Identification of Two Novel Mutations of ABCD1 Gene in Pedigrees With X‐linked Adrenoleukodystrophy and Review of the Literatures

Bingzi Dong
The Affiliated Hospital of Qingdao University
Wenshan Lv
The Affiliated Hospital of Qingdao University
Lili Xu
The Affiliated Hospital of Qingdao University
Yuhang Zhao
The Affiliated Hospital of Qingdao University
Xiaofang Sun
The Affiliated Hospital of Qingdao University
Bingfei Cheng
The Affiliated Hospital of Qingdao University
Yangang Wang (wangyg1966@126.com)
The Affiliated Hospital of Qingdao Univerisity https://orcid.org/0000-0003-3411-4025

Research

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Identification of two novel mutations of *ABCD1* gene in pedigrees with X-linked adrenoleukodystrophy and review of the literatures

*Dong Bingzi, *Lv Wenshan, Xu Lili, Zhao Yuhang, Sun Xiaofang, Cheng Bingfei, Wang Yangang $

Department of Endocrinology and Metabolism, The Affiliated Hospital of Qingdao University, Qingdao 266003, China.

Corresponding authors:

Prof. Wang Yangang

Department of Endocrinology and Metabolism, the Affiliated Hospital of Qingdao University

No. 16 Jiangsu Road, Qingdao, 266003, China

E-mail: wangyg1966@126.com

Tel: +86-0532-82911383 Fax: +86-0532-82911999

*Dong Bingzi and Lv Wenshan contributed equally to this work.

Abstract:

Background: X-linked adrenoleukodysrophy (ALD) is an inherited peroxisomal metabolism disorder, results from the loss-of-function mutation of ATP-binding cassette protein subfamily D1 (*ABCD1*) gene. The dysfunction of ALD protein, a peroxisomal ATP-binding cassette transporter, results in the excessive saturated very long chain fatty acids (VLCFAs) accumulation in organs including brain, spine and adrenal cortex. X-ALD is characterized as the childhood, adolescent, adult cerebral ALD, adrenomyeloneuropathy (AMN), adrenal insufficiency, and asymptomatic phenotypes, exhibiting a high variety of clinical neurological manifestations with or without adrenocortical insufficiency. **Results:** In this study, we reported two cases of X-ALD, which were firstly diagnosed as adrenal insufficiency (Addison's disease) and treated with adrenocortical supplement. However, both of the cases progressed as neurological symptoms and signs after decades. Elevated VLCFAs level, brain MRI scan and genetic analysis confirmed final diagnosis. In addition, we identified two novel mutations of *ABCD1* gene, c.874_876delGAG (p.Glu292del) and c.96_97delCT (p.Tyr33Profs*161) in exon 1 of *ABCD1* gene. Sanger sequencing confirmed that the proband's mother of the first case was hemizygous carrying the same variant. Adrenal insufficiency-only type is very rare, however, it may be the starting performance of X-ALD. In addition, we summarized reported mutation sites and clinical manifestations to investigate the correlationship of phenotype-genotype of X-ALD. **Conclusions:** The early warning manifestations should be noticed, and the probability of X-ALD should be considered. This report could be beneficial for the early diagnosis and genetic counseling for patients with X-ALD.

Keyword :X-linked adrenoleukodystrophy (ALD), *ABCD1*, very long chain fatty acids (VLCFAs), adrenal insufficiency

Background:

X-linked adrenoleukodystrophy (X-ALD) (OMIM#300100) is a X-linked inherited peroxisome metabolic neurodegenerative disorder, resulted from the defects in ATP-binding cassette protein subfamily D1 (*ABCD1*) gene. *ABCD1* gene locates in chromosome Xq28, contains 10 exons, and codes the adrenoleukodystrophy protein (ALDP), a member of the ABC transporter superfamily. ALDP expresses in the peroxisome membrane, containing 745 amino acids. It plays a key role in VLCFAs transporting into peroxisome for β-oxidation and degradation [1]. The defective ALDP results in the impaired degradation of saturated very long-chain fatty acids (VLCFAs). Excessive VLCFAs accumulate in plasma and tissues including adrenal cortex, cerebral white matter, or spinal cord [2] to exhibit clinical manifestations. Till now, there are more than thousand variants have been reported (X-ALD Mutation Database http://www.x-ald.nl/) [3].

Based on the age of onset and the extent of organs affected, X-ALD is classified into seven clinical subtypes: childhood cerebral, adolescent cerebral, adult cerebral, adrenomyeloneuropathic (AMN), cerebellar, adrenal insufficiency-only (Addison's disease), and asymptomatic or pre-symptomatic types. X-ALD exhibits a high variety of clinical characteristics [4]. Childhood cerebral ALD (CCALD) and adult-onset AMN are the two most common phenotypes [5]. The clinical symptoms include progressive dysfunction of central and peripheral nervous systems, such asparalysis, behavioral disturbance, abnormalities of sphincter control, sensory loss or paresthesia, with or without adrenal insufficiency. Brain MRI shows a typical pattern of symmetrical enhanced T2WI signal in the bilateral white matter around the lateral ventricle, cerebellum, and the genu of the corpus callosum [6]. Significantly elevated level of VLCFAs is the important diagnostic clue for the individuals with ALD. There are three parameters of VLCFAs to analyze, the concentration of C26:0, the ratio of C24:0/C22:0, and the ratio of C26:0/C22:0 [5]. Final diagnosis relays on the genetic analysis to detect *ABCD1* mutation.

Adrenal insufficiency-only type is rare, especially in females less than 1% [7-8]. Some individuals were diagnosed as Addison's disease without symptoms of cerebral or myelopathy ALD for decades. However, the early-onset Addison's disease may gradually progress to exhibit neuropsychiatric symptoms, showing a time-dependent fellow-up [9].

In this study, we reported two pedigrees of X-ALD patients firstly diagnosed as Addison's disease for decades, and gradually exhibited variable clinical neuropsychiatric manifestations in their late twenty's, and showed rapid progression and poor prognosis. In addition, we identified two novel mutations of *ABCD1* gene, c.874_876delGAG (p.Glu292del) and c.96_97delCT (p.Tyr33Profs*161), in the two pedigrees of X-ALD. Further study of genotype-phenotype correlation analysis is needed to provide deeper insights into X-ALD.

Methods and Materials:

Patients' recruitment

The diagnosis of X-ALD depends on clinical symptoms, biochemical parameters, neurological imaging and genetic analysis. This study was approved by the ethics committee of the Affiliated Hospital of Qingdao University.

