RESEARCH ARTICLE

Mutational Spectrum of the Succinate Semialdehyde Dehydrogenase (*ALDH5A1*) Gene and Functional Analysis of 27 Novel Disease-Causing Mutations in Patients With SSADH Deficiency

Shinjiro Akaboshi,[1,2] Boris M. Hogema,[1,3,4] Andrea Novelletto,[5] Patrizia Malaspina,[6] Gajja S. Salomons,[3] George D. Maropoulos,[7] Cornelis Jakobs,[3] Markus Grompe,[1] and K. Michael Gibson[1]*

[1]Departments of Molecular and Medical Genetics and Pediatrics, Oregon Health and Science University, Portland, Oregon; [2]Division of Child Neurology, Institute of Neurological Sciences, Tottori University, Tottori, Japan; [3]Metabolic Unit, Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands; [4]Department of Biochemistry, Erasmus University Medical Center Rotterdam, the Netherlands; [5]Department of Cell Biology, University of Calabria, Rende, Italy; [6]Department of Biology, "Tor Vergata" University of Rome, Rome, Italy; [7]Department of Biochemistry, General Hospital of Athens, 'Laiko,' Greece

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Succinate semialdehyde dehydrogenase (SSADH; ALDH5A1) deficiency, a rare metabolic disorder that disrupts the normal degradation of GABA, gives rise to a highly heterogeneous neurological phenotype ranging from mild to very severe. The nature of the mutation has so far been reported in patients from six families world wide and eight different mutations were described. Here we report the mutational spectrum in 48 additional unrelated families of different geographic origin. We detected 27 novel mutations at the cDNA level, of which 26 could be attributed to changes at the genomic level. Furthermore, six mutations were detected that did not strongly affect SSADH activity when expressed in HEK 293 cells and are considered nonpathogenic allelic variants. Twenty of the mutations were only found in one family. The spectrum of disease-causing mutations from all patients sequenced thus far consists of 25 point mutations, four small insertions, and five small deletions. Seven of these mutations affect splice junctions, seven are nonsense mutations, and 12 are missense mutations. Although there were no mutational hotspots or prevalent mutations responsible for a significant number of cases, 14 out of 37 (38%) of the missense alleles were present in exon 4 or 5. With one exception, the missense mutations we consider to be causative of SSADH deficiency reduced the SSADH activity to less than 5% of the normal activity in our in vitro expression system. This indicates that residual expression is not likely to be an important factor contributing to the large phenotypic differences observed among different families and even among siblings, suggesting that other modifying factors are of great importance in disease pathology. Hum Mutat 22:442–450, 2003. © **2003 Wiley-Liss, Inc.**

KEY WORDS: succinate semialdehyde; GABA metabolism; aldehyde dehydrogenases; GHB; ALDH5A1; SSADH

DATABASES:
ALDH5A1 – OMIM: 271980; GenBank: AL031230 (genomic), Y11192 (cDNA)

INTRODUCTION

Deficiency of succinate semialdehyde dehydrogenase (SSADH; ALDH5A1; EC 1.2.1.24; MIM# 271980) is a rare autosomal recessively inherited metabolic disorder that results in accumulation of 4-hydroxybutyrate (GHB; Fig. 1). GHB possesses a number of unusual neuropharmacologic properties primarily mediated via the dopaminergic system, and is used to treat cataplexy and drug-withdrawal syndromes, to induce absence seizures in animal models, and as a drug of abuse linked to its putative euphoric effects [Mason and Kerns, 2002]. SSADH is a mitochondrial, NAD^+-dependent enzyme that catalyzes the oxidation of succinate semialdehyde to

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*Correspondence to: K. Michael Gibson, Ph.D., FACMG, Department of Molecular and Medical Genetics, Biochemical Genetics Laboratory, Oregon Health and Science University, Mail Code MP-350, 2525 SW 3rd Avenue, Suite 350, Portland, OR 97201.
E-mail: gibsonm@ohsu.edu

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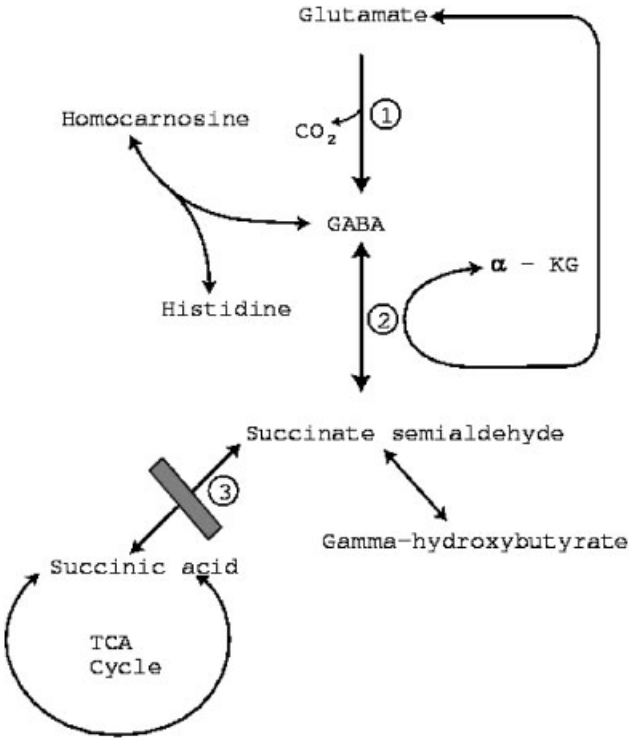

