RESEARCH ARTICLE

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A Novel *ALDH5A1* Mutation Is Associated With Succinic Semialdehyde Dehydrogenase Deficiency and Severe Intellectual Disability in an Iranian Family

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Succinic semialdehyde dehydrogenase (SSADH) deficiency is a disorder of the catabolism of the neurotransmitter gamma-aminobutyric acid (GABA) with a very variable clinical phenotype ranging from mild intellectual disability to severe neurological defects. We report here on a large Iranian family with four affected patients presenting with severe intellectual disability, developmental delay and generalized tonic–clonic seizures. Molecular genetic analysis revealed a missense mutation c.901A>G (p.K301E, RefSeq number NM_001080) in *ALDH5A1* co-segregating with the disease in the family. The missense mutation affects an amino acid residue that is highly conserved across the animal kingdom. Protein modeling showed that p.K301E most likely leads to a loss of NAD^+ binding and a predicted decrease in the free energy by 6.67 kcal/mol furthermore suggests a severe destabilization of the protein. In line with these in silico observations, no SSADH enzyme activity could be detected in patient lymphoblasts.

Key words: SSADH deficiency; *ALDH5A1*; intellectual disability; Iranian; autosomal recessive

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INTRODUCTION

Succinic semialdehyde dehydrogenase (SSADH) deficiency (OMIM #271980) is a rare autosomal recessive inherited metabolic disorder resulting in the accumulation of γ-hydroxybutyrate (GHB) in the brain [Mason and Kerns, 2002; Pearl et al., 2003]. In patients with SSADH deficiency, brain concentrations of GHB are increased 30-fold and concentrations of γ-amino-butyric acid (GABA) are increased two- to fourfold as compared to healthy

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individuals [Gibson et al., 1998]. Concordant with this phenotype, SSADH deficient mice (*ALDH5A1*$^{-/-}$) show a 60-fold increase of GHB and a 2-fold increase of GABA levels in the brain [Hogema et al., 2001; Jansen et al., 2008].

The major inhibitory neurotransmitter of the brain, GABA, is derived from the major excitatory neurotransmitter glutamate. GABA is in turn converted into succinic semialdehyde that is then oxidized to succinate by the NAD^+-dependent succinic semialdehyde dehydrogenase SSADH. During the conversion from GABA to succinic semialdehyde by GABA transaminase, an amino group is removed from GABA and added to alpha-ketoglutarate from the Krebs cycle, thus providing an equal amount of glutamate for each molecule of GABA converted to succinic semialdehyde ("GABA shunt") [Hassel et al., 1998]. Alternatively, succinic semialdehyde can be converted to GHB by succinic semialdehyde reductase [Maitre, 1997; Kelly et al., 2002; Gibson et al., 2003]. In SSADH deficiency the final step of the GABA degradation pathway is shifted towards the production of the alternative by-product GHB [Gropman, 2003]. GHB has effects on multiple neurotransmitter systems, such as dopamine, serotonine, acetylcholine and GABA [Gibson et al., 2003]. High levels of GHB and GABA desensitize postsynaptic GABA(B) receptors resulting in a decrease in G-protein coupled inwardly rectifying potassium (GIRK) channel function, which is believed likely to cause the seizures in affected individuals and the *ALDH5A1*$^{-/-}$ mouse model [Vardya et al., 2010].

The clinical phenotype of SSADH deficiency has a high intra- as well as interfamilial variability ranging from mild delayed intellectual, motor, speech and language development to severe neurological defects including seizures, hypotonia, ataxia and behavioral problems [Jakobs et al., 1993; Gibson et al., 1997; Pearl et al., 2003]. Therapeutic concepts in human and murine SSADH deficiency have been reviewed [Knerr et al., 2007; Kim et al., 2011]. Recently seizures were treated successfully with high-dose phenobarbital in a young SSADH deficiency patient [Yamakawa et al., 2012]. The GABA(B) receptor antagonist SGS-742 showed positive effects in a Phase II double-blind, placebo-controlled clinical trial in patients with mild cognitive impairment [Froestl et al., 2004]. Pearl et al. [2009] could show that SGS-742 significantly improves the spike-wave duration in a dose dependent manner and also controls absence seizures.