Genetic analysis and Prediction of pathogenicity

Genomic DNA was extracted from peripheral blood sample using the QIAamp Blood DNA Mini Kit (QIAGEN, USA) according to the manufacturer's protocol. After amplification using 2X polymerase chain reactions (PCR) MasterMix polymerase (Tiangen, China) by ABI9700 PCR (Life technology, USA), the products were captured and purified with Panel probe (illumine Inc. USA), then directly sequenced on the ABI 3500 automated DNA sequencer (Life technology, USA). Mutations of *ABCD1* were detected using next generation sequencing (NGS) and subsequently confirmed using

Sanger sequencing. The databases including ESP6500, 1000 Genomes and dbSNP and Uniprot were used for elimination of polymorphisms. Prediction of mutations were performed with PROVEAN (Protein Variation Effect Analyzer, http://provean.jcvi.org/) and Mutation Taster (http://www.mutationtaster.org) to predict the possible impacts of amino acid substitution on the structure and function. The PROVEAN score represented the normalized probability that the amino acid change is deleterious. The cutoff of PROVEAN score was -2.5. The PROVEAN score <-2.5 was predicted to be deleterious. In addition, the following forty genes associated with congenital adrenal hypoplasia or glucocorticoid deficiency were investigated for case 2, including *MC2R, MRAP, AAAS, AIP, PRKAR1A, PDE11A, PDE8B, PRKACA, MEN1, RET, GNAS, ARMC5, NF1, NR3C1, LMNA, SOX3, PCSK1, PROP1, LHX3, HSD11B1, CYP21A2, CYP17A1, CYP11B1, HSD3B2, STAR, MCM4, TBX9, POR, POMC, AIRE, NR5A1* and so on.

Three-dimensional structure prediction of protein and the potential dysfunction

We identified the alteration of the *ABCD1* encoded ALD protein structure and potential dysfunction using the SWISS-MODEL work-space (http://swiss-model.expasy.org).

Case presentations and biochemistry results:

Case 1: A 27-year old male was presented in hospital with weakness, fatigue, poor academic performance, exhibiting skin and mucous membrane pigmentation, especially in areolae, joints and axilla for two decades. The cortisol at 8am was reduced 3.58 nmol/L (range 171-536 nmol/L), and adrenocorticotropic hormone (ACTH) was increased 499.30 pg/mL (range 7.2-63.6 pg/mL). After diagnosed as primary adrenal insufficiency (Addison's disease), he was administrated with glucocorticoid replacement (hydrocortisone 20mg per day) for one year, and the skin pigmentation and

weakness were improved. In recently half a year, the patient acted mild cognitive impairment, slurred speech, ataxia, and rapidly progressed as walking instability, incontinence, and paroxysmal blurring vision. Physical examination detected scanty scalp hair, V$^-$grade muscle strength, hypomyotonia, tendon hyperreflexia (+++), positive Barbinski's sign and Gordon's sign. The light and deep reflex were normal. The heel-to-knee test, alternating movement test, and Romberg's test were negative. The respiratory, cardiovascular, and abdominal examinations were unremarkable. Brain MRI showed symmetrical distribution of hyperintensity long T1 and long T2 signal in of bilateral splenium of corpus callosum, and widened fissures and sulci (Figure 1A). Serum VLCFA levels were significantly increased in the proband, and also elevated in the proband's mother (Table 1, Figure 2A II-10). Until now, his mother did not show manifestations of cerebral disorders, myeleterosis, or adrenal insufficiency. His maternal grandmother (Figure 2A, I-2) died at her 55-year-old due to encephalopathy. However, family members were unable to provide valid information, and no blood samples could be obtained for testing. Other siblings of his mother did not showed neuropsychiatric manifestations. Genetic analysis revealed variants c.874_876delGAG of *ABCD1* gene in exon 1. According to the clinical manifestations and genetic analysis, a pedigree of X-ALD was constructed. The patient was administrated with hydrocortisone 20mg per day, mecobalamine, and compound vitamin B. During the fellow-up, the proband showed rapidly progressed neuropsychiatric manifestations including blurred vision, instability of gait, and incontinence within six months.

Case 2: A 31-year-old male was presented with adrenal crisis, exhibiting severe fatigue, anorexia, nausea and vomiting five years ago. The patient had significantly generalized skin pigmentation especially in the gingiva, areolae, and creases of the hands. Laboratory examination revealed severe hyponatremia (serum sodium 108~125 mmol/L, normal range 145-155 mmol/L), markedly decreased cortisol level (24.83-45.40-35.13 nmol/L) and increased ACTH level (1522-1071-1750 pg/mL) at 8am-4pm-

0am, respectively. The adrenal CT scan showed bilateral adrenal atrophy. At first, the patient was diagnosed as adrenal insufficiency, without exhibiting central or peripheral neuropsychiatric symptoms. After therapy with hydrocortisone (20mg at 8am,-10mg at 4pm), symptoms of fatigues and vomiting were improved. The patient showed incorrigible severe hyponatremia, only corrected into normal by treated combination with fludrocortisone (0.05mg per day). The serum sodium levels were maintained in normal range for five years. However, in recent eight months, the patient showed significantly aggravated walking instability, cognitive dysfunction, intellectual disabilities, emotional disturbance, memory loss and incontinence. Neurological physical examination revealed positive Barbinski's sign and Hoffman's sign, hyperactive tendon reflexes, and abnormal heel-to-knee-to-toe test. He spoke unclearly with dysarthria, while the vision and hearing were normal. Brain MRI revealed long T1 and long T2 signals in white matter of bilateral frontal lobes, and around the anterior of lateral ventricle, and corpus callosum (Figure 1B). The patient was sickness and showed poor learning ability during childhood. His brother (Figure 2C, III-3) also showed sickness in childhood, and died at his eight-year-old. His maternal uncle (Figure 2C, II-5) was weak and fatigued in his youth, and showed disturbance of consciousness from 25-year-old, and died at 28-year old. None of the other family members exhibited neuropsychiatric symptoms and signs of X-ALD. To confirm the diagnosis, genetic sequencing of the proband detected deletion mutation c.96_97delCT in *ABCD1* gene. Thus, the pedigree of X-ALD was suspected. During the fellow-up, the patient was treated with hydrocortisone and fludrocortisone, and serum sodium level was maintained in normal range. However, he showed rapidly progressed symptoms of incontinence and consciousness dysfunction.

Genetic analysis and Prediction of pathogenicity

Genetic sequencing of *ABCD1* gene was performed on the probands of two cases and the familial relatives of the proband in case one. In Case one, genetic sequencing

detected heterozygous mutation of c.874_876delGAG (p.Glu292del) in *ABCD1* gene Exon 1 (Figure 2A and 2B), leading to the deletion of translation product glutamate and accumulation of VLCFAs (Table 1). Sanger sequencing of *ABCD1* gene was performed on his mother, and confirmed the hemizygous point mutation for the same variant. In case two, gene sequencing showed a heterozygous deletion mutation c.96_97delCT in *ABCD1* gene (Figure 2C and 2D), leading to the frameshift and premature transcription termination of amino acid p.Tyr33Profs*161. His immediate family members (parents and his sister) refused to perform genetic sequencing.

The frequency of those variants was examined in the reference database. Both novel mutations were not detected in the database including ESP6500, 1000 Genomic, or dbSNP (Table 2). The potential pathogenicity of both mutations were investigated using prediction bioinformatics, suggesting both mutations were possible to be disease causing (Table 2).

