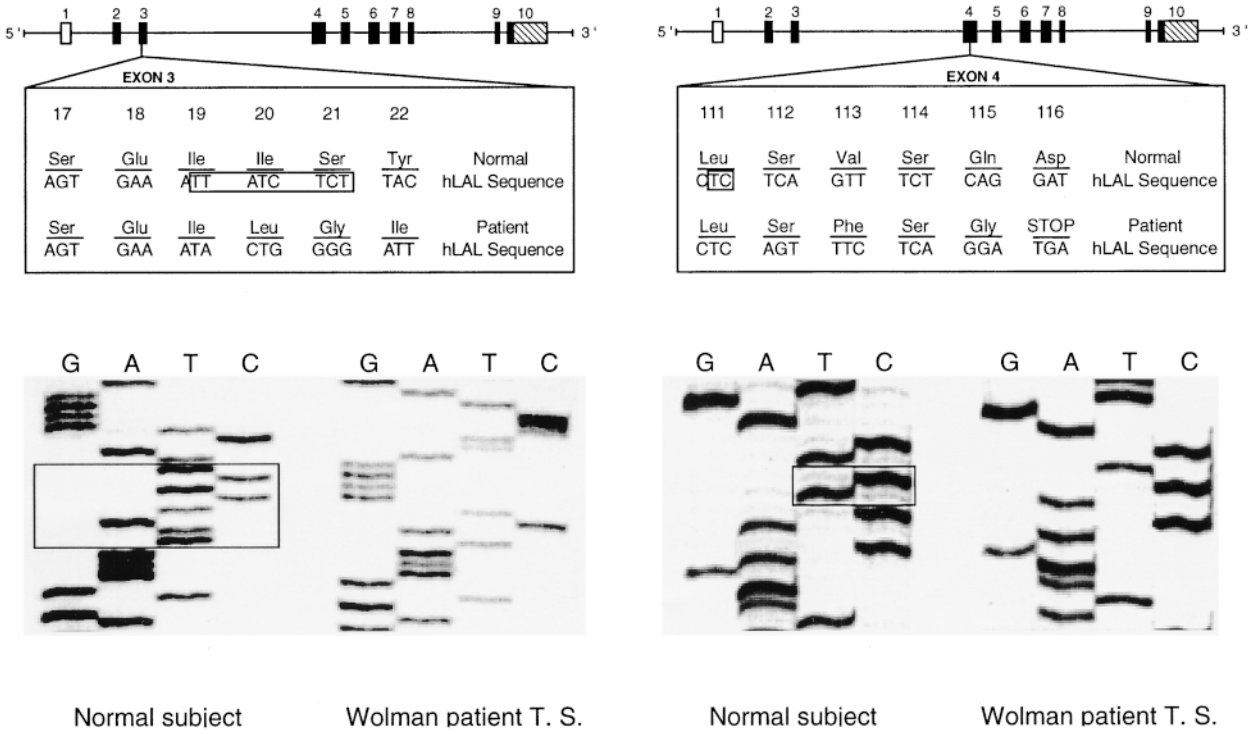

Fig. 1. Sequence analysis of hLAL gene exons 3 and 4 in Wolman patient T. S. The upper panels illustrate the genomic structure and the location of the 8-bp and 2-bp deletions (boxed) within the third and fourth exon of the proband's hLAL gene, respectively. The lower panels contain the corresponding autoradiograms of sequencing gels of DNA from a normal subject (left side) and from the patient (right side). The deleted nucleotides are boxed.