Even though approximately 50 mutations in the SSADH encoding aldehyde dehydrogenase 5a1 gene (*ALDH5A1*; OMIM 610045) are associated with SSADH deficiency to date (HGMD Professional 2012.1; for a recent review on disease causing mutations in *ALDH5A1* see Kim et al. [2011]), only one has so far been reported to affect NAD^+ binding.

Here, we present now a new homozygous missense mutation, c.901A>G (p.K301E), in *ALDH5A1* and provide experimental evidence that this change causes SSADH deficiency in affected individuals, most probably owing to disturbed NAD^+ binding and molecular destabilization of the gene product, as shown by protein modeling.

PATIENTS AND METHODS

Patients

This study was carried out in accordance with the ethical standards of the appropriate national and institutional committees. An Iranian family with severe ID and a history of generalized tonic–clonic seizures without hyperactivity or attention deficit was ascertained and written informed consent obtained from the parents. The family pedigree and facial aspects of affected individuals are shown in Figure 1A,B respectively. All affected individuals were born full-term after uneventful pregnancies with birth weights between 3,000 and 3,500 g. There is a history of hypotonia during the neonatal period in this family. Nystagmus was not observed in any patient, but there is a history of unilateral glaucoma in V-9, which has started at the age of 11 years. All affected members had developmental delay and speech delay (4–6 years), walking started at 3.5 years of age. The index patient V-11 suffers from severe ID (IQ 35) like both his affected sisters (V-9, IQ 30, and V-12, IQ 35) (Table I).

Apart from epilepsy, the patients suffered from no other neurologic disorders, and they had no neuropsychiatric problems. Seizures were successfully treated with carbamazepine. Brain MRI scans or metabolic tests were not performed in this family.

The ethnically matched control panel included 94 unrelated healthy individuals. Additionally, 124 unrelated healthy German individuals were also directly screened for the mutation. Samples from patients as well as from controls were obtained after receiving informed consent. Moreover, we used the publically available sequencing data from the Exome Variant Server (NHLBI Exome Sequencing Project (ESP), Seattle, WA, URL: http://evs.gs.washington.edu/EVS/), 200 Danish individuals [Li et al., 2010] and 185 genomes of healthy individuals made available by the 1,000 Genome Project (URL: http://www.1000genomes.org/) to further extend the control cohort.

SNP Array

DNA was extracted from peripheral blood of the patients (V-9, V-11, and V-12), one healthy sibling (V-2), their parents (IV-3 and IV-4) and their affected cousin (V-1) using standard procedures. Karyotype analysis by G-banding was performed. The karyotype of all patients was normal and fragile X-syndrome was excluded. Genotyping (SNP analysis) was performed using the Human 610-Quad BeadChip (Illumina) following the protocol of the manufacturer. Linkage analysis was performed using the Merlin software [Abecasis et al., 2002]. Details of data quality controls and linkage analysis have been published elsewhere [Garshasbi et al., 2006].

Mutation Screening and Evaluation

Mutation screening of *ALDH5A1* exons and exon–intron boundaries was performed in the index patient in 11 independent PCR reactions from genomic DNA (Table SI). We employed the PyMOL software (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC) to model the *ALDH5A1* protein structure based on the wildtype crystal structure (PDB 2w8o) available in the Protein Data Bank PDB [Berman et al., 2000] and performed an in silico analysis to estimate the effect of p.K301E on protein stability using Concoord/PBSA [Benedix et al., 2009; Potapov et al., 2009]. In silico prediction of pathogenic effects of p.K301E were performed with SIFT [Ng and Henikoff, 2001], PolyPhen2 [Adzhubei et al., 2010], Mutation Taster [Schwarz et al., 2010], and