Three-dimensional structure prediction of protein and the potential dysfunction

The *ABCD1* gene encoded ALD protein contains transmembrane domains and three segments on the peroxisome side. We identified the predicted alteration of the ALD protein structure and its potential dysfunction induced by mutations c.874_876delGAG (p.Glu292del) and c.96_97delCT (p.Tyr33Profs*161) (Figure 3). The amino acid changes in ALD protein due to those deletion mutations of *ABCD1* gene might affect function, leading to the impaired degradation of VLCFAs and inflammation environment.

Discussion:

X-ALD is a demyelinating and neurodegenerative disorder, due to the loss-of-function mutation of *ABCD1* gene. *ABCD1* gene codes the peroxisome transporter protein ALDP, which plays an important role in regulating peroxisome oxidation and

degradation of VLCFAs [5]. The dysfunction of ALDP, a fatty acid transporter, results in the impaired VLCFA oxidation and excessive VLCFAs accumulation in tissues [5, 10]. Excessive VLCFAs in cytoplasm leads to mitochondrion dysfunction and ER stress, finally caused cell death. In addition, accumulated VLCFAs arouses inflammation, results in demyelinating and neurodegeneration.

In this study, we reported two Chinese X-ALD pedigrees. Both of the probands were diagnosed as adrenal insufficiency at beginning without neuropsychiatric symptoms for decades, but developed into neurological disorders at their late 20's, and showed rapidly progression and poor prognosis. Two novel mutations in exon 1 of *ABCD1* gene were detected and suggested to be associated with X-ALD.

According to the previous reports, the majority is cerebral ALD among those seven types of ALD [11]. CCALD is early onset with a feature of rapidly progressed neuro-inflammatory of cerebral demyelination. Neurological symptoms of AMN commonly occur from the ages of 20s, with manifests as a chronic progressive paraparesis, accompanied with sensory and motor disturbances [12]. Due to the variable clinical manifestations, some are difficult to make the diagnose at early stage [13]. Adrenocortical insufficiency was the reported as the first clinical manifestation of 38% X-ALD, greater than the group of spinal cord 25% and cerebral disorders 14.5% [9]. To be noticed, some of those male patients diagnosed as adrenal insufficiency may develop with cerebral or myeloneuropathy ultimately, and show a rapid progressive neurological symptoms and poor prognosis. On the contrast, female ALD patients rarely show adrenocortical insufficiency or cerebral ALD. Most of female patients suffer from lately-onset myelopathy [10, 14]. We reported two cases both were firstly diagnosed as Addison's disease, and then the therapy focused on the adrenocortical replacement. However, after the neurological symptoms and signs become apparent, both of the cases were rapidly progressed. The therapy including fatty acid restriction or neurotrophic treatment did not show therapeutic effect. Therefore, at diagnosis of congenital adrenal insufficiency, the probability of ALD

should be fully considered especially for male patients [8, 15]. To make the early diagnosis, family history, MRI scan of brain or spinal cord, VLCFAs measurement, and genetic screening may be helpful for those patients with subtle early neurological manifestations.

We identified two novel point mutations in exon 1 of *ABCD1* gene in the pedigree of first case (c.874_876delGAG, p.Glu292del) and in second case (c.96_97delCT, p.Tyr33Profs*161). The genetic sequencing demonstrated nucleotides deletion, resulting in those frameshift mutations. Based on the clinical symptoms and positive neurologic signs, family history, imaging, biochemical characteristics and the results of pathogenicity prediction using bioinformatics tools, we suggest those mutations as pathogenic variants. However, the biopsy of brain, spinal cord, and adrenal gland were not done. Based on the elevated plasma VLCFAs, the deleterious change of ALDP impaired the cellular beta-oxidation of fatty acid [16].

There are more than thousand mutations have been reported in ALD database (http://www.x-ald.nl/). Among them, 67.57% of them are pathogenic mutations, 9.65% are synonymous, 2.16% are benign, 18.65% are VUS (variant of undetermined significance), and 1.87% are status unknown mutations detected during the screening of newborns (Figure 4A). More than 77.5% are point mutations, other types including 12.93% deletion, 1.97% del-insertion, 4.22% insertion, and 3.37% duplication (Figure 4B). Most of the mutations locate in the exon (92.94%), others in the intron (6.18%), 5'UTR (0.61%) and 3'UTR (0.26%), respectively [4]. In the database, nearly half of the mutations were reported anchoring in exon 1 (Figure 4C), which encodes the transmembrane domain (TMD) of the protein, contains ligand-specific binding sites and plays an important role in the localization of ALDP [17]. Another clustering of mutations occurs in exon 6, which encode the ATP binding domain of ALDP [18]. ALDP locates in the peroxisome membrane, to transport VLCFAs from cytosol into the peroxisome for degradation. Pathogenic *ABCD1* mutations may induce defective

the stability of ALDP transmembrane structure region and ATPase activity, to affect the localization of VLCFAs to the peroxisome correctly. As a result, VLCFAs cannot be transported into peroxisomes for β-oxidation, then accumulate mainly in the white matter, neuron axons of central nervous system, and adrenal cortex. Excess VLCFAs accumulation leads to destructive progression by triggering oxidative stress and mitochondrial dysfunction, chronic inflammation, lipid-induced neuron apoptosis, and degenerative changes in the nervous system, and finally leading to neurodegeneration and adrenal insufficiency [3, 19]. Though some variations may not affect protein translation or function, they still represent polymorphisms. The genotype-phenotype correlationship have not been identified clearly [20-21]. To further investigated the relationship between genotype and phenotype, we searched the database of X-ALD Mutation (https://adrenoleukodystrophy.info/) and Uniprot, and summarized the reported mutation sites and clinical manifestations including childhood and adolescent cerebral ALD, adult ALD, AMN, adrenal insufficiency-only, asymptomatic or pre-symptomatic types, and complicated ALD which cannot be classified. (Figure 5). X-ALD has high genetic heterogeneity. The clinical manifestations are highly variable even in the pedigree carrying same variant. The main phenotypes differ from different periods of the disease. The same variants can exhibit different clinical features. Thus, further effects may be needed to clarify the diversity in clinical manifestations and the correlationship of genotype-phenotype.

