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The Molecular Basis of Sjögren-Larsson Syndrome: Mutation Analysis of the Fatty Aldehyde Dehydrogenase Gene

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Summary

Sjögren-Larsson syndrome (SLS) is an autosomal recessive disorder characterized by ichthyosis, mental retardation, spasticity, and deficient activity of fatty aldehyde dehydrogenase (FALDH). To define the molecular defects causing SLS, we performed mutation analysis of the FALDH gene in probands from 63 kindreds with SLS. Among these patients, 49 different mutations—including 10 deletions, 2 insertions, 22 amino acid substitutions, 3 nonsense mutations, 9 splice-site defects, and 3 complex mutations—were found. All of the patients with SLS were found to carry mutations. Nineteen of the missense mutations resulted in a severe reduction of FALDH enzyme catalytic activity when expressed in mammalian cells, but one mutation (798G→C [K266N]) seemed to have a greater effect on mRNA stability. The splice-site mutations led to exon skipping or utilization of cryptic acceptor-splice sites. Thirty-seven mutations were private, and 12 mutations were seen in two or more probands of European or Middle Eastern descent. Four single-nucleotide polymorphisms (SNPs) were found in the FALDH gene. At least four of the common mutations (551C→T, 682C→T, 733G→A, and 798+1delG) were associated with multiple SNP haplotypes, suggesting that these mutations originated independently on more than one occasion or were ancient SLS genes that had undergone intragenic recombination. Our results demonstrate that SLS is caused by a strikingly heterogeneous group of mutations in the FALDH gene and provide a framework for understanding the genetic basis of SLS and the development of DNA-based diagnostic tests.

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Introduction

Sjögren-Larsson syndrome (SLS [MIM 270200]) is an autosomal recessive disease characterized by ichthyosis, mental retardation, and spastic diplegia or tetraplegia (Sjögren and Larsson 1957; Rizzo, in press). Ichthyosis is usually apparent at birth and has a generalized distribution. The neurological symptoms appear during the 1st or 2d year of life and result in delay in achievement of motor and cognitive milestones. Spastic diplegia prevents or impedes walking in most patients with SLS. Seizures occur in ~40% of patients. Retinal abnormalities, such as glistening white dots in the perimacular region and pigmentary changes, are often seen in patients with SLS, and photophobia is a common complaint (Jagell et al. 1980). Many patients have short stature as a result of growth delay and leg contractures (Jagell and Heijbel 1982).

Patients with SLS have deficient activity of fatty aldehyde dehydrogenase (FALDH) (E.C. 1.2.1.48; Enzyme Nomenclature Database) (Rizzo and Craft 1991), a microsomal nicotinamide-adenine-dinucleotide (NAD)–dependent enzyme that is necessary for the oxidation of long-chain aliphatic aldehydes to fatty acids (Kelson et al. 1997). Because FALDH is a component of the fatty alcohol:NAD^+ oxidoreductase (FAO) complex, which catalyzes the oxidation of fatty alcohol, patients with SLS show deficient activity of both FALDH and FAO (Rizzo et al. 1988). Measurement of either enzyme provides a specific diagnostic test for this disease. Tissue accumulation of fatty alcohol, fatty aldehyde, and related lipid metabolites is thought to be responsible for the symptoms, but the pathogenic mechanisms are unclear (Rizzo, in press).

SLS is caused by mutations in the FALDH gene on chromosome 17p11.2 (De Laurenzi et al. 1996). The FALDH gene (also called "ALDH10") consists of 11 exons and is widely expressed in tissues (Chang and Yoshida 1997; Rogers et al. 1997). The major FALDH transcript in all tissues examined is composed of 10 exons and encodes a protein of 485 amino acids. Alternative splicing of the gene results in a minor transcript that includes an additional sequence (exon 9′) that gives rise to a larger protein of 508 amino acids with a variant

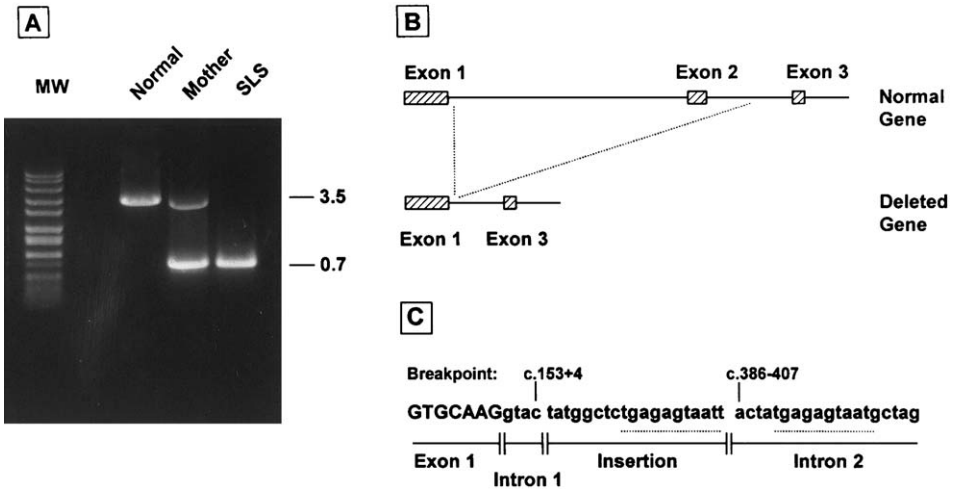

Figure 1 Large deletion mutation (153+5→386–408del+ins19bp) in a homozygous SLS patient. *A,* PCR amplification of a fragment of the FALDH gene spanning exons 1–3 in a normal control patient, in the mother of the patient, and in the homozygous SLS patient (SLS). MW = molecular-weight markers. The normal PCR product is 3.5 kb, whereas the product of the deletion mutation is 0.7 kb. *B,* Illustration of the normal and deleted FALDH gene. *C,* DNA sequences at the deletion breakpoints. A duplicated portion of intron 2 has been inserted and is underlined with a dotted line. Nucleotides flanking the deletion/insertion are numbered according to the FALDH cDNA nomenclature.

carboxy-terminal sequence (Rogers et al. 1997). The carboxy-terminal domain of the more abundant FALDH protein is necessary for anchoring of the enzyme to the microsomal membrane (Masaki et al. 1994), but the function of the minor protein isoform is as yet unknown. In addition, utilization of three distinct polyadenylation sites gives rise to transcripts that differ in length (2.0, 4.0, and 4.2 kb).