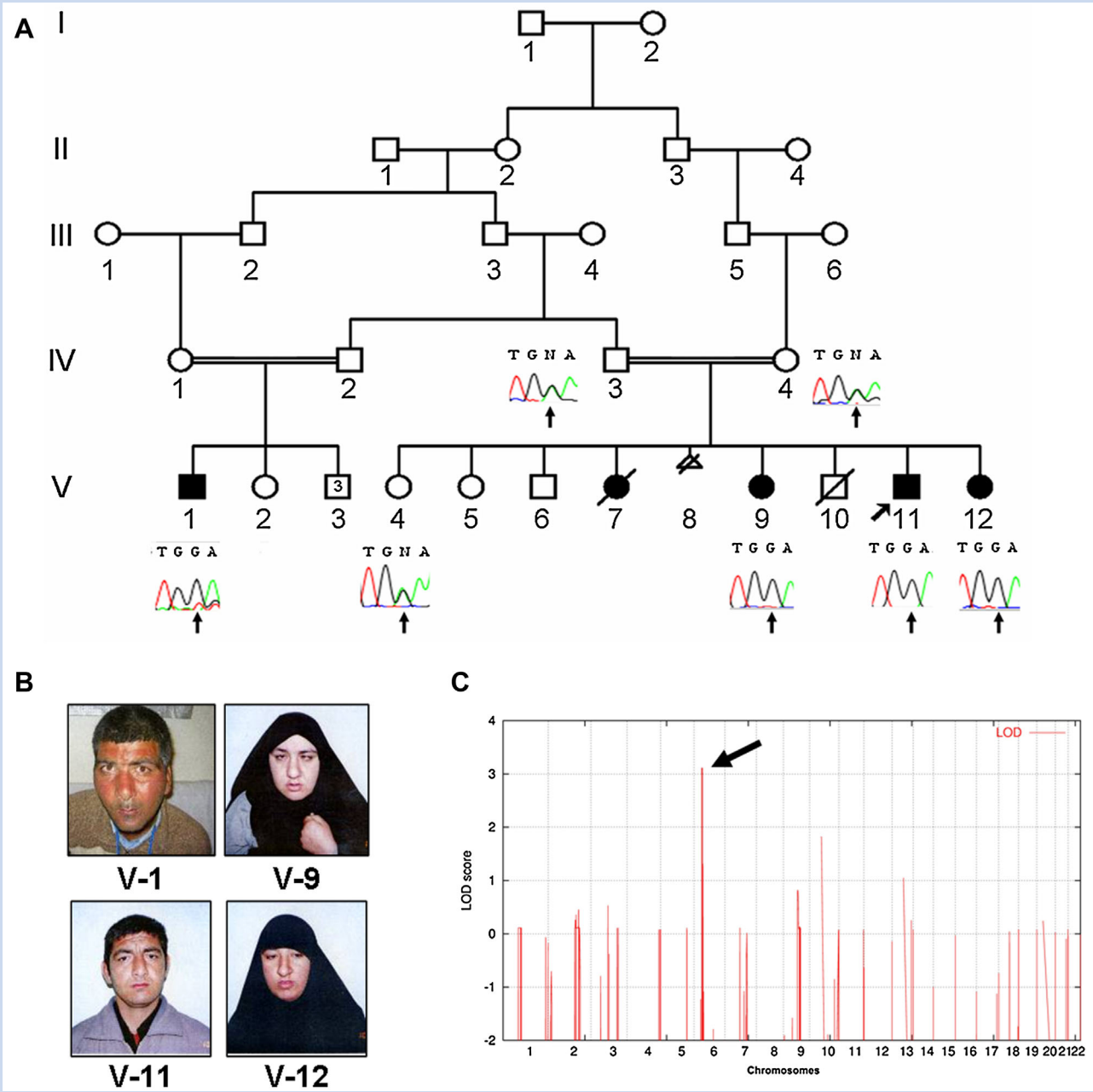

FIG. 1. A: Pedigree of the family 8600485. Full symbols denote affected individuals with ID. Sequence chromatograms next to all examined individuals show c.901A>G in *ALDH5A1* in a heterozygous (IV:3, IV:4, and V:4) or homozygous (V:1, V:9, V:11, and V:12) state. B: Affected individuals with SSADH deficiency. V-11 is the index patient. C: Result of whole genome parametric linkage analysis (Merlin software), showing a linkage interval with significant LOD score of 3.1 on chr.6p22-p21.32 (arrow).