Therapeutic strategies include adrenocortical replacement, some of the adrenal insufficiency patients may require both glucocorticoid and mineralocorticoid to maintain water electrolyte balance. For severe adrenal insufficiency, or even adrenal crisis, timely initiation of steroid replacement is essential. Nutrition intervention, for instance, low-fat diet and Lorenzo's Oil may reduce VLCFAs level [22]. In this way, the risk of development with cerebral disorders may be reduced. However, they lack the evidence to improve the symptoms and progression. Another efficiency approach is hematopoietic stem cell transplantation (HSCT), especially in the early stage of the

disease [23]. Transplantation with autologous bone marrow transfected in vitro with *ABCD1*gene modification has been performed [24-25]. Bone marrow transplant has been reported in CCALD therapy, most of the patients achieved improved prognosis. However, the pre-existed severe symptoms may influence the outcome after transplantation [26]. In animal experiments, Abcd1^{-} mice show reduction of the mitochondrial biogenesis driven by PGC-1α/PPARγ pathway [27]. Oxidative stress is involved in the physiopathological mechanism of neurodegenerative cascade and mitochondrial depletion [28]. Pioglitazone, a PPARγ/PGC-1α axis activator may modulate the anti-inflammatory and anti-oxidant response. Recently, pioglitazone is under phase II Clinical trials for adrenoleukodystrophy patients, reveals a potential for clinical translation [29]. Therapy options are limited, in addition, some therapeutic approaches relay on the early diagnosis and intervention. Therefore, gene sequencing analysis may be an important tool for newborn screening and genetic counseling.

Conclusions:

In this study, we reported two novel mutations of *ABCD1* gene in men with X-ALD. These two cases were both considered as adrenal insufficiency at first, and finally diagnosed with X-ALD when neurological manifestations occurred after several years. In these two pedigrees, we identified two novel mutations of *ABCD1* gene, c.874_876delGAG (p.Glu292del) and c.96_97delCT (p.Tyr33Profs*161). Screening test for ALD, such as VLCFA concentration and brain MRI scan, should be considered in the early-onset Addison's disease patients with family history to screen X-ALD early.

Figure legends

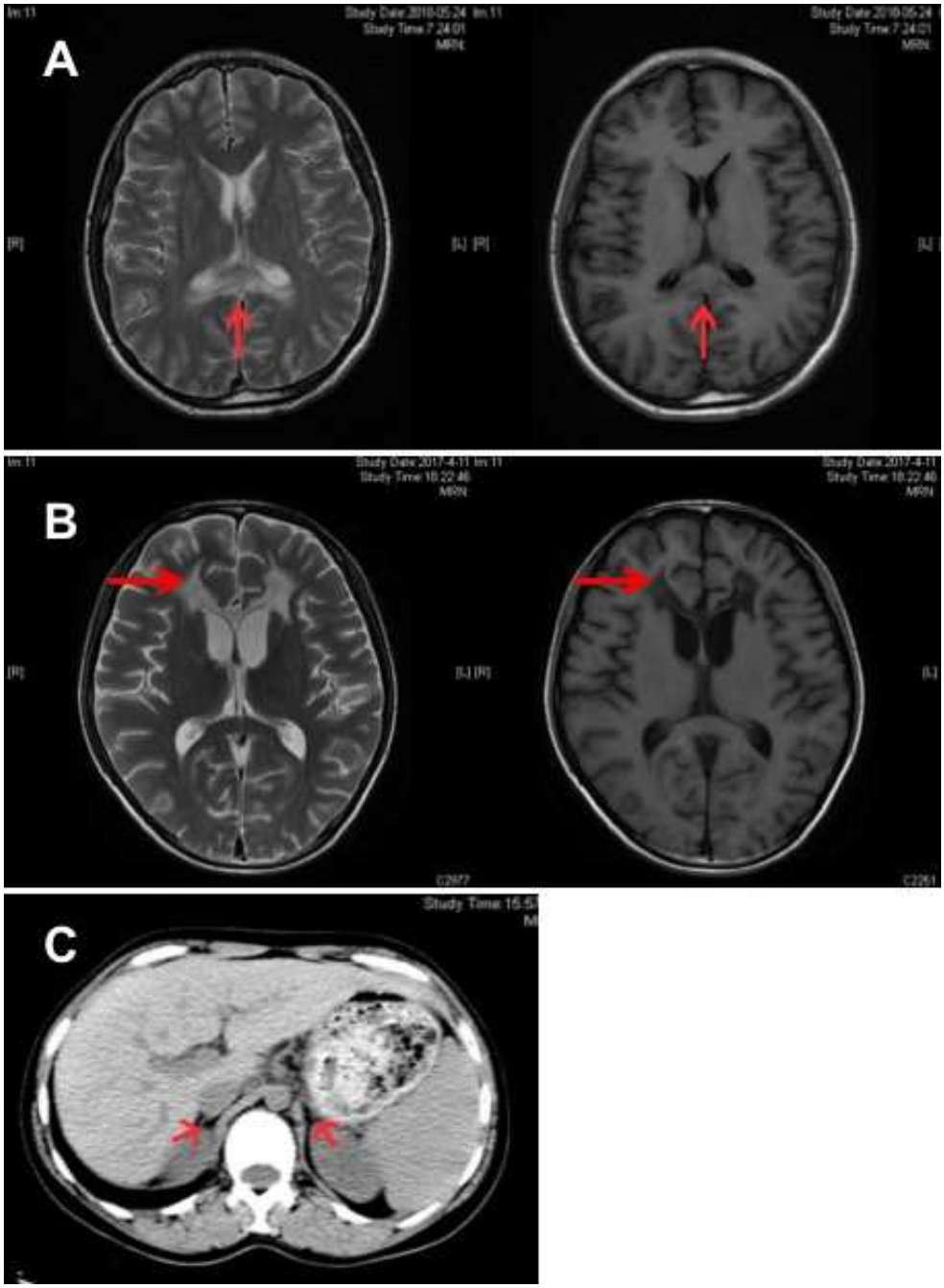


Figure 1. Brain magnetic resonance imaging (MRI) scan of patients. A. Symmetric bands with high T2 low T1 abnormal signal in bilateral genu of the corpus callosum detected in case one. **B.** MRI scan of case two revealed long T1 and long T2 signals in white matter of bilateral frontal lobes, and around the anterior of lateral ventricle, and splenium of the corpus callosum. **C.** Computed tomography (CT) scan of the adrenal gland of case one, to show bilateral adrenal atrophy. Red narrow to show lesions.