Several mutations in the FALDH gene have been reported in patients with SLS (De Laurenzi et al. 1996, 1997; Rizzo et al. 1997; Sillén et al. 1997, 1998; Tsukamoto et al. 1997; Willemsen et al. 1999). Two mutations (943C→T and 1297–1298delGA) are common among European patients (De Laurenzi et al. 1997; Rizzo et al. 1997; Sillén et al. 1997). However, the spectrum of mutations that can cause SLS has not yet been well defined, especially among patients of non-European origin, and only one mutation (943C→T) has been expressed to confirm that it actually destroys enzymatic activity. We therefore performed mutation analysis of 63 probands with SLS, representing kindreds from throughout the world. Our results demonstrate a striking mutational heterogeneity in SLS and provide insight into the nature and origin of the molecular defects causing this disease.

Material and Methods

Patients with SLS and Control Subjects

Probands with SLS, from 63 kindreds, were studied. None of the probands were known to be genetically related. All patients with SLS were confirmed to have FALDH deficiency in cultured skin fibroblasts (Rizzo and Craft 1991). A control population consisted of 66 normal subjects (51 Europeans, 7 African Americans, 4 American Indians, 3 Asians, and 1 individual from the Middle East). This research was approved by the Committee on the Conduct of Human Research at the Medical College of Virginia.

Cells

Cultured skin fibroblasts were grown in Dulbecco's minimal essential medium containing 10% fetal bovine serum, penicillin, and streptomycin, at 37°C in an atmosphere of 5% CO_2. Chinese hamster ovary cells deficient in FALDH (FAA-K1A) were kindly provided by Dr. R. A. Zoeller, Boston University School of Medicine (James and Zoeller 1997), and were grown in F12 medium containing 5% fetal bovine serum.

DNA Isolation

Genomic DNA was purified from blood or cultured fibroblasts, by means of the Wizard Genomic DNA Purification Kit (Promega). Alternately, DNA used for PCR was obtained from buccal brushings or fibroblast pellets, as described elsewhere (Richards et al. 1993).

FALDH Assay

Cultured skin fibroblasts were harvested by trypsinization, and FALDH was assayed with octadecanal as substrate (Rizzo and Craft 1991; Kelson et al. 1997). In experiments measuring expression of mutant FALDH, transfected Chinese hamster ovary cells were homoge-

nized in buffer containing 0.1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride, rather than in the usual homogenization buffer (Rizzo and Craft 1991).

Mutation Analysis

The FALDH gene was screened for mutations by use of direct DNA sequencing of PCR-amplified exons. FALDH exons (GenBank) were amplified from genomic DNA, with use of the primer pairs shown in table 1. All reactions were performed in 50 μl final volume with 1 U *Taq* polymerase (PE Biosystems), 2.5 mM $MgCl_2$, 0.5 mM each dNTP, 5 pmol each oligonucleotide primer, and a variable amount of purified DNA or buccal-brushing DNA. Reactions were initiated by hot start with addition of the *Taq* polymerase. The thermocycling conditions used for amplification of exons 2–10 consisted of an initial denaturation at 94°C for 5 min; then 35 cycles of denaturation at 94°C for 30 s, annealing at 62° for 30 s, and extension at 72°C for 1 min; and then a final extension at 72°C for 5 min. The coding sequence of exon 1 was amplified as described above, with the use of denaturation steps at 96°C and an annealing temperature of 65°C. PCR products were sequenced by use of the BigDye Terminator Cycle Sequencing Kit (PE Biosystems), with the same primers that were used for exon amplification, and the products were then analyzed on

Table 1

Oligonucleotide Primers Used to Amplify Exons in the FALDH Gene from Genomic DNA

Exon or Intron and Primer[a]	Sequence
Exon 1:	
HF21S (forward)	5′-CCTGGGCGAGTGAATTGTGGCT-3′
HF20Ai (reverse)	5′-TCTGTATCGCTGGAGATCATACTC-3′
Exon 2:	
HF15Si (forward)	5′-CAGTTGTTGTCACTACAGGTGTACCTG-3′
INT 2− (reverse)	5′-GGTGTAAACAAAGGCAAATCACC-3′
Exon 3:	
INT 2++ (forward)	5′-GAGCTGCAGAAATAATTGGGAGTACCTAGC-3′
INT 3/2− (reverse)	5′-GAACTTCTTTTGCTAAGCCAACTGTTGAAG-3′
Exon 4:	
HF14Si (forward)	5′-TATTTGGCAGTGCAAGAGTTTGT-3′
HF14Ai (reverse)	5′-ACAAAAGAGTAACACTGTCAGGAAAAGTC-3′
Exon 5:	
HF18Si (forward)	5′-ATTATATAGCTGTTCTGGATGTTTTC -3′
INT 5/1− (reverse)	5′-GGGAGAAACAAATGGGGATGCTGC-3′
Exon 6:	
HF16Si (forward)	5′-GCATGGCTGGATTTTGTACTTACTG-3′
INT 6− (reverse)	5′-GTGGCGTCATTCAATACACAGAAAC-3′
Exon 7:	
HF13Si (forward)	5′-GCACAGTTCATCCACGTGCTCAG-3′
HF17Ai (reverse)	5′-GGAACATGACTCTTGACAGTTGCTTATG-3′
Exon 8:	
INT 7++ (forward)	5′-TTGACACATAACTGAGCACACAGCCCTC-3′
HF15Ai (reverse)	5′-AGCAGCCCATACAATCCACTCATGA-3′
Exon 9:	
HF17Si (forward)	5′-CAGAAGTAGCTTGCATCATCTACAGTG-3′
HF16Ai (reverse)	5′-CTAGAATATGCATCTGGCAGCCATC-3′
Exon 10:	
INT 9/2+ (forward)	5′-TGTACAATGCATGTAGAGTCTCTTCAG-3′
FALDH 3′− (reverse)	5′-GGCAATGACTTTTAAGTTTGATCACAGAGTGTTTG-3′
Exon 9′:	
HF19Si (forward)	5′-TGGGCAATAGAGCGAGACTCCATC-3′
HF19Ai (reverse)	5′-CCACTTGGCCCACCTTACATATAG-3′
Intron 1 SNP:	
HK8+ (forward)	5′-GAGGCCCTGCGGAGGATGGTGCA-3′
C153(+39) (reverse)	5′-GTCCACAAGTGCGCG<u>T</u>CGG-3′
T153(+39) (reverse)	5′-GTCCACAAGTGCGCG<u>T</u>CGA-3′
Exon 10 SNP:	
INT 9/2+ (forward)	5′-TGTACAATGCATGTAGAGTCTCTTCAG-3′
A1446 (reverse)	5′-CAGGATCATTCTTCAGTAATA<u>C</u>TCT-3′
T1446 (reverse)	5′-CAGGATCATTCTTCAGTAATA<u>C</u>TCA-3′

[a] Primers were generated from flanking intron (Rogers et al. 1997) or exon (De Laurenzi et al. 1996) sequences (GenBank). Primers used to detect SNPs incorporated nucleotide mismatches (underlined) to improve allele specificity.

an ABI 377A sequencer. All mutations were confirmed by sequencing the exons with both sense and antisense primers and by digestion with restriction enzymes whenever mutations altered a restriction site. In some complex mutations and in certain compound heterozygotes, PCR products were cloned into plasmid pNoTA/T7 (Prime PCR Cloner System; 5 Prime→3 Prime, Inc.) prior to the sequencing.