Panther Classification System [Thomas et al., 2003; Thomas and Kejariwal, 2004] software tools.

Enzymatic Assay

An EBV immortalized lymphoblastoid cell line was established from the blood of the index patient (V-11) using standard procedures. Cells derived from 6 age and sex matched controls as well as the index patient were used to measure the level of SSADH enzyme activity. The method described by Gibson et al. [1991], based on the fluorometric assay by Cash et al. [1977], was modified for measuring NADH fluorescence in a microplate reader.

Cell pellets (4×10^7 cells/ml) were resuspended in 100 mM Tris–HCl (pH 8.6) and lysed by sonication twice for 2 cycles of 10 sec at maximum power with a pause between the bursts. The final assay

TABLE I. Features of Affected Individuals in Family 8600485

Affected individual	Sex	Age at examination	IQ	OFC (cm)	Height (cm)
V-1	Male	27	—[a]	55	181
V-9	Female	27	30	51.5	163
V-11	Male	23	35	55.5	181
V-12	Female	21	35	52	157

OFC, occipitofrontal circumference.
[a]Diagnosed as severely intellectually disabled without IQ testing.

contained 100 µl cell lysate (approxiamtely 0.6–0.7 mg of total protein). SSADH activity was assayed for 30 min at 37°C in a final volume of 300 µl in buffer containing 100 mM Tris–HCl (pH 8.6), 0.1 mM EDTA, 20 mM β-mercaptoethanol, 50 mM KCl and 0.1% Triton-X 100 (v/v), 3 mM succinic semialdehyde (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and NAD^+ (Sigma-Aldrich Chemie GmbH, Taufkirchen bei München, Germany) in concentrations ranging from 0.01 to 2 mM. Succinic semialdehyde (Santa Cruz Biotechnology) was used at a concentration of 3 mM. Blank reactions contained water substituted for succinic semialdehyde. The reaction was stopped by heating for 5 min at 100°C. After cooling down on ice for 10 min, probes were centrifuged at 4°C (20,800*g*) to remove cell debris. NADH fluorescence was measured in a POLARstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany; excitation 355 nm, emission 460 nm).

RESULTS

Linkage Analysis and Mutation Screening

Parametric linkage analysis including individuals from both branches of family 8600485 (IV-3, IV-4, V-1, V-4, V-9, V-11, and V12) revealed a single interval of homozygosity on chromosome 6p22-p21.32 with a significant LOD score of 3.1 (Fig. 1C). The linkage interval had a size of 9.67 Mb and was flanked by the heterozygous SNPs rs9379512 (chr6: 23499628, UCSC genome browser March 2006 assembly) and rs10484569 (chr6: 33166930, UCSC genome browser March 2006 assembly). The interval contains 284 genes including *ALDH5A1*. As the patient phenotype was strongly suggestive of SSADH deficiency, we screened *ALDH5A1* for mutations and found an A>G substitution (c.901A>G) in exon 6 (NM_001080) resulting in a change from leucine to glutamic acid at amino acid position 301 (p.K301E; NP_001071) (Fig. 2A). This missense mutation co-segregated with the disease in the core family (Fig. 1A) and was also present in the affected cousin. c.901A>G was absent from the control panel as well as from the 185 healthy individuals studied by the 1,000 Genomes Project and the exomes from 200 Danish individuals and is not listed in the Exome Variant Server (ESP6500).

The affected nucleotide c.901A has a PhyloP score of 5.031 and the lysine at the corresponding amino acid position 301 is highly conserved throughout the animal kingdom (eFig. S1 in Supporting information online). Taken together this evidence suggested strongly that c.901A>G had disease-causing potential. In order to further corroborate this hypothesis we analyzed the effect of the amino acid change caused by the mutation in silico and found that all four programes we used unanimously predicted deleterious consequences for SSADH function.