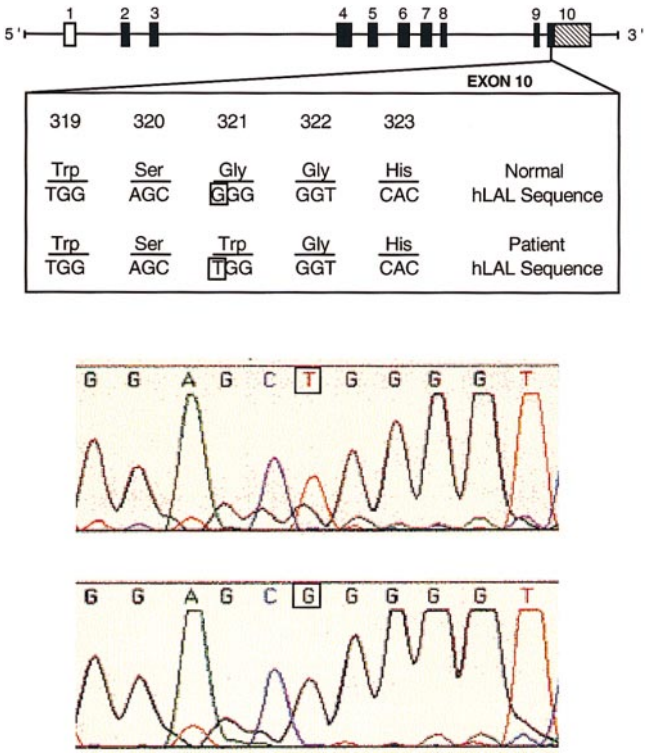

Fig. 2. Sequence analysis of hLAL gene exon 10 in Wolman patient Y. O. A schematic diagram of the hLAL genomic structure (upper panel) shows the position of the G→T nucleotide substitution in exon 10 (indicated by a box) which results in the replacement of the conserved amino acid 321, glycine, with tryptophan. The lower panel presents the corresponding sequence electropherograms of hLAL exon 10 amplification products from the proband (top) and from a normal control (bottom).

condition. A bone marrow aspirate showed increased numbers of foam cells. Thin-layer chromatography of extracts prepared from a liver biopsy revealed a massive accumulation of cholesteryl esters. Deficient hLAL activity in fibroblasts of the patient confirmed the diagnosis of Wolman disease. Anemia and thrombocytopenia became more severe as the disease progressed and the infant died at age 11 weeks due to acute heart failure.

Case history of patient Y. O.

The subject was the second child of Turkish parents whose first child had died in the neonatal period for reasons unknown. Pregnancy was uneventful and spontaneous vaginal delivery occurred in the 40th week of gestation with a birth weight of 3000 g. Shortly after birth, pronounced vomiting developed and at 40 days of age, abdominal distension and hepatosplenomegaly were apparent. An abdominal X-ray disclosed bilateral adrenal calcifications and a tentative diagnosis of Wolman disease was made. On referral to the hospital at 2 months of age, she presented as a pale infant with failure to thrive (weight 3950 g). She had severe anaemia (Hb 5.8 g/dl; haematocrit 14%), thrombocytopenia (99,000/ml), increased tendency to bleed (prolonged PT, PTT, bleeding and coagulation time with normal fibrinogen levels), but normal serum protein concentrations (total protein 6.6 g/dl; albumin 3.7 g/dl). Hepatocellular enzyme levels were increased (SGOT 621 U/l; SGPT 241 U/l; LDH 1179 U/l). Total bilirubin (15 mg/dl), ammonia (>400 mg/dl), total cholesterol (216 mg/dl), and triglycerides (823 mg/dl) were also elevated. Foam cells were observed in the bone marrow aspirate. She received multiple blood and fresh-frozen plasma transfusions during her hospitalization. On the 5th day, she developed disseminated petechia, ecchymoses, and was bleeding from multiple sites. Her condition deteriorated and she became comatose with features suggesting hepatic insufficiency and encephalopathy. Supportive treatment was unsuccessful in controlling hepatic dysfunction and haemorrhagic diathesis as a result of which she expired due to respiratory and cardiac arrest after a convulsive episode. Post mortem findings were in accordance with a lipid storage disease which was especially pronounced in the liver, spleen, and adrenals. Acid lipase activity in cultured fibroblasts was essentially undetectable, using cholesteryl-[1-^{14}C]-oleate as substrate. Thin-layer chromatography of lipids extracted from liver tissue demonstrated a pathological storage of cholesteryl esters.