Amplification of DNA fragments >2 kb (shown in fig. 1) was performed by use of the Expand Long Template PCR System (Roche), according to the manufacturer's instructions. The forward primer was HK8+, and the reverse primer was Int 3/2− (table 1). Thermocycling conditions consisted of an initial denaturation at 94°C for 2 min, then 5 cycles of denaturation at 94°C for 10 s and annealing/extension at 68°C for 2 min, and then 30 subsequent cycles at 94°C for 10 s and at 68°C for 2.5 min with increments of 20 s/cycle. Mutations were described according to the nomenclature of Antonarakis (1998) and are identified by the cDNA nucleotide–numbering system.

Analysis of Splice-Site Mutations

To identify the aberrant transcripts resulting from splice-site mutations, total RNA was isolated from cultured fibroblasts by use of the SV Total RNA Isolation System (Promega), and it was amplified by reverse-transcription–PCR (RT-PCR) (Access RT-PCR System; Promega) by use of exonic primers, as described elsewhere (De Laurenzi et al. 1996). DNA products were separated by agarose electrophoresis, were isolated, and then were either sequenced directly or cloned prior to being sequenced.

To amplify the aberrant FALDH transcripts arising from donor splice-site mutations in exon 5 (shown in fig. 2), the following primers were used for RT-PCR: (forward) 5′-ATCTTGGCAAAGCTTCTCCCTCAGT ATTTAGACC-3′ and (reverse) 5′-GGCCTCATCAG TCTCCCCACCAAAAGCTATC-3′. To amplify the FALDH mRNA shown in figure 3, we used the following primers: (forward) 5′-CTGCTGCCAAGC ATCTGACCCCTGTGAC-3′ and (reverse) 5′-AGG-TTTGGCCACAATTCATGTATTTTCCCCAGG-3′. The cDNA synthesis reaction occurred at 48°C for 45 min, with the use of 200 ng RNA. After initial denaturation at 94°C for 2 min, PCR consisted of 5 cycles of denaturation at 94°C for 0.5 min and annealing/extension at 68°C for 2 min, and then 30 cycles of denaturation at 94°C for 0.5 min, annealing at 64°C for 1 min, and extension at 68°C for 2 min. A final extension time was 5 min at 68°C.

Mammalian Expression of FALDH Mutations

To express FALDH mutations, we constructed an expression vector containing an S-tag attached to the 5′

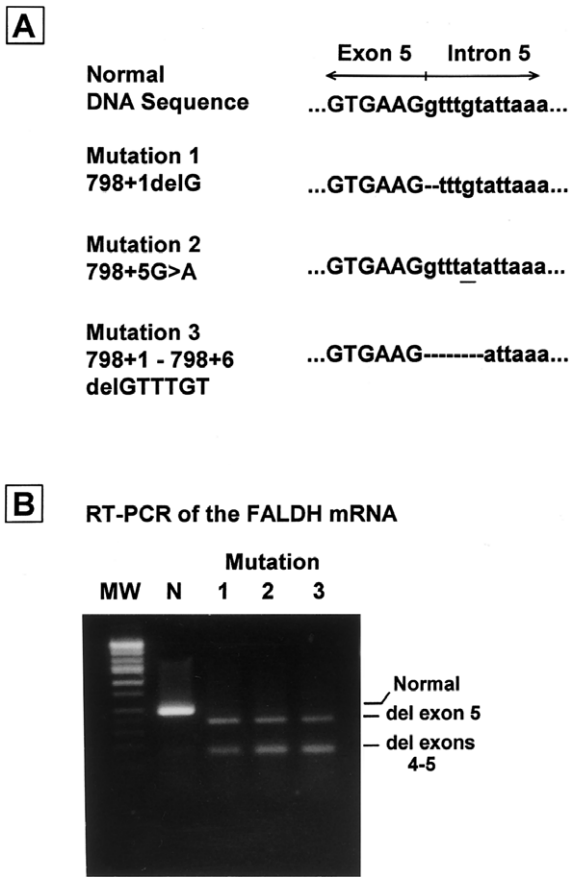

Figure 2 Aberrant splicing caused by three splice-donor mutations at the junction of exon 5 and intron 5. *A*, Normal and mutant DNA sequences. Exon sequence is indicated by uppercase letters, intron sequence by lowercase letters. *B*, Amplification of fibroblast FALDH mRNA by RT-PCR shows that all three mutations result in two abnormal transcripts, one of which skips exon 5 and the other of which skips exons 4 and 5.

end of the FALDH cDNA, by cloning the FALDH cDNA into an S-tag plasmid (Novagen) and by subcloning the S-tag-FALDH construct into the pCIneo (Promega) mammalian expression vector. Mutations were introduced into the S-FALDH-pCIneo expression vector by means of site-directed mutagenesis performed with the use of the Transformer Site-Directed Mutagenesis Kit (Clontech), and they were confirmed by DNA sequencing. Plasmid DNA was purified and transfected into FAA-K1A cells with the use of Fugene-6 reagent (Roche), according to the manufacturer's instructions. FALDH activity in the cells was measured after 2 d. To confirm that loss of enzyme activity did not arise simply from inefficient expression of the mutant FALDH, the concentration of S-Tag protein was measured with the S-Tag Rapid Assay Kit (Novagen), in the same cell homogenates used for FALDH assay. FALDH activity was normalized to S-tag protein, and the enzyme-specific activity measured in cells transfected with the mutant construct