FIGURE 1. **The GABA catabolic pathway and GABA "shunt." In CNS tissue, the "shunt" refers to the regeneration of glutamate, the main GABA precursor, during the transamination reaction which converts GABA to succinic semialdehyde, with stoichiometric production of glutamate from alpha-ketoglutarate. The site of the block in SSADH deficiency is indicated by the solid box. Numbered reactions include: 1, glutamate decarboxylase; 2, GABA-transaminase; and 3, succinate semialdehyde dehydrogenase. Abbreviations: GABA, 4-aminobutyric acid; α-KG, α-ketoglutarate; TCA, tricarboxylic acid.**

succinate, which is the second and final step of the 4-aminobutyric acid (GABA) metabolic pathway. GHB is subsequently formed from succinate semialdehyde by one or more NADPH-linked GHB dehydrogenases [Cash et al., 1979] and is likely to be important in disease pathogenesis [Gibson et al., 1998]. The enzyme deficiency also leads to significant accumulation of GABA, which makes SSADH deficiency a unique disorder, since the levels of two neuroactive compounds are increased. Furthermore, it is unusual in the sense that seizures occur in about 50% of patients even though GABA is increased. Since the description of the first patient [Jakobs et al., 1981] approximately 300 patients have been diagnosed [Gibson and Jakobs, 2001]. The clinical phenotype is highly variable (both interfamilial and intrafamilial) and ranges from mild retardation in language, motor, and mental development to severe neurological defects such as seizures, hypotonia, ataxia, and behavioral problems, especially in older patients [Gibson et al., 1997a]. Although the disease is usually diagnosed during childhood it has been found in adults as well [Jakobs et al., 1990]. EEG and MRI abnormalities have been described in some cases, but proper diagnosis can only be achieved by organic acid analysis of urine or plasma, followed by enzymatic determination of SSADH activity in leukocytes, and recently by DNA sequence analysis [Gibson et al., 1991, 1994; Chambliss et al., 1998; Hogema et al., 2001a; Aoshima et al., 2002].

The ALDH5A1 gene is located on chromosome 6p22 and consists of 10 exons encompassing over 38 kb. The open reading frame consists of 1,605 bp, encoding 535 amino acids, of which the first 47 residues form the mitochondrial targeting sequence [Chambliss et al., 1995, 1998; Trettel et al., 1997]. Recently we described the complete genomic structure of the gene and found an alternatively spliced small exon which may encode a novel isoform of the enzyme with an additional 13 amino acids [Blasi et al., 2002].

Eight different pathologic mutations in the ALDH5A1 gene in patients from six different families have been described in the literature [Chambliss et al., 1998; Hogema et al., 2001a; Aoshima et al., 2002]. Mutant in vitro expression has not been performed in these studies, and the possibility that some of the mutations represent nonpathogenic polymorphisms could not be excluded. In order to gain a better understanding of the molecular basis of the disease, we screened the ALDH5A1 coding region in 61 additional patients from 48 different families by sequence analysis of genomic DNA and/or cDNA. (Fig. 2)

MATERIALS AND METHODS

Human Subjects

All patients analyzed for ALDH5A1 mutations in this study were diagnosed by measurement of SSADH activity in leukocytes after detection of abnormal GHB levels in physiological fluids [Gibson et al., 1990, 1994]. In addition, 56 probands' relatives from 22 different families were evaluated for mutations found in probands. In case families were known to be related only one of the patients (or sibships) is represented in our results because we never found additional mutations or polymorphisms in the related families. All samples submitted to analysis were obtained with informed consent of either the patients or the caregivers.