Single-strand conformation polymorphism (SSCP) analysis

Amplification of hLAL exons 1–10 and subsequent gel electrophoresis on a 10% neutral polyacrylamide gel led to the discovery of additional bands in case of the single-stranded exon 3 and exon 4 products of proband T. S. The same result was obtained with the exon 3 fragment, but not with exon 4 DNA on high resolution 0.5× MDE gels without glycerin. The denatured exon 10–5′-fragment of patient Y. O. also displayed an abnormal mobility pattern that was slightly shifted towards the cathode on 0.5× MDE gels with glycerin (data not shown). No additional SSCP anomalies were observed for any of the other exons in either of the subjects.

DNA sequence analysis

In order to elucidate the molecular basis of Wolman disease in these two patients, we assessed the structural integrity of their hLAL genes. DNA sequence analysis of the coding and non-coding exons and of the flanking intron regions revealed the presence of two small deletions in subject T. S. The first encompassed nucleotides 159–166 (TTATCTCT; numbering according to ref. 4) in exon 3 (**Fig. 1**), the second nucleotides 435/436 (TC) or 437/438 (TC) in exon 4 (Fig. 1). Both deletions destroy the reading frame and lead to premature termination of protein translation at amino acid positions 24 and 116 of the mature enzyme. In the case of proband Y. O., a single G_{1064}→T substitution was found in hLAL exon 10 (**Fig. 2**), replacing glycine (GGG) at position 321 with tryptophan (TGG).

Restriction fragment length polymorphism analysis

Software (PC/GENE; IntelliGenetics, Mountain View, CA)-assisted screening for polymorphic restriction enzyme

sites revealed the loss of a Fau I site (5′-CCCGC(4/6)-3′) and the presence of a new cleavage site for Alu I (5′-AG/CT-3′) in exon 10 as a consequence of the G→T replacement. The 8-bp deletion in exon 3 resulted in the loss of a restriction site for the enzyme Tsp509 I (5′-/AATT-3′). The deletion of the two nucleotides in exon 4, in contrast, did not alter the computer-generated restriction pattern. We therefore decided to synthesize the mutation detection primer AL-119 containing an incorporated partial Taq I site (5′-T/CGA-3′) which is completed by T_{435} of the normal allele and destroyed by A_{433} of the mutated allele.

Agarose gel electrophoresis of Tsp509 I-digested exon 3 amplification products and of PCR-amplified exon 4 fragments digested with Taq I demonstrated that patient T. S. was a heterozygote for the 8-bp deletion in exon 3 and the TC-deletion in exon 4 (**Fig. 3**). Subject Y. O., in contrast, was homozygous for the G_{1064}→T substitution in exon 10, as shown by restriction enzyme digest with Alu I (**Fig. 4**).

Allele-specific cDNA amplification

To confirm that the two deletions are on separate chromosomes, total RNA of patient T. S. was reverse transcribed with 3′-oligonucleotide AL-27 in exon 5 and PCR-amplified with primer pair AL-27 and AL-116 (5′-GAAT GTGAGTGAAATTATCTCT-3′), the latter encompassing the 8 bp deletion in exon 3. Subsequent sequence analysis of the amplification product revealed the presence of the 2-bp deletion in exon 4, whereas the exon 3 mutation was absent (data not shown).

In vitro mutagenesis and transient expression in Cos-7 cells

In order to investigate whether the replacement of tryptophan for glycine at amino acid position 321 abolished hLAL catalytic activity and was the cause for Wolman disease in proband Y. O., we reproduced this mutation in vitro by site-directed mutagenesis of normal hLAL cDNA. After transient expression for 72 h, homogenates of Cos-7 cells transfected with a wild-type or mutant cDNA as well as mock-transfected cell extracts were assayed for substrate hydrolysis. Enzymic activity of cell extracts transfected with hLAL(Gly_{321}→Trp) was 0.4 ± 0.8% (n = 12) using cholesteryl oleate and 0.5 ± 1.4% (n = 12) with triolein when compared with the wild-type enzyme, demonstrating synthesis of a completely inactive enzyme.