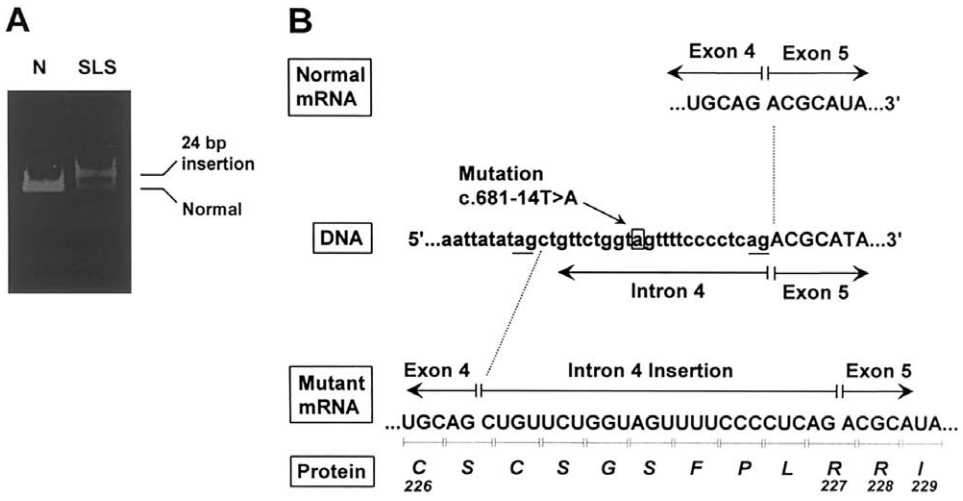

Figure 3 Splice-acceptor–site mutation 681–14T→A and its effect on FALDH mRNA and protein. *A,* RT-PCR products, obtained with the use of primers in exons 4 and 5, separated in a 10% polyacrylamide gel. The left lane shows normal RNA, and the right lane shows patient RNA demonstrating an abnormal larger product arising from a 24-bp insertion in the patient's mRNA. *B,* Mutation and sequence of the abnormally spliced larger transcript. The larger product was amplified from an aberrant FALDH transcript that arose from utilization of a cryptic splice-acceptor site upstream of the mutation, whereas the smaller product represents the normally spliced transcript. Normal and cryptic splice-acceptor sites are underlined. The mutation is denoted by a boxed letter "a."

was expressed as a percentage of the activity measured with the wild-type cDNA vector.

Polymorphism Screening

Two single-nucleotide polymorphisms (SNPs) in the gene altered restriction-enzyme cut sites. To screen for the intron 3 polymorphism at 471+31T/C, a 342-bp genomic DNA fragment was amplified by PCR, with the use of exon 3 primers (table 1), and the DNA (0.5 μg) was digested overnight with 20 U *Nde*I in a 25-μl reaction volume, according to the manufacturer's instructions. The 471+31T allele was cut with *Nde*I, producing 224-bp and 118-bp products, whereas *Nde*I did not cut the alternate C allele. In a similar fashion, a 422-bp fragment containing the intron 6 polymorphism (940+53G/C) was amplified with primers for exon 6 (table 1) and was digested with 10 U *Hph*I. The 940+53G allele was cut by *Hph*I, generating 281-bp and 141-bp products, whereas 940+53C was not cut. For both polymorphisms, DNA from control individuals who were homozygous or heterozygous for the polymorphic variants was simultaneously treated, to verify complete digestion. The digestion products were separated on a 2% agarose gel and were stained with ethidium bromide.

Two other polymorphisms, 1446A/T in exon 10 and 153+39C/T in intron 1, were detected by means of allele-specific PCR. As an internal control, multiplex PCR was performed by coamplification of exon 6. The PCR contained 1 U *Taq* polymerase, 2.5 mM $MgCl_2$, 0.5 mM each dNTP, 2 pmol each exon 6 primer (table 1), buccal or fibroblast DNA, and polymorphism-specific primers, in a final volume of 50 μl. To selectively coamplify the 1446A allele, the PCR also contained 5 pmol forward primer for exon 10 (table 1, primer Int 9/2+) and 10 pmol A1446 allele-specific reverse primer (table 1). The cycling conditions were the same as those described above for amplification of exons 2–10. To selectively amplify the 1446T allele, the A1446 primer was replaced with 10 pmol antisense T1446 primer, and an annealing temperature of 63°C was used. To screen for the 153+39C/T polymorphism in intron 1, the PCR mixture was modified from that described above, in that it contained 1 pmol each exon 6 primer, 15 pmol exon 1 forward primer (table 1), and 15 pmol reverse primer C153(+39) or T153(+39) (table 1). The cycling conditions were as follows: annealing temperatures of 60°C for the C allele and 62°C for the T allele. PCR products were separated on either a 1.5% or 2% agarose gel and were visualized with ethidium bromide. There was complete concordance between results of DNA sequencing and those for allele-specific PCR screening for the intron 1 (n=37) and exon 10 (n = 24) polymorphisms.

Results

Among 63 probands with SLS, we detected 49 different mutations, including 10 deletions, 2 insertions, 22 amino acid substitutions, 3 nonsense mutations, 9 splice-site mutations, and 3 complex mutations (table 2). All

Table 2

Mutations Detected in Probands with SLS

Type of Mutation and Exon	Nucleotide Change[a]	Protein or Transcript Change	No. of Probands with Mutation (Ethnic or Geographic Origin)
Deletion:			
2	153+5→386–408del+ins19bp	Delete exon 2 and frameshift→truncation	1 (Turkish[b])
2	286–296del	Frameshift→truncation	1 (Middle Eastern[b])
2	338delA	Frameshift→truncation	1 (Spanish)
2	374–378del	Frameshift→truncation	1 (Middle Eastern)
3	469delC	Frameshift→truncation	1 (Polish, Swiss)
6	821–822delAA	Frameshift→truncation	1 (European)
7	968delC	Frameshift→truncation	1 (Turkish)
7	1100delA	Frameshift→truncation	1 (English, Irish, Polish, American Indian)
9	1291–1292delAA	Frameshift→truncation	1 (Japanese[b])
9	1297–1298delGA	Frameshift→truncation	16 (European[b])
Insertion:			
4	620–621insG	Frameshift→truncation	1 (Italian)
9	1311–1312insACAAA	Frameshift→truncation	1 (French[b]) 1 (French, Irish, American Indian)
Complex:			
5+6	[733G→A, 901G→C, 906delT, 909T→G]	D245N, A301P, frameshift→protein truncation	4 (European[b])
7	941–943delCCC+ins21bp	A314G+insert 6 amino acids+P315A	2 (Lebanese[b]) 1 (Greek[b]) 1 (Middle Eastern, European)
8	1108–1116del+ins ACAG	Frameshift→truncation	1 (Italian)
Missense:			
1	133A→T	I45F	1 (German)
2	191T→A	V64D	1 (African American)
2	317T→G	L106V	1 (Middle Eastern[b])
2	341C→T	P114L	1 (Turkish[b])
2	362C→T	P121L	1 (African American)
4	551C→G	T184R	1 (Tunisian[b])
4	551C→T[c]	T184M	1 (German), 2 (European[b]) 1 (European, Middle Eastern)
4	554G→C	G185A	1 (French, Irish, American Indian)
5	682C→T[c]	R228C	2 (Palestinian[b]), 2 (Middle Eastern[b])
5	710G→A	C237Y	2 (Arab-Jewish, Syrian-Jewish[b])
5	733G→A[c]	D245N	1 (European)
5	798G→C	K266N; unstable transcript	1 (German)