In Silico Modeling of SSADH p.K301E

To gain insights into the structural consequence of the p.K301E mutation in SSADH, we performed structure-based in silico modeling of the mutant protein. In the wild type structure, Lys301 connects two alpha helices and one beta strand, which are involved in the binding of NAD^+ (Fig. 2B, eFig. S2 in Supporting information online). The non-conservative change from a positively charged lysine to the negatively charged glutamine (Fig. 2C) likely destabilizes this area of the protein core. We have quantified this effect by performing in silico energy calculations with the Concoord/PBSA method [Benedix et al., 2009], which calculates stability changes upon mutation. The simulation revealed a decrease in stability of 6.67 kcal/mol, which is more than the overall stability of most proteins [Dill, 1990]. Thus we hypothesized that the p.K301E mutation would severely reduce or even abolish SSADH activity.

SSADH Activity Measurement

Therefore, we next measured lymphoblast SSADH activity (blood, liquor, and urine were not available) in 6 age and sex matched controls and the index patient (V-11). Measurement of SSADH activity was performed in triplicate for each NAD^+ concentration on two consecutive days, respectively. We found SSADH activity to increase relating to elevated amounts of NAD^+ in the range of 0.01–0.1 mM in the control cell lines, whereas in the patient cell line no SSADH activity could be detected (Fig. 3). At even higher NAD^+ concentrations (0.5–2 mM), SSADH was found to be still inactive in the patient cell line, whereas in the control cell lines a dose dependent decrease in SSADH activity was observed (not shown). Day-to-day variability in our study was in line with assay variability published by others [e.g., Gibson et al., 1991].

DISCUSSION

We present a novel missense mutation that is associated with SSADH deficiency in an Iranian family and leads to undetectable levels of SSADH activity. The affected individuals are from a large consanguineous family and present with developmental delay as

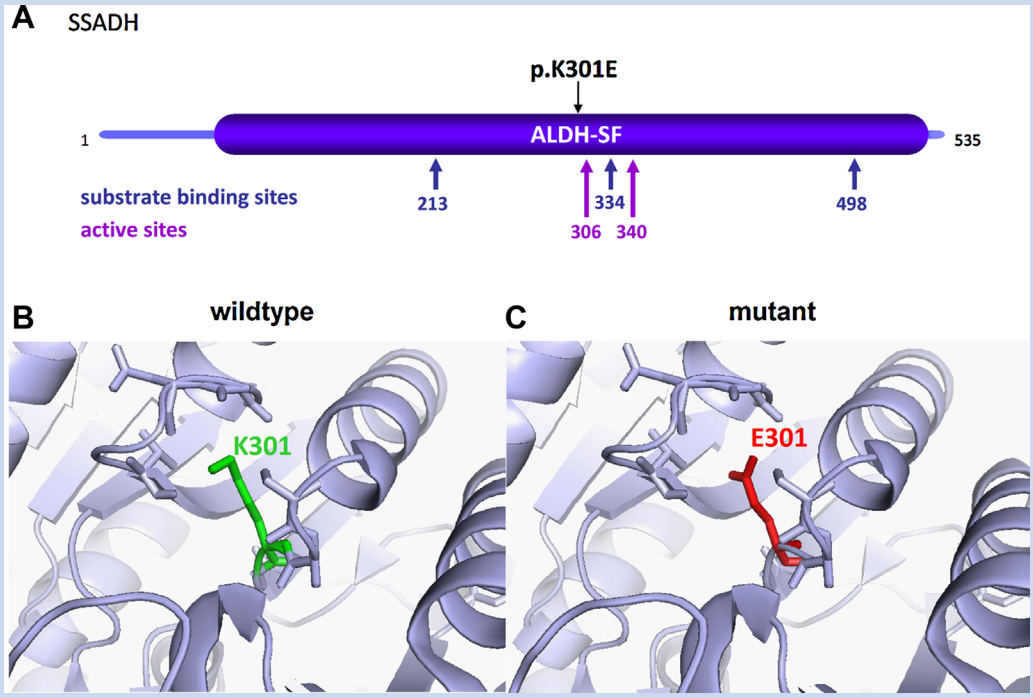

FIG. 2. A: Schematic representation of the *ALDH5A1* gene product indicating the position of the change from lysine to glutamic acid at position 301 as well as the positions of the residues of the active sites and substrate binding sites. B: The residue affected by the missense mutation is presented as stick model with green color in the wildtype and red color in the mutant protein (C) respectively.

well as speech delay, severe ID, tonic–clonic seizures, and hypotonia during the neonatal period.