Mutation Screening

Total RNA and genomic DNA were extracted from lymphoblastoid or epithelial cell lines derived from patients or from fresh lymphocytes or whole blood using standard procedures. RT-PCR was performed using Gibco reverse transcriptase and Taq polymerase according to the instructions of the manufacturer, and in order to improve the low yields caused by the high GC-content of the 5′ end of the gene, betaine (1 M) was added to the reactions. Even then we could not amplify the first 258 bp of the open reading frame by RT-PCR. For that reason we analyzed the first exon by sequence analysis from genomic PCR products generated using platinum Taq polymerase (Gibco, Grand Island, NY) in the presence of 1 M betaine. Other genomic PCR reactions were done using regular Taq polymerase (Gibco), using the PCR primer pairs listed in Table 1. PCR amplification was performed using PCR mixes as specified by the manufacturer. The PCR conditions were: denaturation 94°C for 4 min followed by 30 cycles (94°C for 45 sec, annealing at 53°C for 1 min, extension at 72°C for 2 min), followed by a further extension at 72°C for 5 min. PCR products were purified using the Qiagen PCR cleanup kit and sequenced using BigDye dideoxy terminators (Perkin Elmer, Boston, MA) and an ABI automated sequencer. If two different size bands were detected in RT-PCR reactions, both bands were sequenced to verify which part of the coding region was missing.

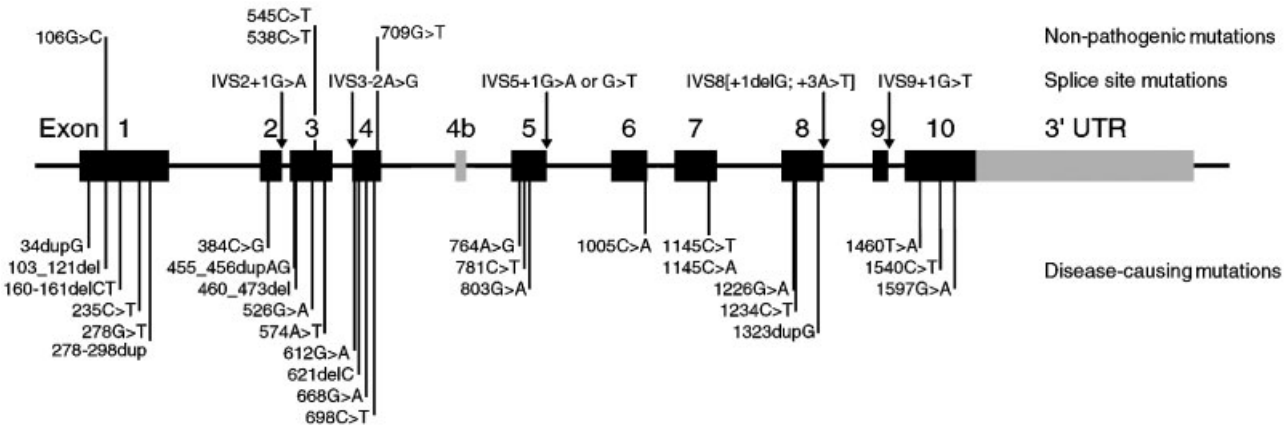

FIGURE 2. **Summary of mutations and polymorphisms in *ALDH5A1*. Splice site mutations and nondeleterious mutations are shown above the diagram of the intron/exon structure and disease-causing mutations are indicated below, all in terms of cDNA sequence. The figure is drawn to scale with the size of the introns reduced to 5% of the actual length for clarity.**

TABLE 1. Primer Pairs Used for Amplifying the ALDH5A1 Coding Regions

Primer name	Primer sequence (5′–3′)	Product length (bp)
RT-PCR F	TCTGGGCATGGTAGCCGACTG	1477
RT-PCR R	GGACTGGATGAGTTCTGAAAAATTC	
Exon 1 F	TGCTCTGTGGCTCTGCAACCTTCCG	655
Exon 1 R	CCCTTGTTGCTCTGACTTTCCAC	
Exon 2 F	CACGGACATACCCAGAAATGATG	348
Exon 2 R	GCGGCTTTCCCAAGTTTAGTG	
Exon 3 F	TTTTCCCCACTCTATGGGTATCG	361
Exon 3 R	TGTTTCCCAACTCCCTATCCAATC	
Exon 4 F	GGTTTGTCAATCAGTTGTGC	475
Exon 4 R	ATAGATACCATTTACAGTAGG	
Exon 4B F	TGTGACTTCCTTAAATTGGGC	301
Exon 4B R	AGGGGGAGCTACTACATCA	
Exon 5 F	GTAAATTGTTGGCACATGTTTG	271
Exon 5 R	TGGTGATCAGGATGAAATAG	
Exon 6 F	CCAGCCTCTTCCCAATGTTTC	393
Exon 6 R	ACATGCACACGCAAACACACATC	
Exon 7 F	CACCGAGGGAAGTGTTTCAC	330
Exon 7 R	TGCTGATTAAGCTTTTCATATTG	
Exon 8 F	CTGAATCTCTGCAAATGTGGTTCC	410
Exon 8 R	CTCTTTCAGGGTTTCCTATG	
Exon 9 F	CTGAGTTATAACGGGGAATGGT	470
Exon 9 R	TGCACCTTCTCTTCTTCCCACA	
Exon 10 F	TCATCAATGGTGCCCTCATC	415
Exon 10 R	GGACTGGATGAGTTCTGAAAAATTC	

Mutations found in RT-PCR products were always verified by sequencing the corresponding exon amplified from genomic DNA. If no mutation or only one mutated allele was found with the RNA-based method all exons were sequenced.