6	835T→A	Y279N	1 (European)
7	943C→T	P315S	8 (Northern European, Swedish[b])
7	984G→C	M328I	1 (Mixed European[b])
7	1094C→T[c]	S365L	2 (German[b]) 1 (German, English, Scottish)
9	1216G→A	G406R	1 (Pakistani[b])
9	1231C→T	H411Y	1 (Egyptian[b])
9	1244G→A	S415N	1 (German[b])
9	1256T→C	F419S	1 (Middle Eastern[b])
9	1268G→A[c]	R423H	1 (Middle Eastern)
9	1339A→G	K447E	1 (Japanese[b])
Nonsense:			
1	24–25CC→TT	R9Ter	1 (Turkish)
1	28C→T	Q10Ter	1 (Spanish)
2	233G→A	W78Ter	1 (Italian[b]), 1 (French)
Splice-site:			
Exon 3/intron 3 junction	471+1delG	Skips exons 2 and 3→in-frame deletion of amino acids 52–157	1 (Italian), 1 (Polish, Swiss)
Exon 3/intron 3 junction	471+2T→G	Skips exons 2 and 3→in-frame deletion of amino acids 52–157	1 (Mixed European)
Intron 3/exon 4 junction	472−2A→G	Skips initial 33 bp in exon 4 and uses downstream cryptic acceptor site in exon 4→deletion of amino acids 158–168	1 (Austrian)
Intron 4/exon 5 junction	681−2A→G	Skips exon 5→frameshift and premature termination	1 (French)
Intron 4/exon 5 junction	681–14T→A	Causes use of a new cryptic acceptor site in intron 4, adding 24 bp between exons 4 and 5→insertion of 8 amino acids between C226 and R227; skips exon 5→frameshift and premature termination	1 (German)
Exon 5/intron 5 junction	798+5G→A	Skips exon 5→frameshift and premature termination; skips exons 4 and 5→deletion of amino acids 158–266	1 (Lebanese[b])
Exon 5/intron 5 junction	798+1delG	Skips exon 5→frameshift and premature termination; skips exons 4 and 5→deletion of amino acids 158–266	1 (European[b]), 1 (Japanese[b])
Exon 5/intron 5 junction	798+1−798+6delGTTTGT	Skips exon 5→frameshift and premature termination; skips exons 4 and 5→deletion of amino acids 158–266	1 (Hispanic[b])
Intron 7/exon 8 junction	1108−1G→C	Skips exon 8→frameshift and premature termination; skips exons 8–9→in-frame deletion of amino acids 370–481	1 (Brazilian[b])

[a] Mutation numbers refer to the cDNA position.
[b] Denotes mutations carried in a homozygous state by one or more probands.
[c] Transition mutation at CpG dinucleotide.

of the probands carried FALDH mutations. Thirty (48%) of the probands were homozygous for their mutation. Thirty-seven of the mutations were each seen in only one proband.

Exonic Deletions, Insertions, and Complex Mutations

Of the 10 deletions, 8 involved one or two nucleotides, and 1 deletion consisted of 11 nucleotides (table 2). All of these mutations cause a frameshift and a premature stop codon. All four deletions that involved two or more nucleotides were located at short palindromic sequences. Two of the deletions in exon 9 (1291–1292delAA and 1297–1298delGA) involved the same 9-bp palindromic sequence (AAGAGAGAA). With the exception of 1297–1298delGA, which was found in 16 probands, all of European origin, each deletion was carried by a single proband. As shown in figure 4, the deletions were not clustered in any region of the gene.

One large deletion was found in a homozygous state in a consanguineous Turkish patient. Using PCR, we amplified DNA across this deletion and generated a 0.7-kb product in the patient and a 3.5-kb product in normal control individuals (fig. 1*A*). Both PCR products were generated with the use of DNA from the patient's mother. Sequencing the patient's DNA fragment identified the deletion breakpoints beginning at nucleotides (nt) 153+5 in intron 1 and extending through nt 386–408 in intron 2 (fig. 1*B* and *C*). Interestingly, at the break site, the patient also had a 19-bp insertion that included a 10-bp duplication of normal intron 2 sequence. This mutation results in both deletion of exon 2 and, in exon 3, a frameshift associated with a premature stop codon.

Two small insertion mutations, each causing a frameshift, were found in our patients (table 2). The 1311–1312insACAAA mutation is actually a small duplication of a 5-bp sequence located immediately upstream of the insertion site.

Three complex mutations involved a combination of deletions, insertions, and nucleotide substitutions. One of these (941–943delCCC+ins21bp) was seen in four probands with Middle Eastern or Greek ancestry. This mutation had previously been reported in a consanguineous patient of mixed European origin (De Laurenzi et al. 1996), who was later discovered to have some Lebanese ancestry. A second complex mutation involving a single nucleotide deletion and two transversions (901G→C, 906delT, and 909T→G) in exon 6 was associated with 733G→A in exon 5 on the same allele. This mutant allele was seen in four European probands, including one who was homozygous for the allele. A single Italian patient carried a third deletion/insertion mutation (1108–1116del+insACAG) in exon 8.

Missense Mutations

Twenty-two missense mutations were detected (table 2). The amino acid substitutions were scattered across the protein, but none involved the amino- or carboxy-terminal 35 amino acids (fig. 4). Five of the missense mutations were transitions that occurred at CpG dinucleotides (table 2). Two different mutations (551C→G and 551C→T) involved the same nucleotide and are predicted to cause substitution of Thr184 by Arg or Met, respectively. Interestingly, the 733G→A mutation, which was associated with a second complex mutation on the same allele, was seen in combination with a second complex mutation, was also found as an isolated homozygous mutation in one proband.

To determine whether the missense mutations were actually destructive to enzyme catalytic activity, we ex-

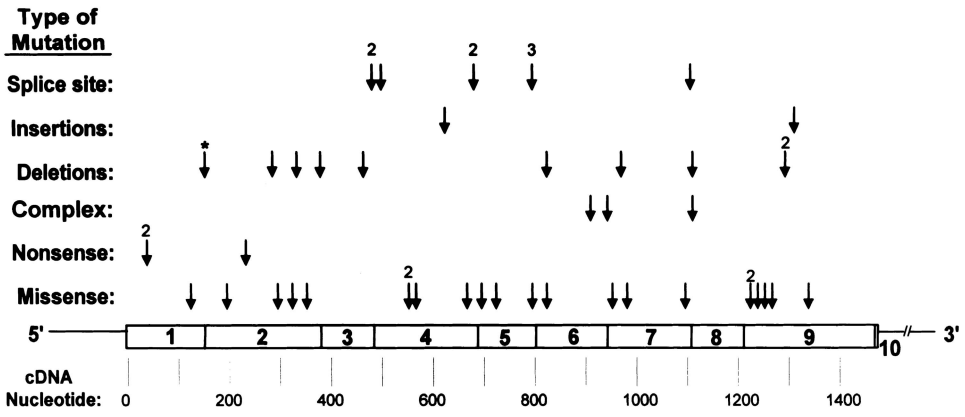

Figure 4 Location of mutations within the FALDH cDNA. The major FALDH cDNA containing exons 1–10 is shown. Exon 9′, which is spliced between exons 9 and 10 in a minor FALDH transcript, contains no mutations and is not illustrated. Arrows indicate mutation locations. The numbers above some arrows refer to the number of different mutations located within several base pairs at the same location. An asterisk (*) above an arrow indicates the beginning of the large deletion mutation encompassing intron 1 and extending through exon 2 and most of intron 2. All splice-site mutations alter intron sequences.