Protein secondary structure predictions

The replacement of glycine by tryptophan at residue 321 occurred at a position absolutely conserved in hLAL, human gastric lipase, rat lingual lipase, and murine lysosomal acid lipase (22), and in close vicinity of the catalytic triad residue aspartic acid at position 324 (20).

Computer modeling (PC/GENE) of the normal and mutant enzymes demonstrated that the replacement of

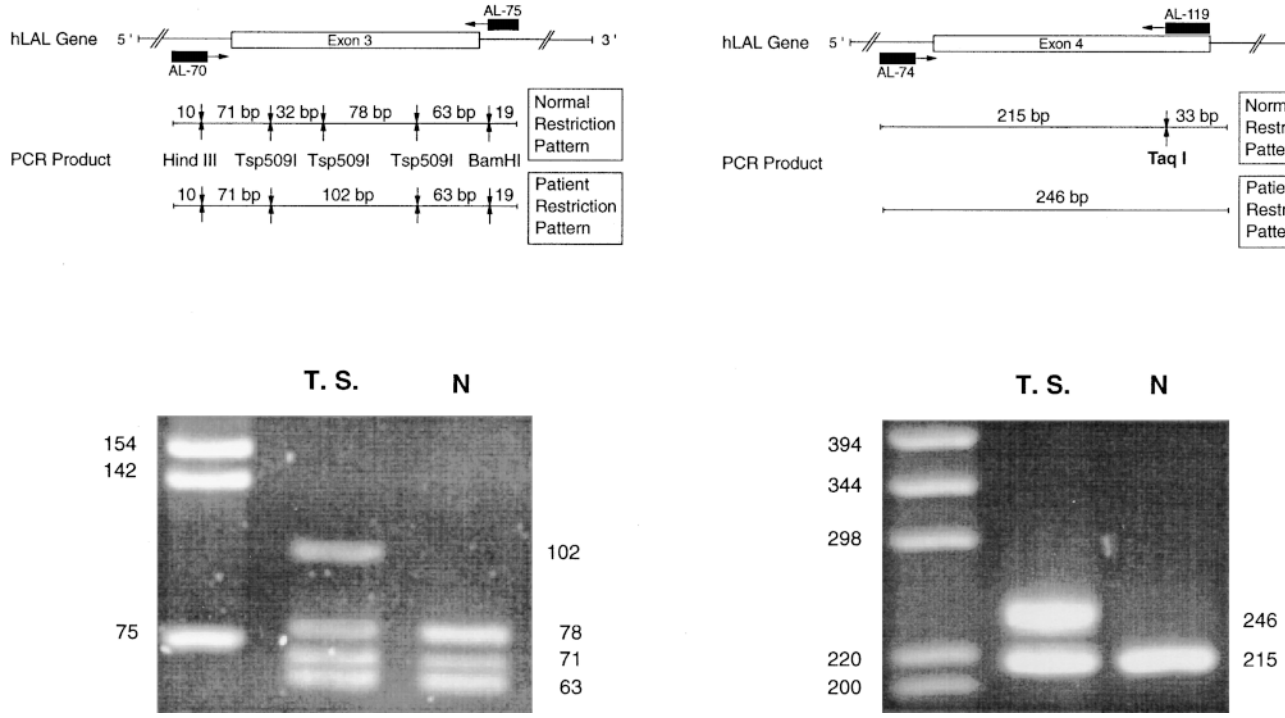

Fig. 3. Restriction enzyme digestion of PCR-amplified hLAL exon 3 and exon 4 products from proband T. S. and from a control subject. Primer combinations AL-70 + Al-75 and AL-74 + AL-119 were used to amplify the regions of the mutations within the third and fourth exon of the hLAL gene, respectively, as shown in the upper panel. The exon 3 products were cut with restriction endonucleases BamH I, Hind III, and Tsp509 I, while the exon 4 products were digested with the enzyme Taq I. The positions of the cleavage sites and the sizes of the resulting fragments are also given. The lower panel illustrates the electrophoretic analysis of the PCR products from a control subject (N; right lane) and from the patient (middle lane) which confirms that proband T. S. was a compound heterozygote for both nucleotide substitutions. A nucleic acid size standard was loaded in the left lane and the size of the fragments is given on the left side. The sizes of the uncut and digested products are indicated on the right side.