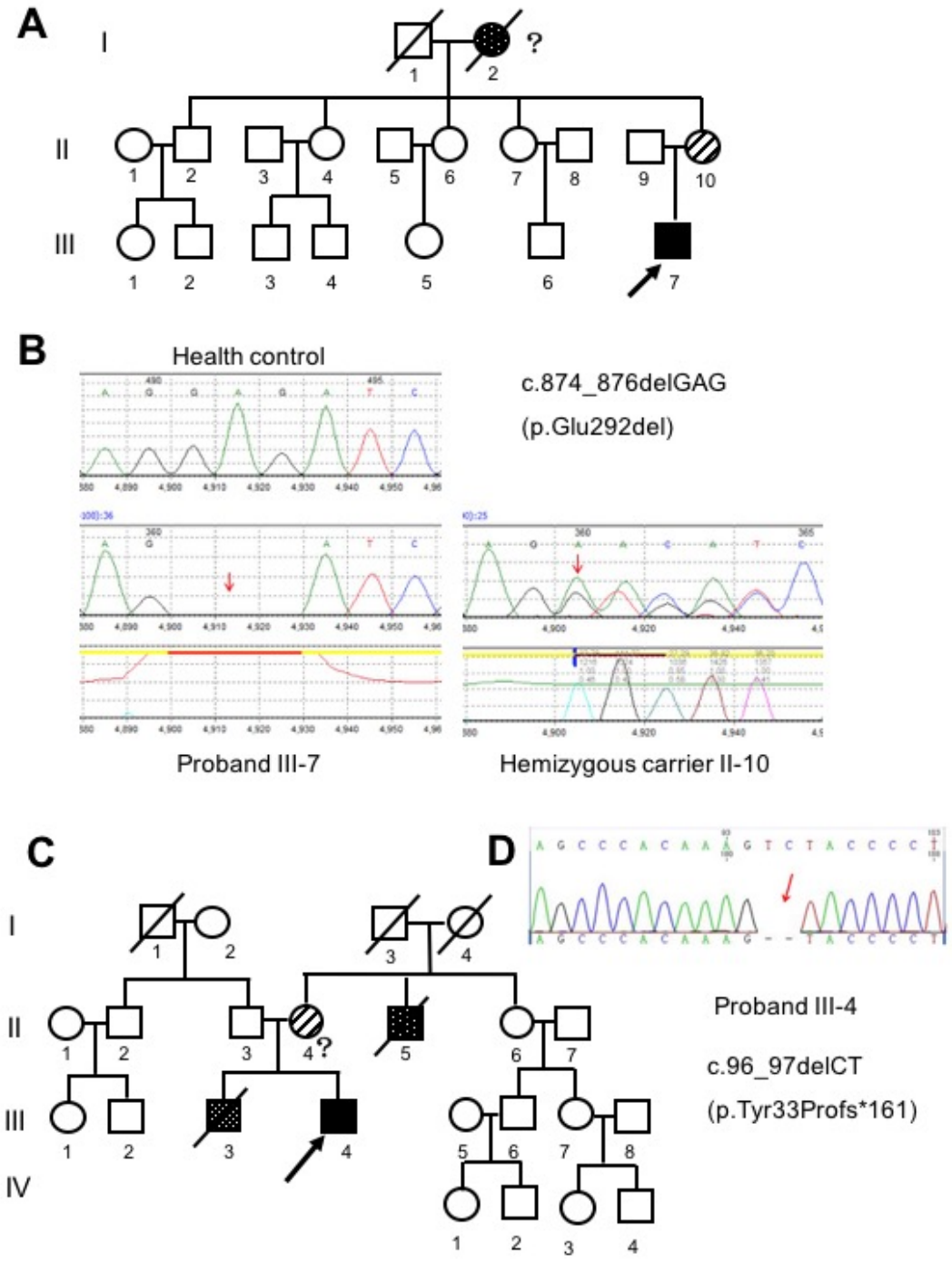


Figure 2. Pedigree diagrams and genetic analysis of two cases. A and C. Pedigree diagrams of the case one and case two with X-linked ALD, respectively. Empty square and circle to show health male and female. Dark square represents patients with X-ALD. Diagonal stripes to show hemizygous carrier. Arrow represents proband. **B.** Genetic analysis revealed novel mutation of c.874_876delGAG (p.Glu292del) of

ABCD1 gene in patient one (proband III-7) and his mother (II-10). **D.** Novel mutation c.96_97delCT (p.Tyr33Profs*161) was detected in patient two (Proband III-4).

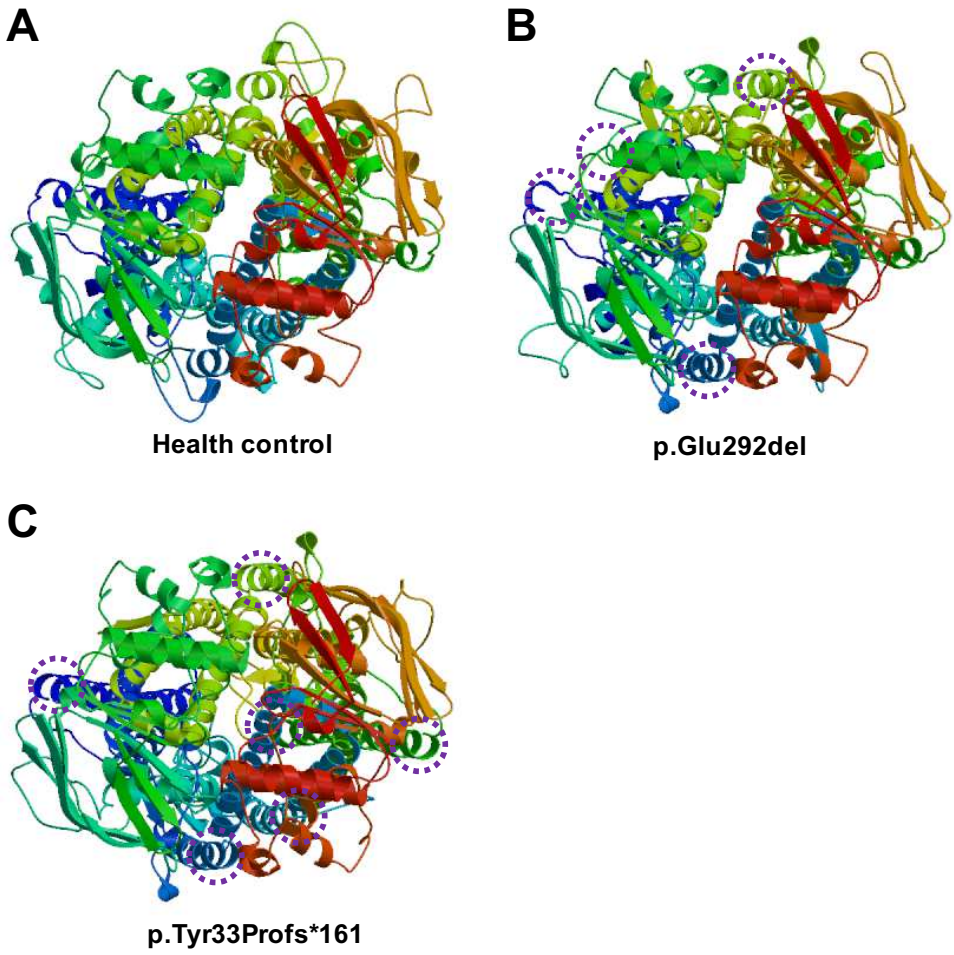


Figure 3. Three-dimensional structure prediction of adrenoleukodystrophy (ALD) protein. A. The modeled structure of health control. The visible alteration of ALD protein structure induced by mutations **B.** c.874_876delGAG (p.Glu292del) and **C.** c.96_97delCT (p.Tyr33Profs*161) were indicated in circles. It indicated that those mutations might lead to alteration of the protein structure and potential dysfunction.