pressed 20 of these mutations in Chinese hamster ovary cells that were genetically deficient in FALDH activity. As shown in table 3, 19 of the mutant proteins had little or no detectable FALDH activity, but the protein encoded by 798G→C (K266N) possessed considerable residual activity (55% of normal). As noted below, however, this mutation seemed to have a greater destructive effect on mRNA stability than on enzyme catalytic activity.

Splicing Mutations and Mutations That Cause Unstable mRNA

Nine splice-site mutations were found (table 2). Analysis of fibroblast FALDH mRNA showed that all of these mutations resulted in an abnormal mRNA. Five of the mutations altered splice-donor sites. Three of these mutations (798+5G→A, 798+1delG, and 798+1–798−6delGTTTG) involved the splice-donor site at the junction of exon 5 and intron 5 and were carried by homozygous patients. As shown in figure 2, amplification of their fibroblast mRNA by RT-PCR revealed two smaller aberrant transcripts that had deletions of exon 5 or of exons 4 and 5. Similarly, the 471+1delG and 471+2delT deletions at the splice-donor site of exon 3 resulted in a shortened transcript in which exons 2 and 3 were skipped.

In contrast to the splice-donor–site mutations, two splice-acceptor–site mutations resulted in utilization of cryptic acceptor sites. The 681−14T→A mutation was carried by a German patient who was a compound heterozygote for 1297–1298delGA in exon 9. RT-PCR amplification and sequencing of an mRNA fragment containing exon 9 indicated that the GA deletion was not represented, suggesting that this deletion transcript was rapidly degraded (Maquat 1996). Amplification of the mRNA across exons 3–6, which includes the region potentially altered by the 681−14T→A mutation, demonstrated both an abnormally short product arising from skipping of exon 5 and a product that appeared slightly larger than the normal product (data not shown). When the mRNA was amplified with primers in exons 4 and 5 that were closer to the mutation, two distinct products—one of normal size and one slightly larger—were observed (fig. 3*A*). After cloning and sequencing of the products, the more abundant longer transcript was found to arise from aberrant recognition of a cryptic acceptor site, located 24 bp upstream of exon 5, in intron 4 (fig. 3*B*). This cryptic acceptor site was not preceded by the typical pyrimidine-rich region, and the mutation itself generated a consensus AG that was not utilized as an acceptor site. The mutant transcript results in an in-frame insertion of eight amino acids in the FALDH protein, between Cys226 and Arg227.

The 472−2A→G mutation altered the consensus AG of the splice–acceptor site of exon 4. Analysis of the mRNA showed utilization of the next available downstream AG as a cryptic acceptor site. This resulted in an in-frame deletion of the first 33 nucleotides within exon 4 of the mRNA and a predicted loss of 11 amino acids from the FALDH protein.

As noted above, expression of the 798G→C (K266N) missense mutation resulted in an enzyme with 55% residual catalytic activity. Since this mutation altered the last nucleotide in exon 5 and lowered the splice-donor score from 80% to 67% (Shapiro and Senapathy 1987), we wondered whether the transcript arising from this allele might have altered splicing or mRNA instability. A patient with SLS who was a compound heterozygote for 943C→T in exon 7 carried the mutation. When the patient's FALDH mRNA was amplified by RT-PCR, the 943C→T mutation appeared to be homozygous and the 798G→C mutation could not be detected in the mRNA (data not shown). No abnormally spliced transcripts were seen. These results are compatible with decreased stability of the mRNA arising from the 798G→C allele.

Table 3

Expression of FALDH Missense Mutation in Mammalian Cells

Mutation	Protein Change	FALDH Activity[a] (% of Wild Type)
Wild type	None	100
133A→T	I45F	9
191T→A	V64D	<1
317T→G	L106V	<1
341C→T	P114L	<1
362C→T	P121L	<1
551C→G	T184R	<1
551C→T	T184M	<1
554G→C	G185A	1
682C→T	R228C	9
710G→A	C237Y	<1
733G→A	D245N	1
798G→C	K266N	55
835T→A	Y279N	<1
943C→T	P315S	<1
984G→C	M328I	NT
1094C→T	S365L	3
1216G→A	G406R	NT
1231C→T	H411Y	<1
1244G→A	S415N	<1
1256T→C	F419S	3
1268G→A	R423H	1
1339A→G	K447E	1

NOTE.—Mutations were introduced into expression vector pCIneo-S-FALDH and were transfected into FALDH-deficient Chinese hamster ovary cells. Enzyme activity was measured after 48 h and was normalized to the amount of S-protein, to determine the specific activity. Each mutation was expressed in two or three experiments, and data shown are the mean percentage of the mutant FALDH-specific activity compared with wild-type enzyme.

[a] NT = not tested.

Nonsense Mutations

Three nonsense mutations were found within exons 1 and 2. One of these was an unusual dinucleotide transition (24–25CC→TT) that involves codons 8 and 9 and that causes no amino acid change in Val8 but that results in a termination at codon 9.

Polymorphisms and Sequence Variants

During these studies we identified four SNPs within the FALDH gene. Three SNPs were located within intronic sequence (introns 1, 3, and 6), and one was a previously reported silent polymorphism (1446A/T) that involved the third nucleotide of the codon for Ala482 (Chang and Yoshida 1997; Sillén et al 1998). The allele frequencies of these SNPs were established in a population of normal subjects and in the probands with SLS (table 4). Interestingly, in the patients with SLS, the allele frequencies of three SNPs differed from those in the normal population. This difference, however, probably reflected an overrepresentation of patients carrying common mutations, because the SNP-allele frequencies in a subgroup of probands carrying only private mutations appeared to be similar to those in the control population.

In addition to the SNPs, we detected a sequence variation within intron 2, at 386−6A→G, in two patients with SLS who carry the 1311–1312insACAAA mutation. This nucleotide change was near the splice-acceptor site at exon 3, but no splicing errors were detected in the patients' fibroblast mRNA. We concluded that 386−6A→G was an innocuous variation.