Nomenclature and Sequence Analysis

Mutation nomenclature follows the guidelines from den Dunnen and Antonarakis [2000]. Numbering of nucleotide and amino acid positions refers to the complete cDNA sequence (GenBank accession Y11192) including the mitochondrial leader sequence and starting from the first ATG or amino acid. Sequences were aligned using the DNAstar program Megalign and sequence motifs were analyzed using the PFAM and Prosite databases.

Functional Analysis of ALDH5A1 Alleles

Missense mutations detected in patients were introduced into a pAD1/RSV-driven expression vector described previously [Blasi et al., 2002] using the 'quickchange' site-directed mutagenesis kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. Resulting mutations were confirmed by sequence analysis. Expression was measured 48 hr after transfection of HEK293 cells with the Effectene transfection reagent (Qiagen, Valencia, CA) by using the fluorometric assay as described [Gibson et al., 1991].

RESULTS

Genotyping

The results of sequence analysis of ALDH5A1 cDNA and/or genomic DNA of patients from 54 families that are not known to be related in whom SSADH deficiency was diagnosed are reported in Table 2. This table includes the six families of whom the mutations were previously published [Chambliss et al., 1998; Hogema et al., 2001a; Aoshima et al., 2002]. Only three alleles (in families 19, 25, and 29) could not be attributed to specific mutations at the genomic level, showing that the methods employed by us resulted in a high mutation detection rate (97%). In one of these three cases (family

TABLE 2. Identification of the Mutations and Polymorphisms in *ALDH5A1* in 54 Different Families

Family no.	Mutation 1[a]	Mutation 2	Genotype at c.538	Additional polymorphisms	Ethnic origin	Reference/remarks
1	c.34dupG	c.612G>A	C/C	c.1216G>A	British	
2	c.103_121del	c.1460T>A	n.d.[b]		Japan	Aoshima et al. [2002][c]
3	c.235C>T	c.235C>T	T/T	c.106G>C+ c.106G>C+ c.545C>T+ c545C>T	New Caledonia	Gibson et al. [1997b]
4	c.278_298dup	c.1145C>T	C/T		Greek	
5	c.278G>T	c.278G>T	C/C		Turkey	Jakobs et al. [1981]
6	c.278G>T	IVS5+1G>T	C/T		India	Aligianis et al. [2002]
7	c.278G>T	c.1597G>A	C/C		Unknown	
8	c.384C>G	c.384C>G	C/C		Inuit	Gibson et al. [1997a]; patient P.F.
9	c.455_456dupAG	c.455_456dupAG	C/C		Unknown	
10	c.460_473del	c.460_473del	T/T		Hispanic	Gibson et al. [1988]
11	c.526G>A	c.526G>A	C/C		India	Gibson et al. [1997a]; patient P.V.
12	c.526G>A	c.1226G>A	C/C		Greek	
13	c.612G>A	c.612G>A	C/C		American-European	
14	c.612G>A	c.612G>A	C/C		Unknown	DeVivo et al. [1988]
15	c.612G>A	c.803G>A	C/T		American-European	Hogema et al. [2001a][c]; 612G>A not expressed
16	c.612G>A	c.1005C>A	C/C		American-European	The 612G>A allele was not expressed
17	c.612G>A	c.1234C>T	C/C		Australian	Hogema et al. [2001a][c]
18	c.612G>A	c.1540C>T	C/C		American-European	The 612G>A allele was not expressed
19	c.612G>A	?	C/T		French	No RT-PCR product
20	c.621delC	c.621delC	C/C		Turkey	
21	c.668G>A	c.668G>A	C/C	c.709G>T+ c.709G>T	Unknown	
22	c.698C>T	c.698C>T	T/T		Turkey	
23	c.698C>T	c.698C>T	T/T		Turkey	Gibson et al. [1997a]; patient K.S.
24	c.698C>T	c.698C>T	T/T		Unknown	Jakobs et al. [1990]; no RT-PCR product detected
25	c.764A>G	?	C/T	c.106G>C+ c.545C>T	Lebanese	Gibson et al. [1997a]; patient T.A.
26	c.781C>T	c.803G>A	C/T		Swedish	No RT-PCR product detected
27	c.803G>A	c.1234C>T	C/T		American-European	Gibson et al. [1989]; 1234C>T not expressed
28	c.803G>A	c.34dupG	C/T		United Kingdom	The c.34dupG allele was not expressed
29	c.803G>A	r.EX2_EX5del	T/L.O.H.[d]	c.1389T>C	Swedish	
30	c.1145C>A	c.1145C>A	C/C		Unknown	
31	c.1226G>A	c.1226G>A	C/C		Greek	Hogema et al. [2001a][c]
32	c.1226G>A	c.1226G>A	C/C		Dutch	Gibson et al. [1997a]; patient E.G.
33	c.1226G>A	c.1226G>A	C/C		Croatian	
34	c.1226G>A	IVS2+1G>A	C/T	c.545C>T	Greek	
35	c.1234C>T	c.1234C>T	C/C		American-European	Hodson et al. [1990]
36	c.1234C>T	c.1234C>T	C/C		Pakistan	No RT-PCR product detected
37	c.1234C>T	c.1234C>T	C/C		American-European	Gibson et al. [1997a]; patient M.W.
38	c.1234C>T	c.1234C>T	C/C		Unknown	
39	c.1323dupG	c.1323dupG	C/T		Dutch	
40	c.1540C>T	c.160_161delCT	C/C		Unknown	
41	c.1540C>T	c.1540C>T	C/C		Unknown	
42	c.1597G>A	c.160_161delCT	C/C		Italian	The 160_161delCT allele was not expressed
43	IVS3_2A>G	c.574A>T	C/T		Nova Scotia	
44	IVS3_2A>G	c.1234C>T	C/C		American-European	
45	IVS5+1G>A	IVS5+1G>A	C/C		Malta	Chambliss et al. [1998][c]; patient A.C.
46	IVS5+1G>T	IVS5+1G>T	T/T		Turkey	Gibson et al. [1997a]; patient Y.A.
47	IVS6−2A>C	c.1226G>A	C/T		Haitan/Spanish	
48	IVS8+1delG; IVS8+3A>T	IVS8+1delG; IVS8+3A>T	C/C		Palestinian-Lebanese	Gibson et al. [1997a]; patient A.E-M.
49	IVS8+1delG; IVS8+3A>T	IVS8+1delG; IVS8+3A>T	C/C		Pakistan	Gibson et al. [1997a]; patient M.S.
50	IVS9+1G>T	IVS9+1G>T	T/T		Turkey	Chambliss et al. [1998][c]; patient Z.Z.
51	IVS9+1G>T	IVS9+1G>T	T/T		Turkey	
52	IVS9+1G>T	IVS9+1G>T	T/T		Syrian	Gibson et al. [1997a]; patient B.H.
53	IVS9+1G>T	IVS9+1G>T	T/T		Unknown	
54	IVS9+1G>T	IVS9+1G>T	T/T		Unknown	