SSADH deficiency is a rare autosomal recessive disorder that has been reported in no more than about 450 affected individuals to date [Pearl et al., 2011]. Parental consanguinity is observed in approximately 40% of the cases [Pearl et al., 2003]. The SSADH deficient patient database maintained by the Department of Neurology at Children's Medical Center in Washington DC is based on systematic questionnaire data of 60 patients. These data suggest that developmental delay, as well as ID are global findings in affected individuals (100%), and that hypotonia (82%), ataxia (77%) as well as tonic–clonic seizures (53%) are common clinical symptoms [Pearl et al., 2009, 2011]. All of these features, except for ataxia, are present in the affected individuals in our study and are consistent with the typical characteristics of human SSADH deficiency (as reviewed, e.g., by Kim et al., 2011; Pearl et al., 2011; or Knerr et al., 2008). Accompanying neuropsychiatric symptoms such as, for example, inattention, anxiety, aggressive behavior or autistic features, which occur in 72%, 40%, 21% or 12% of cases respectively [Kim et al., 2011; Pearl et al., 2011], were not obvious in our patients.

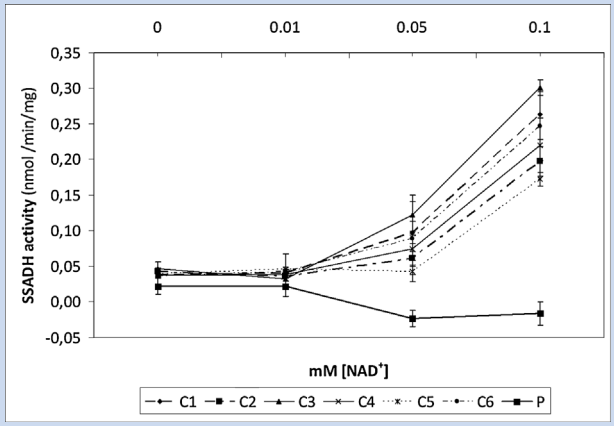

FIG. 3. Lymphoblast SSADH activity in cell lines of the affected individual and 6 controls at different NAD^+ concentrations (0–0.1 mM). SSADH activity is given as nmol/min/mg protein. Data were normalized against background activity. Each data point is the mean value of two separate experiments that were each performed in triplicate. Error bars represent the standard deviation.

The murine knockout model (*ALDH5A1*$^{-/-}$) is a viable phenocopy of the human SSADH deficiency and presents the most severe outcome of this disease. The clinical symptoms of the knockout mice comprise progressive ataxia, seizures and failure to thrive. Around postnatal Day 14 absence seizures appear, followed by tonic–clonic seizures around postnatal Day 20 and status epilepticus leading to rapid death during the fourth postnatal week [Hogema et al., 2001; Cortez et al., 2004; Gupta et al., 2004]. The substrate of SSADH, succinic semialdehyde, is derived from GABA after reuptake from the synaptic cleft into the presynaptic neuron. SSADH oxidizes succinic semialdehyde to succinate and

simultaneously reduces the coenzyme NAD^+ to NADH. The Lysine affected by the p.K301E mutation we describe here is located in close spatial neighborhood to a glutamine at position 306 and a cysteine at position 340, which together constitute the active site environment. Under oxidized conditions SSADH is inactive and a disulfide bond is formed between the catalytic residues p.C340 and p.C342. A catalytic loop including residues 334–344 blocks the binding sites for succinic semialdehyde as well as for NAD^+ [Kim et al., 2009]. In silico analysis provided evidence that p.K301 is part of the protein core and involved in binding of NAD^+ and that p.K301E might lead to a destabilization of SSADH. This led us to the hypothesis that p.K301E severely reduces SSADH activity either by loss of protein stability through misfolding and subsequent degradation or, as the affected p.K301 connects two alpha helices and one beta strand that are involved in the binding of NAD^+, by reduced NAD^+ binding capacity.