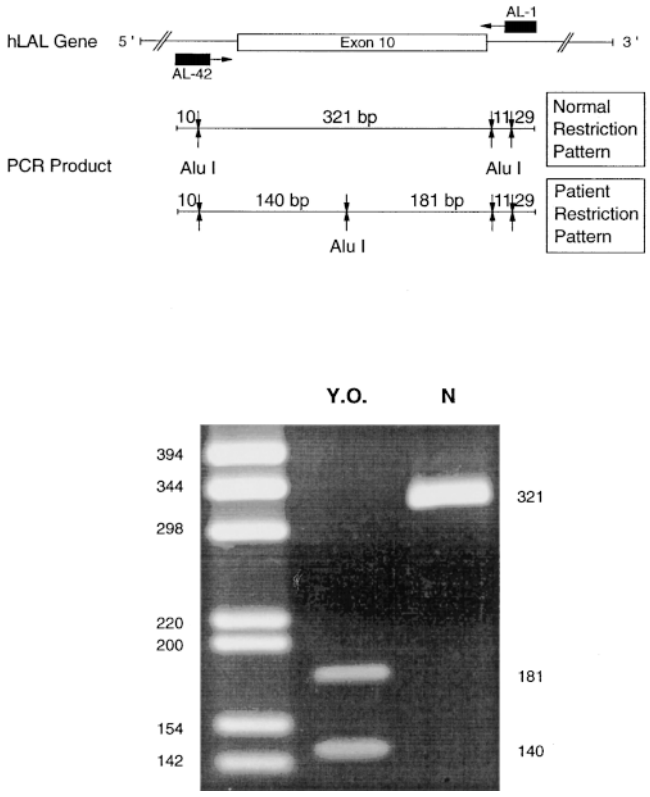

Fig. 4. Restriction enzyme digestion of PCR-amplified hLAL exon 10 products from proband Y. O. and from a control subject. The coding region of hLAL exon 10 was amplified by PCR with oligonucleotides AL-42 and AL-1. The products were then digested with Alu I and the fragments were separated on a 2% low melting point agarose gel together with a nucleic acid size standard (lower panel, left lane). The enzyme is expected to cleave the patient's 371 bp amplification product four instead of three times, leading to the production of two abnormally sized fragments of 140 and 181 bp in length. In the proband (middle lane), only these two smaller fragments were visible, confirming that she was a homozygote for this mutation. The normal subject (N; right lane), in contrast, had only the uncleaved PCR product of 321 bp.

the aliphatic glycine by the hydrophobic and aromatic amino acid tryptophan decreases the chain flexibility (23) of the surrounding region. Protein secondary structure prediction by the rules of Garnier, Osguthorpe, and Robson (24) indicated that the substitution at position 321 changes the conformation of the catalytically active Asp_{324} from coil to turn and shortens the consecutive stretch of amino acids in extended conformation (residues 325–344) by four amino acids. The GGBSM method (25), in contrast, predicted a change of residue 323 from a coil to a helical conformation, while amino acid 324 was unaffected by the Gly_{321}→Trp mutation.

DISCUSSION

In order to investigate the molecular basis of Wolman disease and CESD and to establish hLAL structure–function relationships, we have obtained DNA samples and, in some cases, fibroblast cultures from 26 different patients affected with these disorders through collaboration with clinicians all over Europe.