[a]Nucleotides are numbered from the start codon according to the cDNA sequence of GenBank accession Y11192.
[b]n.d., not determined.
[c]Mutation analysis in these patient has been reported previously.
[d]L.O.H.: loss of heterozygosity.

19), no RT-PCR product was obtained. In the patient from family 29 we found a heterozygous deletion of exons 2–5 in the RT-PCR product, but no mutations were found in the parts of the genomic DNA we sequenced. The complex haplotype of Patient 25 will be discussed later.

The features of all currently known mutations, including the eight mutations found in six different families that have been published previously [Chambliss et al., 1998; Hogema et al., 2001a; Aoshima et al., 2002] are summarized in Table 3. Patients from 23 of the 55 different families were compound heterozygotes and in 32 families patients were homozygous. This probably results from the rather high percentage (approximately 40%) of consanguinity in families with SSADH deficiency [Gibson et al., 1997a]. The most common mutation (W204X) was found in eight different families (in 8 out of 10 cases in compound heterozygous patients), all with European ancestry, suggesting a founder effect for this mutation. The second most common mutation (R412X) was detected in seven families (four homozygous patients) from various geographic origins.

Table 3 also shows the patients' genotypes for the common c.538C>T nucleotide substitution, which results in the H180Y amino acid substitution. The associations of the various mutations and the allele at pos. 538 could be resolved unequivocally with the exception of families 4, 25, 34, and 47. One patient (family 29) showed loss of heterozygosity at pos. 538, due to deletion of exons 2–5 in which this position resides. This patient also carried the c.1389T>C mutation, a silent third-base substitution affecting codon D463.

Six patients showed additional sequence variations, which were not considered causative of SSADH deficiency as shown in Table 2, column 5. It is interesting to see that three of them carried both the G36R (c.106G>C) and the P182L (c.545C>T) substitutions. The patient of family 3 is homozygous for both substitutions, showing that they were transmitted in *cis*. This would also be compatible with the genotype of Patients 25 and 34. In our limited population survey [Blasi et al., 2002], we did not detect this type of chromosome, showing that it is rare at least in the European population. However, we detected chromosomes carrying both 538(T) and 545(T) with a frequency of about 10% [Blasi et al., 2002].

In order to check whether changes in the recently discovered exon 4B, which codes for an additional 13 amino acids, could be involved in disease pathogenesis, we sequenced genomic DNA surrounding this area from 35 patients (70 chromosomes) and found no changes.