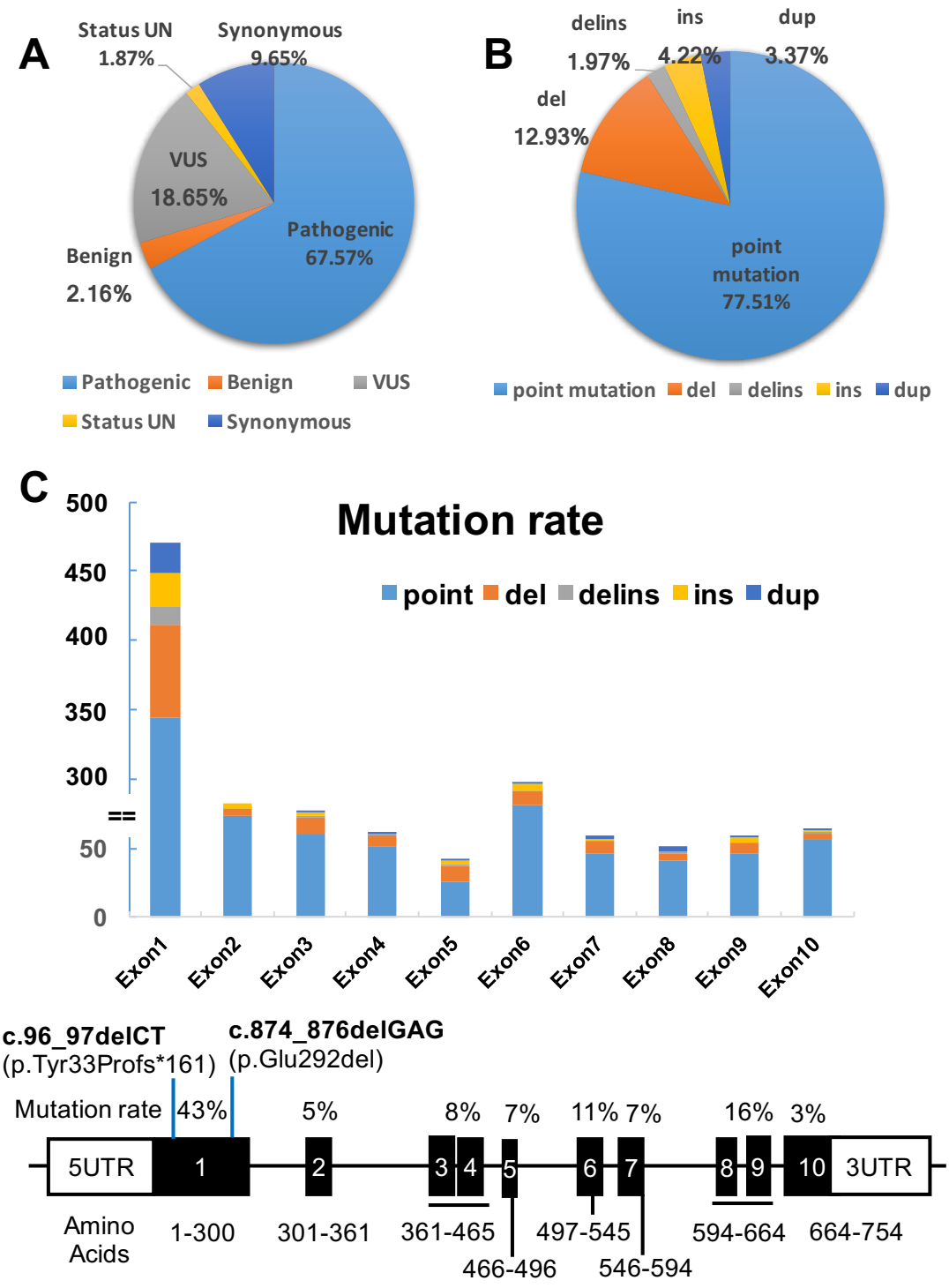
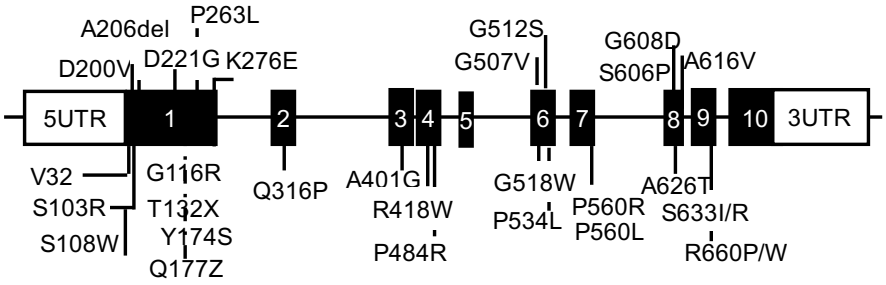


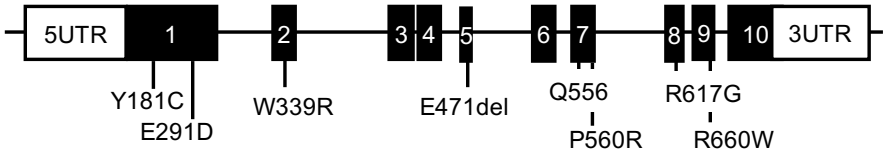
Figure 4. *ABCD1* gene reported from ALD mutation database. A. Types of variants of *ABCD1* gene, including pathogenic, synonymous, benign, variants of undetermined significance (VUS) and screening of newborns state of unknown (UN). **B.** The content of *ABCD1*mutations, including points mutations, deletion, del-insertion, insertion, and duplication. (http://www.x-ald.nl/). **C.** The distribution of

mutations, including point mutation, deletion, del-insertion, insertion, and duplication. Most of the mutations harboring in the Exon 1. The lower panel shows the mutation rate in each exon. Bold to show the location of two novel mutations indicated in this study.