In this report, we present the genetic defects of two Wolman probands of German (T. S.) and Turkish (Y. O.) origin. DNA sequence and restriction fragment length polymorphism analysis as well as allele-specific amplification demonstrated that T. S. was a compound heterozygote for an 8-bp and a 2-bp deletion in exon 3 and 4, respectively, while Y. O. was homozygous for an amino acid substitution in the carboxyl-terminal region of the enzyme, replacing the absolutely conserved glycine, residue 321, with tryptophan. The two out-of-frame deletions result in the formation of premature stop codons of protein translation at amino acid positions 24 and 116 and in the production of truncated, inactive enzymes. The Gly_{321}→Trp replacement, in contrast, is located close to the catalytically active aspartic acid residue at position 324 (20) and appears to interfere with the secondary structure of the region between amino acids 321 and 328, as predicted by computer-assisted modeling. This may result in a disturbance of the three-dimensional conformation of the catalytic triad and in a nearly complete loss of enzymatic activity towards neutral lipids, as observed by in vitro expression of the mutated enzyme in Cos-7 cells. Our data confirm previous observations that Wolman disease is due to a more than 99% reduction of cholesteryl ester and triglyceride hydrolysis, thereby also lending support to the threshold hypothesis which postulates that the only difference between Wolman disease and CESD is the level of residual hLAL activity (5, 10, 13).

T. S. appears to be the very rare example of a compound heterozygote for Wolman disease, as, in our seven other Wolman cases, the subject was either homozygous for the respective mutation or, in those cases where the proband was not available for genetic analysis, both parents had inherited the same genetic defect on one of their chromosomes (P. Lohse, M. Elleder, A. W. Eriksson, R. Gatti, W. Storm, and M. T. Zabot, unpublished results), strongly suggesting that the disease manifests itself in almost all cases as a consequence of consanguineous marriage and that patients with a double defect are extremely rare. This is not surprising in light of the fact that the allele frequency of hLAL deficiency is probably very low. In addition, most mutations appear to have arisen independently and to be unique to a single family.

Our observations are in agreement with the results of other case studies reported thus far in the literature. In five Wolman probands, homozygosity for the respective mutation has been observed (10–14), and only the patient FeD, studied by Anderson et al. (5), was a compound heterozygote for a frameshift mutation and an amino acid substitution.

In CESD patients, in contrast, a single genetic defect, the hLAL exon 8 splice junction mutation (E8SJM), is highly prevalent and has been found on at least one of the chromosomes in 11 of the 17 probands studied thus far (10, 13, 26–36). Among our patients who are of Czech, German, Irish, Italian, and Turkish origin, seven are homozygotes and eight are compound heterozygotes (P.

Lohse, M. Elleder, E. Keller, R. Gatti, J. Kirk, and Y. S. Shin-Podskarbi, unpublished results) for the G→A substitution affecting the last nucleotide of exon 8 (position 934 of the hLAL cDNA) which results in the skipping of exon 8 from the hLAL mRNA transcript and in the loss of 24 amino acids (Δ254–277) from the mature enzyme. This mutation allows for approximately 3% of normal splicing to occur (10, 37), thereby ensuring survival of the affected individuals.

Only one of our patients, who is of Italian ancestry and identical with subject PG described by Pagani et al. (13), had not inherited the exon 8 splice site defect. This is interesting in the context that at least two of the five CESD probands known not to possess the E8SJ mutation are also from southern Italy (27, 32), while two others are of Canadian-Norwegian (31) and Swiss (36) descent, suggesting that the frequency of this common genetic defect may vary among different geographic locations and that the E8SJ mutation may have had its origin in Central Europe.

In summary, the molecular basis of Wolman disease has been elucidated in two subjects of German and Turkish origin. Two frameshift and one missense mutation completely disrupt enzyme function and result in a more than 99% reduction of hLAL-catalyzed substrate hydrolysis, thereby establishing a clear genotype–phenotype correlation. Our combined analyses also demonstrate that homozygosity for unique pathologic mutations in the hLAL gene is very common among Wolman patients. In CESD patients, in contrast, the hLAL exon 8 splice site mutation accounts for approximately 70% of all mutant chromosomes analyzed so far, indicating that CESD is much less heterogeneous in nature than Wolman disease.

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