TABLE 3. Summary of Disease-causing Mutations in *ALDH5A1*

Exon	Type of mutation	Nucleotide change [a]	Change in protein	No. of families	No. of alleles
1	Insertion	**c.34dupG**	p.A12fsX123	2	2
1	Deletion	c.103_121del[b]	p.S35fsX49	1	1
1	Deletion	**c.160_161delCT**	p.S55fsX79	2	2
1	Nonsense	**c.235C>T**	p.Q79X	1	2
1	Missense	**c.278G>T**	p.C93F	3	4
1	Insertion	**c.278_298dup**	p.C93_R99dup	1	1
Exon 2/Exon 5	?	**r.EX2_EX5del**	p.E119_K290del	1	1
2	Nonsense	**c.384C>G**	p.Y128X	1	2
Exon 2/Intron 2	Splice site	**IVS2+1G>A (r.spl?)**	?	1	1
3	Insertion	**c.455_456dupAG**	p.A153fsX12	1	2
3	Deletion	**c.460_473del**	p.H154fsX10	1	2
3	Missense	**c.526G>A**	p.G176R	2	3
3	Nonsense	**c.574A>T**	p.K192X	1	1
Intron 3/Exon 4	Splice site	**IVS3−2 A>G (r. 439_452del)**[c]	p.K148fsX16	2	2
4	Nonsense	c.612G>A	p.W204X	8	10
4	Deletion	**c.621delC**	p.S208fsX2	1	2
4	Missense	**c.668G>A**	p.C223Y	1	2
4	Missense	**c.698C>T**	p.T233M	3	6
5	Missense	**c.764A>G**	p.N255S	1	1
5	Nonsense	**c.781C>T**	p.R261X	1	1
5	Missense	c.803G>A	p.G268E	5	5
Exon 5/Intron 5	Splice site	IVS5+1G>A (r.EX5del)[c]	p.L243_K290del	1	2
Exon 5/Intron 5	Splice site	**IVS5+1G>T (r.EX5del)**[c]	p.L243_K290del	2	3
6	Missense	**c.1005C>A**	p.N335K	1	1
Intron 6/Exon 7	Splice site	**IVS6−2A>C (r.spl?)**	?	1	1
7	Missense	**c.1145C>T**	p.P382L	1	1
7	Missense	**c.1145C>A**	p.P382Q	1	2
8	Missense	c.1226G>A	p.G409D	6	9
8	Nonsense	c.1234C>T	p.R412X	7	11
8	Insertion	**c.1323dupG**	p.P442fsX18	1	2
Exon 8/Intron 8	Deletion	**IVS8+1delG; IVS8+3A>T (r.EX8del)**[c]	pV392fsX10	2	4
Exon 9/Intron 9	Splice site	IVS9+1G>T (r.EX9del)[c]	p.F449fsX53	5	10
10	Missense	c.1460T>A[b]	pV487E	1	1
10	Nonsense	**c.1540C>T**	p.R514X	3	4
10	Missense	**c.1597G>A**	p.G533R	2	2
?		One allele not detected (see text)		2	2

[a]Variants not published previously are in boldface. Nucleotides are numbered from the start codon with GenBank accession number Y11192 as reference.
[b]Published by Aoshima et al. [2002].
[c]For splice site mutations the resulting change in RNA (as determined by sequencing RT-PCR product) is indicated when known.

Nevertheless, preliminary evidence suggests that the relative expression level of the splice variant including exon 4B could be variable, since it was undetectable in most of the sequenced RT-PCR products from patient material. However, in several cases it was visible as noise below the stronger signal of the normal sequence. Because it is likely that the splice variant lacks activity [Blasi et al., 2002], it is possible that a higher relative expression level of exon 4B contributes to disease pathology.

Mutation Features

In the current study we characterized 27 mutations in the ALDH5A1 gene that have not been reported before. The overall number of SSADH deficiency-causing mutations detected (including the eight mutations previously published) is now 35. The spectrum of mutation includes five deletions at the genomic level, four insertions, seven splice site mutations (one of which is caused by a 1-bp deletion), seven nonsense, and 12 missense mutations. Overall, eight mutations cause a shift in the reading frame with premature truncation of the polypeptide (listed in Table 3, column 4) and three mutations cause an in-frame insertion or deletion. Of the 19 single nucleotide substitutions in the reading frame, 13 are transitions and 6 are transversions. Finally, in two instances the same nucleotide positions (IVS5+1 and C1145) were the target of two mutational events.