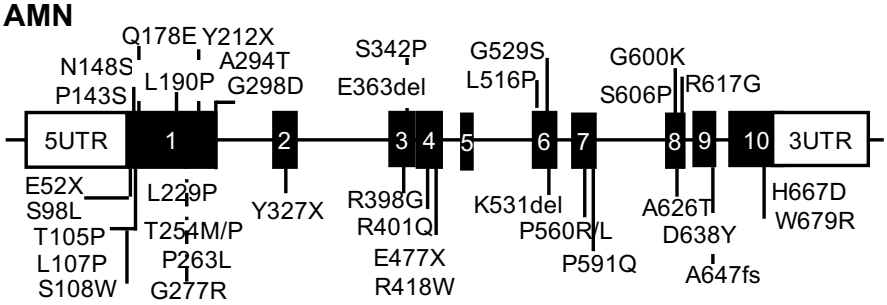
CCALD and adoles ALD



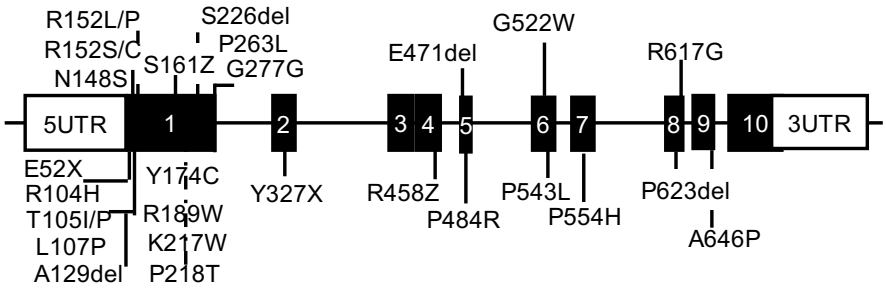
Adult ALD



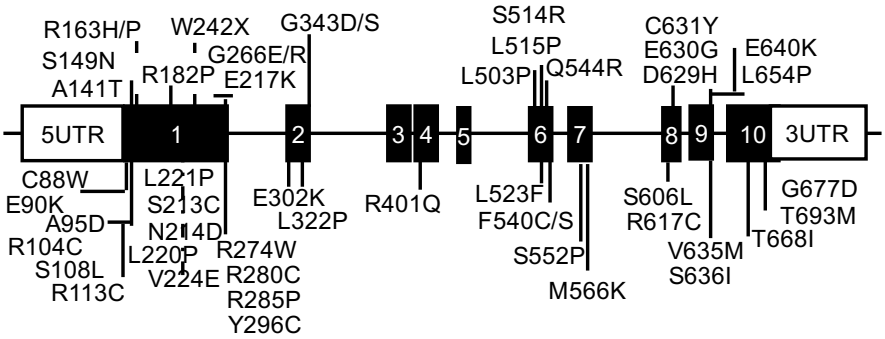
AMN



AIO



ALD



Asymptomatic or pre-Asmp

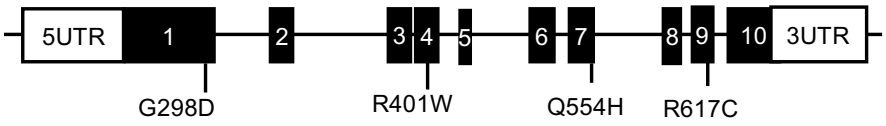


Figure 5. Summary of the reported mutation sites and clinical manifestations from the database of Uniprot (https://www.uniprot.org/uniprot/) and X-ALD Mutation (https://adrenoleukodystrophy.info/). The clinical types include childhood and adolescent cerebral ALD (CCALD and adoles ALD), adult ALD, AMN (adrenomyeloneuropathy), adrenal insufficiency-only (AIO), complicated ALD which cannot be classified, and asymptomatic or pre-symptomatic types.

Table 1. Clinical characteristics and genetic analysis results of the patients.

No.	Sex	Family history	C24:C22	C26:C22	Cortisol (nmol/L)	ACTH (pg/mL)	Sodium (mmol/L)	Potassium (mmol/L)	Corticoid dosage	Mutation	Exon
Case 1	M	+	1.75	0.089	3.58	499.3	128	5.1	Hydrocortisone 20mg/d	c.874_876delGAG	1
Case 1's mother	F	+?	1.06	0.033	ND	ND	138	4.8	None	c.874_876delGAG	1
Case 2	M	-	ND	ND	45.4	1071	125	5.3	Hydrocortisone 30mg/d, fludrocortisone	c.96_97delCT	1
Reference range			< 1.39	< 0.023	171-536	7.2-63.6	135-145	3.5-5.5			

Table 2. Mutations of *ABCD1* gene in the pedigrees of X-ALD.

No.	Nucleotide mutations	Amino acid variants	Exon	Variation type	Inheritance	ESP6500	1000G	dbSNP	PROVEAN score	Mutation Taster
Case 1	c.874_876delGAG	p.Glu292del	1	deletion	Familial	ND	ND	ND	-13.987 (Deleterious)	Disease causing
Case 2	c.96_97delCT	p.Tyr33Profs*161	1	deletion	Familial	ND	ND	ND	-6.7 (Deleterious)	Disease causing

ND, not detected.

PROVEAN score (cutoff= -2.5)

Declarations:

Ethic approval and consent to participate:

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Affiliated Hospital of Qingdao University with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication:

The clinical data, images and individual details were obtained from the probands and their family members. All the participants gave their written consent for publication.

Availability of data and materials:

All data analyzed during this study are included in this published article.

Competing interests:

All authors declare that they have no competing of interest.

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Authors' Contributions:

Bingzi Dong and Wenshan Lv carried out the molecular genetic studies, the design of this study, and drafted the manuscript. Lili Xu participated in the sequence alignment and coordination of this study. Yuhang Zhao, Xiaofang Sun and Bingfei Cheng provided helpful suggestion. Yangang Wang conceived the study, and take responsibility of this study. All authors read and approved the final manuscript.

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Abbreviations:

X-ALD: X-linked adrenoleukodystrophy

ABCD1: ATP binding cassette subfamily D member 1;

ALDP: adrenoleukodystrophy protein;

VLCFAs: Very long chain fatty acids;

AMN: Adrenomyeloneuropathy;

ACTH: Adrenocorticotropic hormone;

MRI: Magnetic Resonance Imaging;

ER: endoplasmic reticulum;

ROS: reactive oxygen species;