Patients with SLS Who Have Undetected or Uncertain Mutations

In two compound-heterozygous patients, only one of the two mutations could be detected after repeated sequencing of the coding regions of all exons amplified from genomic DNA. These patients, both of whom were of European origin, carried the common 1297–1298delGA mutation on one allele. In both patients, exon 9′, which is spliced into a small proportion of FALDH transcripts, was sequenced and was found to be normal.

One Middle Eastern patient carried a missense mutation (1268G→A) in exon 9, along with a single-nucleotide sequence variation (1494G→A) 36 bp from the stop codon in the 3′-UTR of exon 10. No other DNA changes were found within the coding region of the gene. We did not have access to other family members for testing, but the 1494G→A change was not seen in other patients with SLS or in normal controls. To determine whether the 1494G→A allele was expressed at the RNA level, we amplified the FALDH mRNA from the patient's fibroblasts by use of RT-PCR. When sequenced, the 1268G→A missense mutation appeared to be homozygous and 1494G→A was not detected. Moreover, the patient was heterozygous for the 1446A/T polymorphism in exon 10 when testing of genomic DNA was done, but only the 1446A allele was detected in the mRNA. These results suggest that the transcript originating from the 1494G→A allele (also containing the 1446T polymorphism) was unstable or was not expressed. Nevertheless, rigorous proof that the 1494G→A change causes disease and is not simply linked to another undetected mutation will require expression studies.

Table 4

Allele Frequencies of SNPs in the FALDH Gene

Intron or Exon and FALDH Polymorphism	Normal Controls (n = 132 chromosomes)	All SLS Probands[a] (n = 122 chromosomes)	SLS Probands Carrying ⩾1 Common Mutation (n = 88 chromosomes)	SLS Probands Carrying Private Mutations Only (n = 34 chromosomes)
Intron 1:				
153+39T	.29	.55[b]	.63[b]	.35
153+39C	.71	.45[b]	.37[b]	.65
Intron 3:				
471+31C	.27	.55[b]	.63[b]	.29
471+31T	.73	.45[b]	.37[b]	.71
Intron 6:				
940+53G	.16	.22	.20	.26
940+53C	.84	.78	.80	.74
Exon 10:				
1446A	.51	.22[b]	.16[b]	.38
1446T	.49	.78[b]	.84[b]	.62

[a] This group includes 61 probands carrying both common and private mutations. Two probands, including one patient who carried a deletion lacking the intron 1 polymorphism, were not analyzed.

[b] Different from normal controls at $P < .05$ level, by use of χ^2 analysis.

Haplotype Analysis of Common Mutations

Twelve of the mutations were carried by more than one proband. Many of these mutations were found in patients from common geographic or ethnic populations who could share distant ancestors, or the mutations might have recurred independently. To investigate these alternate possibilities, we determined, by using the four SNPs, the FALDH haplotypes of the probands who had identical mutations (table 5). Four of the mutations were found on more than one haplotype. Three of these mutations (551C→T, 682C→T, and 733G→A) were associated with haplotypes that differed at two or more SNPs, suggesting that the mutations arose independently on different genetic backgrounds. Each of these mutations occurs at a CpG dinucleotide. The 551C→T mutation was carried by four European patients, two of whom had homozygous haplotypes that differed at three SNPs. The 682C→T mutation was carried by four patients of Middle Eastern origin and was present on three different FALDH haplotypes. Interestingly, the patient who was homozygous for the isolated 733G→A mutation in exon 5 had a haplotype that, at three SNPs, differed from that of the patients carrying 733G→A in combination with the complex mutation in exon 6 (901G→C, 906delT, and 909T→G). This suggests that the complex mutation did not originate on the 733G→A allele and that this missense mutation originated independently on two occasions. One additional mutation (798+1delG) was carried by two patients, one of European descent and the other of whom was Japanese, who differed at one SNP. All of the other common mutations were each associated with identical FALDH haplotypes among the probands carrying them, which is consistent with existence of a shared ancestor.

Discussion

Our results have uncovered a striking mutational heterogeneity in SLS. Among 63 probands with SLS, 49 different mutations were found. Forty-two of the mutations are novel, and 7 mutations have been previously reported. Thirty-seven of the mutations were each car-

Table 5

SNP Haplotype Associations with Common FALDH Mutations

		SNP Haplotype Associated with SNP Polymorphism			
FALDH Mutation(s)	No. of Probands with Haplotype	Intron 1 153+39	Intron 3 471+31	Intron 6 940+53	Exon 10 1446
233G→A	2	C	T	C	A
471+1delG	1	C/T	C/T	C/G	T
	1	C	T	C/G	A/T
551C→T	2	C	T	G	T
	1	T	C	C	T
	1	C/T	C/T	C/G	T
682C→T	1	C	T	C	A
	1	C	C	C	A
	1	C	C	G	T
710G→A	2	T	C	C	T
733G→A[a]	1	T	T	C	A
733G→A, 901G→C, 906delT, 909T→G	2	C	T	G	T
	1	C/T	C/T	C/G	T
	1	C	T	C/G	A/T
798+1delG	1	T	T	C	T
	1	T	C	C	T
941–943delCCC+ins21bp	4	T	C	C	T
943C→T	6	T	C	C	T
	1	T	C/T	C/G	T
	1	C/T	C/T	C/G	T
1094C→T	2	T	C	C	T
	1	T	C/T	C	A/T
1297–1298 delGA	11	T	C	C	T
	3	C/T	C/T	C	A/T
	1	C/T	C/T	C/G	T
	1	C/T	C	C	T
1311–1312insACAAA	1	C	T	C	T
	1	C/T	C/T	C	T

[a] This mutation, seen in a single patient with SLS, is included for comparison with the patients carrying the 733G→A/901G→C/906delT/909T→G complex allele.

ried by one proband and can be considered private. To our knowledge, this cohort with SLS represents the majority of enzymatically confirmed patients identified to date. With limited clinical information about many of the patients, we did not attempt genotype-phenotype correlations.

Previous studies involving linkage analysis of families of diverse ethnic origins have mapped SLS to chromosome 17p11.2 and have found no evidence for locus heterogeneity (Pigg et al. 1994; Rogers et al. 1995; Lacour et al. 1996). Our results also support a single SLS locus, since all of the probands studied here had mutations in the FALDH gene. This is consistent with biochemical evidence that, except for NAD^+, the active FALDH enzyme requires no additional cofactors or proteins for aldehyde oxidative activity (Kelson et al. 1997).

Mutations in the FALDH gene appeared to be randomly distributed. However, four different mutations—including two deletions, a transition, and a transversion—involved nucleotides at or around nt 798. Three of these mutations disrupted normal splicing, and the remaining missense mutation (798G→C) seemed to result in an unstable mRNA. The clustering of mutations at this site may be fortuitous or may reflect ascertainment bias because of a greater susceptibility to functional impairment. This particular splice-donor site at exon 5 differs from others in the FALDH gene because of its short palindromic character (gtttg), which may be more susceptible to mutation.