Expression Studies

Eighteen different missense variants causing amino acid changes were identified among the SSADH patients (Tables 2 and 4). To establish which of these mutations were pathogenic, 16 mutant alleles were reproduced by site-directed mutagenesis and expressed in HEK 293 cells (Table 4). This analysis displayed two distinct groups of variants (high expression and low expression) with the exception of one allelic variant (N255S) that gave an intermediate expression level of 17%. Nine of the mutations resulted in an almost complete loss of SSADH activity (<5%) and can be considered causative for SSADH deficiency. Four of the mutant alleles detected during the course of this study cause a decrease in SSADH activity of less than 55%, and their occurrence in the general European population has been reported elsewhere [Blasi et al., 2002]. These alleles can be considered to be nonpathogenic polymorphisms since carriers of SSADH deficiency do not have increased GHB levels in bodily fluids, nor do they display clinical symptoms even though SSADH activity is reduced to approximately 50%. The silent D464D substitution (c.1398C>T) can also be included in the group of non-disease-causing mutations. Unlike the H180Y and P182L substitutions that were detected in the general population with a frequency of 23% and 7%, respectively, the other substitutions (G36R and A237S) that did not strongly alter enzyme activity were not detected in controls [Blasi et al., 2002]. Because of the population association found between the H180Y and P182L polymorphisms we also expressed the composite variant. The effects of the two amino acid substitutions appear to be simply multiplicative, without any further synergistic effect. The finding of these sequence variants that have reduced, but still significant enzyme activity (36% of the normal value if the two polymorphisms are combined) in our heterologous expression system may provide an explanation for the high variability in SSADH expression levels in healthy individuals [Gibson et al., 1991]. Because it is not currently known at what level of SSADH activity clinical symptoms arise we do not know whether the reduction of SSADH activity to 36% of the H180Y-P182L variant would cause clinical symptoms if the other allele is affected. Interestingly, two of the patients in whom the H180Y and P182L polymorphisms were detected (families 3 and 25) also shared the G36R substitution that affected activity in our expression system only slightly. Because the resulting amino-acid change lies in the mitochondrial leader sequence we collected the mitochondrial fraction from transfected cells and found that the activity was comparable to the wild-type activity (data not shown), showing that the mitochondrial targeting was not affected.

TABLE 4. SSADH Enzyme Activity of Mutant Protein Containing Missense Mutations Expressed in Mammalian Cells

Nucleotide change(s)	Amino acid substitution(s)	SSADH activity[a]
Control	–	100%
c.106G>C	p.G36R	87%[b]
c.278G>T	p.C93F	3%
c.526G>A	p.G176R	<1%
c.538C>T	p.H180Y	83%[b]
c.538C>T+	p.H180Y+	36%
c.545C>T	p.P182L	
c.538C>T+	p.H180Y+	6%
c.545C>T+	p.P182L+	
c.764A>G	p.N255S	
c.545C>T	p.P182L	48%[b]
c.668G>A	p.C223Y	5%
c.698C>T	p.T233M	4%
c.709G>T	p.A237S	65%[b]
c.764A>G	p.N255S	17%
c.803G>A	p.G268E	<1%
c.1005C>A	p.N335K	1%
c.1145C>T	p.P382L	2%
c.1145C>A	p.P382Q	n.d.
c.1216G>A	pV406I	n.d.
c.1226G>A	p.G409D	<1%
c.1398C>T	p.D464D	Not applicable
c.1597G>A	p.G533R	<1%

[a]Activity is expressed relative to wild-type activity assayed in the same experiment.
[b]Published in Blasi et al. [2002].
n.d., not done.

DISCUSSION

We analyzed the ALDH5A1 genotypes in a large cohort of patients with clinical and biochemical diagnosis of SSADH deficiency and report the characterization of 27 novel mutations in patients from 48 families not known to be related. This increases the total number of different mutations identified in 54 different families to 33 (Tables 2 and 3). Twenty of the mutations were sporadic and except for the IVS5+1G>A allelic variant

and the two alterations detected in the only Japanese SSADH patient currently known, all mutations reported previously were recurrent in this study.

The 10 missense mutations that were confirmed to be causative of SSADH deficiency by expression analysis were distributed rather randomly throughout the protein, except for a short stretch between aa 223–268 in which four of the deleterious amino-acid substitutions were located, accounting for 38% of the missense alleles detected (14 out of 37 missense alleles in a total of 10 families). These are encoded by exons 4 and 5. Our observation then prompts to a higher priority in screening these two exons when searching for mutations in genomic material. The four altered residues between amino acids 223 and 268 were quite conserved between human aldehyde dehydrogenases and between SSADH proteins from the 26 different species of which the (putative) ALDH5A1 gene is sequenced, with the exception of the cysteine residue at position 223 that was mutated to a tyrosine in Patient 21. Surprisingly, all other human aldehyde dehydrogenases contain a tyrosine residue at this position. The importance of the cysteine at this position in SSADH is not only shown by the complete absence of activity of the C223Y mutant protein but is also suggested by the fact that 25 out of 26 (putative) SSADH proteins currently sequenced indeed contain a cysteine at this position. The six SSADH deficiency-causing missense mutations located outside the area from aa 223–268 were also quite conserved between human aldehyde dehydrogenases and between different SSADH proteins with the exception of the C93F and G533R mutations. On the other hand, the five nonpathogenic variants targeted nonconserved residues with the exception of the highly conserved alanine 237.