Loss of SSADH activity leads to accumulation of succinic semialdehyde, which is degraded to GHB by NADPH dependent aldo–keto reductase 7A2 (AKR7A2) [Lyon et al., 2007]. GHB naturally occurs in the mammalian brain at a level of <1% of its parent neurotransmitter GABA [Doherty et al., 1978] and acts on specific GHB receptors [Snead, 2000; Wu et al., 2004] as well as the GABA (B) receptor [Gervasi et al., 2003]. In the CSF of affected individuals, GHB levels are increased 65- to 230-fold, GABA levels are threefold elevated and glutamine is decreased [Gibson et al., 2003]. Comparable to affected humans; *ALDH5A1*$^{-/-}$ mice exhibit elevated levels of GABA and GHB in the brain [Hogema et al., 2001; Jansen et al., 2008]. In human and murine SSADH deficiency it could be observed that absence seizures are related to excessive GHB and GABA(B) mediated activity and that generalized convulsive seizures might be caused by overuse-dependent downregulation of GABA(A) and GABA(B) receptor activity [Buzzi et al., 2006; Wu et al., 2006; Pearl et al., 2011].

The assay we performed to test SSADH enzyme activity in the lymphoblastoid cell line of one affected individual proved our hypothesis to be true, as no SSADH activity could be detected. This is also in line with the results of Akaboshi et al. [2003] who assayed the activity of 27 disease-causing missense mutations observed in affected individuals. Five missense mutations (p.G176R, p.G268E, p.N335K, p.G409D and p.G533R) showed a nearly abolished enzyme activity (<1–1%). While three of these mutations probably affect protein stability (p.G409D), or stability and oligomerization (p.G176R and p.G533R), p.G286E and p.N335K affect the catalytic function of SSADH. The protein residue p.N335 is located on the "dynamic catalytic loop" and probably leads to a severe distortion of the active site environment or reduced dynamics of the "catalytic loop" or both [Kim et al., 2009]. However, only p.G268 seems to be involved in binding of NAD^+, being one of the residues creating the binding pocket for the adenine base of NAD^+. Thus p.G268E might lead to a loss of NAD^+ binding ability and consequently to a loss of SSADH activity [Kim et al., 2009]. To our knowledge the p.K301E mutation we present here is now only the second missense mutation with a potential effect on the binding of NAD^+ that results in undetectable SSADH activity. In controls, SSADH activity increased using NAD^+ ranging from 0.01 to 0.1 mM NAD^+. Higher NAD^+ concentrations led to a decrease of enzyme activity in these samples. This could be explained by the fact that during oxidation of succinic semialdehyde to succinate, NAD^+ is reduced to NADH, which is known to exhibit an inhibitory effect for several mammalian SSADHs [Duncan and Tipton, 1971; Blaner and Churchich, 1979; Rivett and Tipton, 1981; Kang et al., 2005]. Moreover, as a consequence of this, succinic semialdehyde is accumulated leading in turn to substrate inhibition of SSADH [e.g., Kammerat and Veldstra, 1968; Blaner and Churchich, 1979].

In conclusion, our study identified a novel homozygous missense mutation in *ALDH5A1* that is associated with SSADH deficiency and severe ID. The mutation is located near the active site and is thus only the second mutation identified to date that might not necessarily cause a loss of SSADH activity solely through protein misfolding and subsequent degradation, but rather affect SSADH activity through an impairment of NAD^+ binding.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

FIG. S1. Partial amino acid sequence alignment of the human SSADH protein and its homologues in the animal, plant and fungi kingdom. The position of the missense mutation is indicated by an arrow. The respective amino acid is identical in the 13 proteins.

FIG. S2. Overview of Lys301 (green) and NAD^+ binding site (yellow) in stick representation. The ADP moiety of a NAD^+ molecule is shown in orange.

TABLE SI. Primers used for amplification of *ALDH5A1*.