Deletions and insertions within exons accounted for 30% of all mutations. In most cases in which more than one nucleotide was deleted or inserted, the mutations occurred at short palindromic sequences and probably arose from errors introduced by DNA polymerase during replication. In the case of the large, 2.8-kb deletion, a short duplication was introduced at the 3′ break site in intron 2, suggesting unequal crossing-over. No complementary sequences or potential hairpin loops that would explain the error were seen at the deletion breakpoints.

Twenty-two missense mutations altering 21 amino acid residues were identified among the patients with SLS. To establish that these were disease-causing mutations, we expressed 20 of the mutations in mammalian cells and found that 19 of them resulted in a severe loss of FALDH enzyme activity. In contrast, the 798G→C (K266N) missense mutation caused a mild reduction of enzyme catalytic activity but a more profound decrease in its mRNA level, which emphasizes the pleiotropic effects of certain missense mutations that can influence mRNA splicing and alter transcript stability (Cooper and Mattox 1997). The 733G→A (D245N) mutation had previously been thought to be an innocuous polymorphism as a result of its association with a second complex mutation on the same allele in some patients with SLS (Sillén et al. 1998). This mutation was the sole nucleotide alteration in one homozygous patient with SLS and was clearly destructive to FALDH catalytic activity when expressed in mammalian cells.

The mutations leading to amino acid substitutions should prove useful for providing the structure-function correlations in FALDH. This enzyme is a member of a large family of aldehyde dehydrogenase (ALDH) proteins that show varying degrees of sequence homology (Perozich et al. 1999). Recently, a closely related cytosolic class 3 ALDH from rat has been crystallized, and the three-dimensional structure has been determined (Liu et al. 1997). When the carboxy-terminal region of human FALDH that is not present in the rat enzyme is excluded, the two proteins share 65% amino acid identity. Eighteen of 21 amino acid positions altered by the missense mutations in FALDH involve residues shared with the rat protein. This rat ALDH should provide a basis for modeling the human FALDH protein and for determination of the structural alterations caused by the missense mutations.

The splice-site mutations, which accounted for 18% of the detected mutations, often caused multiple FALDH transcript abnormalities. Five of these mutations involved splice-donor sites and resulted in skipping of the upstream one or two exons, which commonly occurs with splice-donor mutations (Nakai and Sakamoto 1994; O'Neill et al. 1998). The four mutations involving splice-acceptor sites caused skipping of either all or a portion of the downstream one or two exons. Two of these splice-acceptor-site mutations also caused utilization of cryptic acceptor sites. The 472−2A→G mutation resulted in recognition of a downstream cryptic acceptor site, whereas the 681−14T→A mutation had multiple effects on the mRNA, including exon skipping and utilization of an upstream cryptic acceptor site leading to insertion of intronic sequence into the transcript.

Several common mutations were clustered among patients of European or Middle Eastern descent, suggesting a founder effect. The mutation most frequently seen in our probands with SLS was 1297–1298delGA, which was restricted to patients of European origin. Among the 37 patients with SLS who were of European descent, 1297–1298delGA was carried by 16 probands, and SNP haplotype analysis, although lacking the statistical power of microsatellite markers, is consistent with their being distantly related. Nine European probands carried the 943C→T mutation, which was previously detected in patients with SLS who were from northern Europe and Sweden and who shared a common microsatellite-marker haplotype (De Laurenzi et al. 1997). Among the European probands studied here, 54% carried either 943C→T or 1297–1298delGA, and the two mutations accounted for 36% of all European SLS alleles. Notably, 23 mu-

tations accounted for the remaining European SLS alleles. Among patients from the Middle East, 10 different mutations were identified, and 3 of these (682C→T, 710G→A, and 941–943delCCC+ins21nt) were found in more than one kindred.

Haplotype analysis using the SNPs within the FALDH gene was useful for investigation of the origin of the common mutations and the genetic relatedness of the patients carrying them. In this regard, we found that at least three common mutations (551C→T, 682C→T, and 733G→A), each associated with haplotypes that differ by two or more SNPs, probably originated independently and may represent mutational hotspots. These mutations occur at CpG dinucleotides and might have arisen by deamination of a methylated cytosine. However, the conclusion that these mutations arose on several occasions must be tempered by the contradictory finding that the 551C→T and 682C→T mutations were restricted to European and Middle Eastern patients, respectively. This geographic clustering is not consistent with recurrent mutations, which should arise independent of geographic region, and argues that these two FALDH mutations represent ancestral SLS alleles upon which the different SNP haplotypes arose from rare intragenic recombination. If the haplotypes were products of intragenic recombination, both mutations would require two crossover events to account for the haplotype diversity seen. If it is assumed that 1 Mb is equivalent to a map distance of 1 cM and that the mutations were 4–6 kb away from the flanking SNPs, intragenic recombination between the mutation and one SNP would be expected to occur with a frequency of $\sim 5 \times 10^{-5}$. The likelihood of two crossover events occurring within the gene (25×10^{-10}) is lower than the estimated transition-mutation rate at CpG dinucleotides (368×10^{-10}/bp/germ cell) (Koeberl et al. 1990), which supports the notion that 551C→T and 682C→T are recurrent mutations. If so, the geographic clustering of the two mutations may be fortuitous. A fourth mutation (798+1delG) was associated with two haplotypes that differed by one SNP only; however, this mutation was detected in two patients—one of European descent and the other from Japan—who are unlikely to share common ancestors. Identification of additional SNPs within the FALDH gene should improve the power of haplotype analysis for detection of recurrent mutations.

Our studies provide a foundation for the development of DNA-based methods for the diagnosis of SLS. Since the majority of the FALDH mutations are private and are scattered throughout the gene, with the use of current techniques the DNA-based diagnosis of SLS will be a challenge. Screening methods have been developed for the two common European mutations, by use of either restriction-enzyme digestion of PCR amplicons (De Laurenzi et al. 1997; Sillén et al. 1997) or allele-specific PCR (Rizzo et al. 1997), and similar approaches could be taken for the common Middle Eastern mutations. However, until newer and less costly molecular methods are developed for mutation screening, perhaps on the basis of DNA chip technology, the diagnosis of SLS will probably still depend on enzymatic assays of FALDH activity. In contrast to diagnostic screening, identification of the specific mutations segregating in kindreds will prove invaluable for carrier detection, prenatal diagnosis, and genotype-phenotype correlations in SLS.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Enzyme Nomenclature Database, http://www.expasy.ch/enzyme/ (for FALDH EC 1.2.1.48]

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html (for FALDH cDNA [NM000382] and exons and flanking intron sequences [U75286–U75297])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SLS [MIM 270200])

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