Only one of the missense mutations (N335K) was found in close proximity to the active site glutamate and cysteine residues (E306 and C340, as predicted by the Prosite signature motifs of aldehyde dehydrogenases). In contrast, among the 26 missense mutations in ALDH3A2 responsible for Sjögren-Larsson syndrome, eight were found near the active site (between aa 283 and 365) and not a single mutation was found between aa 223 and 268 where most ALDH5A1 mutations were identified (numbers refer to positions in the SSADH protein after aligning the sequences) [De Laurenzi et al., 1996; Sillen et al., 1998; Rizzo et al., 1999; Willemsen et al., 2001]. Four of the ALDH3A2 mutations were detected near the C-terminus (leucine 535 in SSADH) where also the G533R mutation detected in Patients 7 and 42 are located. It is not known whether the G533K substitution in SSADH affects the stability and/or the activity of the protein, but it is interesting to note that a E487K substitution at 11 amino acids distance from the c-terminus of ALDH2 (mitochondrial aldehyde dehydrogenase) increases the turnover and reduces the activity of the enzyme in a dominant fashion [Xiao et al., 1996]. The E487K mutation is a polymorphism found in approximately 50% of the Oriental population that causes alcohol-induced flushing resulting from acetaldehyde accumulation after alcohol consumption.

Because of the limited availability of clinical data and the low number of patients with identical haplotypes, discussing genotype–phenotype relations is rather premature. One family with an unusual clinical presentation (Family 3) [Gibson et al., 1997b] was included in the current study and was shown to have a complex combination of mutations and polymorphisms, but the fact that one of the mutations was a nonsense mutation argues against the assumption that residual activity might have influenced the clinical manifestations.

Although missense mutations were detected in 20 different families, the observation that only one of these mutations (N255S) has an intermediate effect on the activity of the enzyme activity in our expression system suggests that differences in residual enzyme activity play a very limited role in determining the severity of the disease. This mutation was found in a patient with a complex genotype (Family 25 in Table 2). When expressed, this mutation produced a six-fold reduction of activity (from 100% to 17%). In combination with the H180Y and P182L polymorphisms the activity was further reduced to 6%. It is worth noting that the genotype of this patient is compatible with both arrangements. If the patient actually carried the N255S mutation on a H180Y-P182L background the 6% residual activity could easily explain SSADH deficiency (assuming heterozygosity with another yet unknown mutation). An alternative explanation would be that the N255S mutant form of the protein cannot form active heterotetramers with the H180Y-P182L variant. This would be rather similar to the dominant-negative effect that the polymorphism in the ALDH2 gene has on protein activity [Xiao et al., 1996]. Unfortunately samples from parents or siblings were not available and we were therefore unable to determine the haplotype with certainty.

There are many factors that could influence the clinical features in patients. There is no correlation between GHB levels in physiological fluids and the severity of the disease, yet the heterogeneity in clinical presentation in sibships is less extensive than between unrelated patients [Gibson et al., 1998]. Factors that could potentially cause modulation of the clinical phenotype are polymorphisms in other metabolic enzymes affecting GABA metabolism, differences in the activity of other aldehyde dehydrogenases that have an overlapping substrate specificity, a different rate of formation of GHB from succinate semialdehyde or different concentrations of succinate semialdehyde in the brain. Altered expression of GABA and GHB receptors might also contribute to pathogenesis [Mehta et al., 2002]. SSADH may also play a role in the oxidation and removal of other toxic aldehydes in brain, such as 4-hydroxy-2-nonenal [Picklo et al., 2001a, b]; this observation would be supported by the significant decrease in brain reduced glutathione (GSH) that we have detected in the brain of Aldh5a1 knockout mice (unpublished results). A recent report about an SSADH

patient who developed malignant neuroleptic syndrome after a single dose of haloperidol suggests that SSADH deficiency could affect other metabolic pathways as well [Neu et al., 2002].

The GABA shunt is also active in most peripheral organs, but not much is known about its function. In plants, SSADH may be involved in regulation of cytosolic pH and adaptation to stress. The observation that *Arabidopsis* SSADH (which is 58% identical and 74% homologous to human SSADH) is inhibited by high $NADH/NAD^+$ ratios suggests that the supply of succinate to the Krebs cycle is affected by the reduction potential in the mitochondria [Busch and Fromm, 1999]. Whether these (potential) additional functions of SSADH activity in peripheral organs are involved in disease pathogenesis is currently not known. The recent development of a mouse model for SSADH deficiency provides a valuable tool for studying the neurophysiological consequences of SSADH on various other metabolic pathways, and will hopefully aid in finding modifier genes and other factors affecting disease pathogenesis [Hogema et al., 2001b; Gibson et al., 2002].

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