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Figures

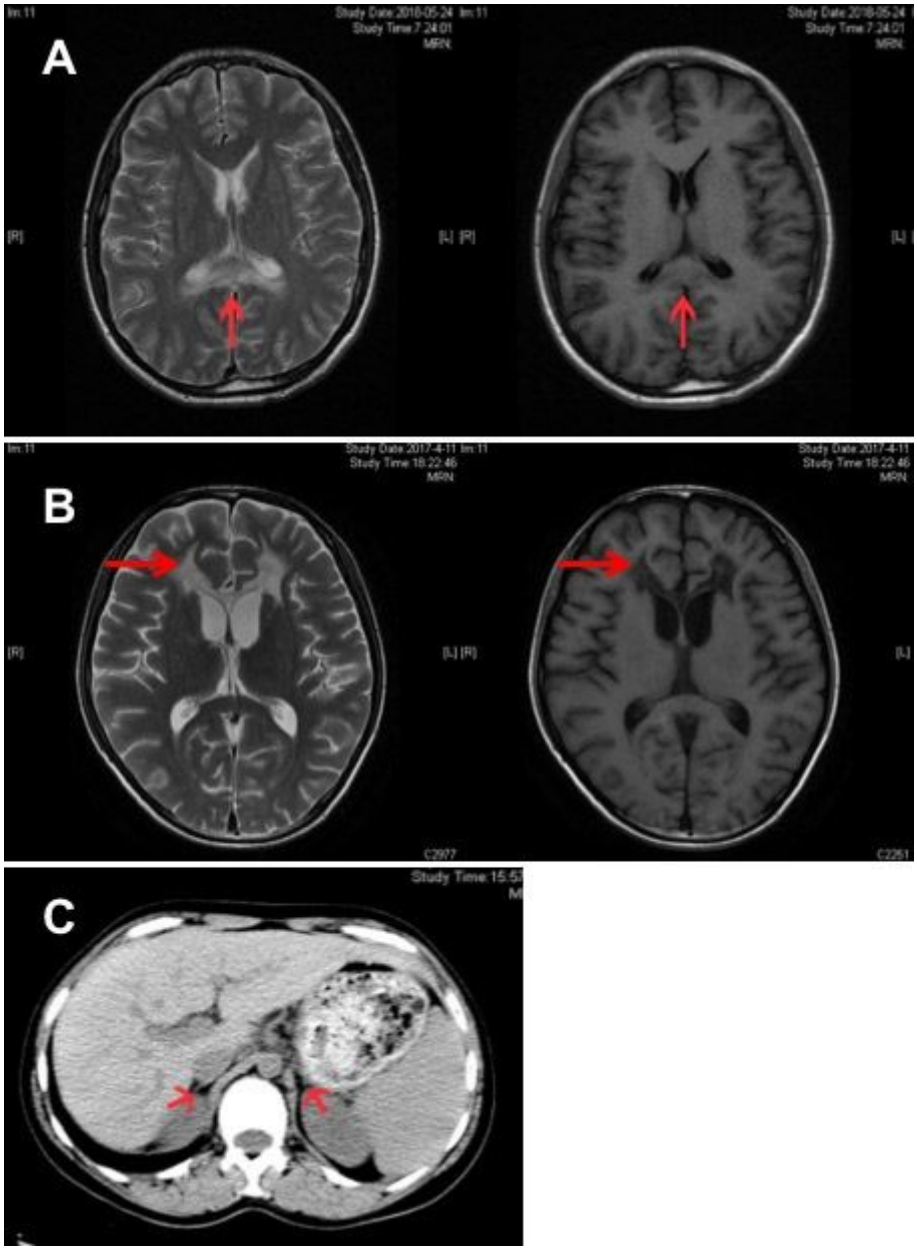

Figure 1

Brain magnetic resonance imaging (MRI) scan of patients. A. Symmetric bands with high T2 low T1 abnormal signal in bilateral genu of the corpus callosum detected in case one. B. MRI scan of case two revealed long T1 and long T2 signals in white matter of bilateral frontal lobes, and around the anterior of lateral ventricle, and splenium of the corpus callosum. C. Computed tomography (CT) scan of the adrenal gland of case one, to show bilateral adrenal atrophy. Red narrow to show lesions.

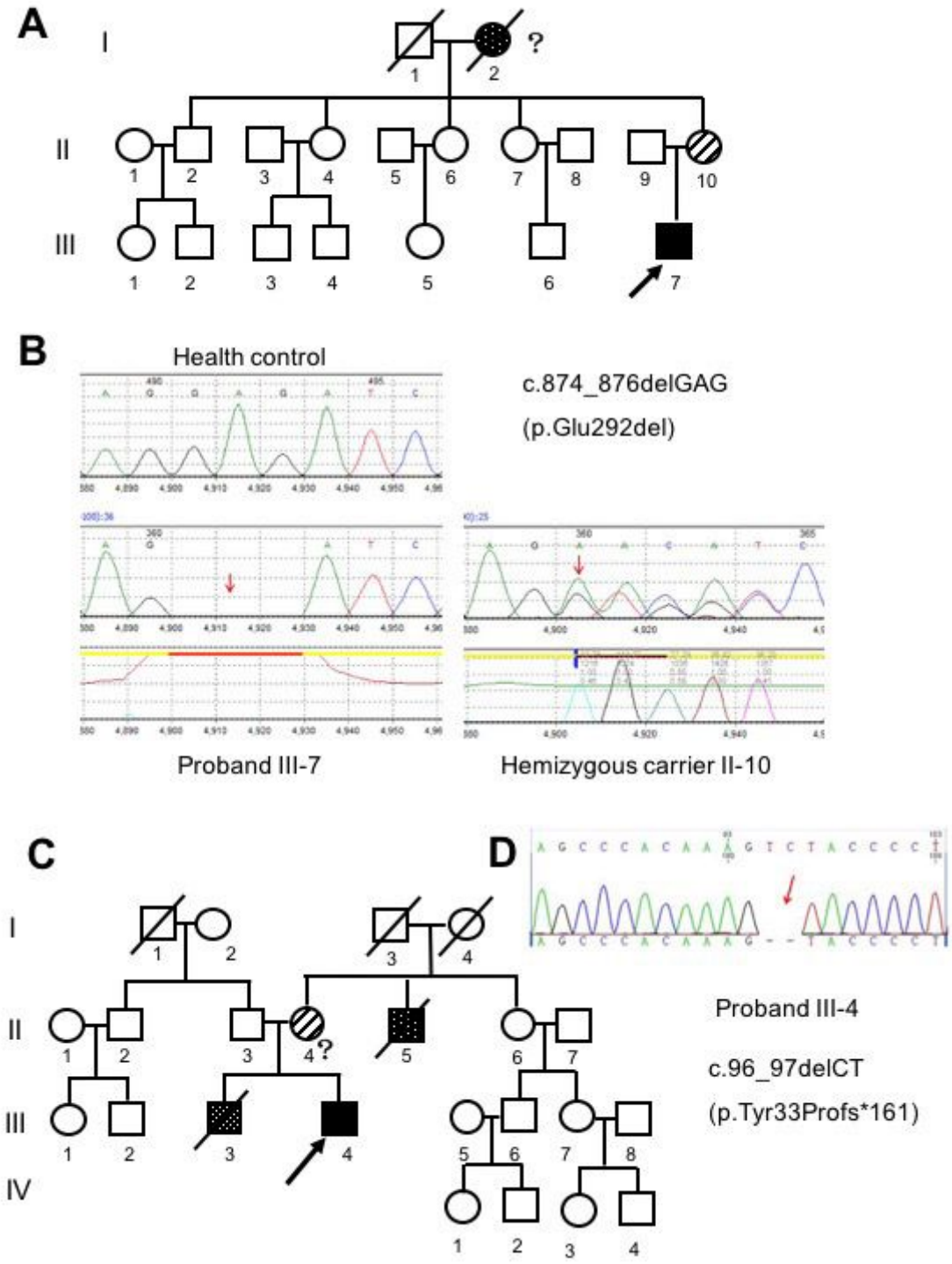

Figure 2

Pedigree diagrams and genetic analysis of two cases. A and C. Pedigree diagrams of the case one and case two with X-linked ALD, respectively. Empty square and circle to show health male and female. Dark square represents patients with X-ALD. Diagonal stripes to show hemizygous carrier. Arrow represents proband. B. Genetic analysis revealed novel mutation of c.874_876delGAG (p.Glu292del) of ABCD1 gene in patient one (proband III-7) and his mother (II-10). D. Novel mutation c.96_97delCT (p.Tyr33Profs*161) was detected in patient two (Proband III-4).

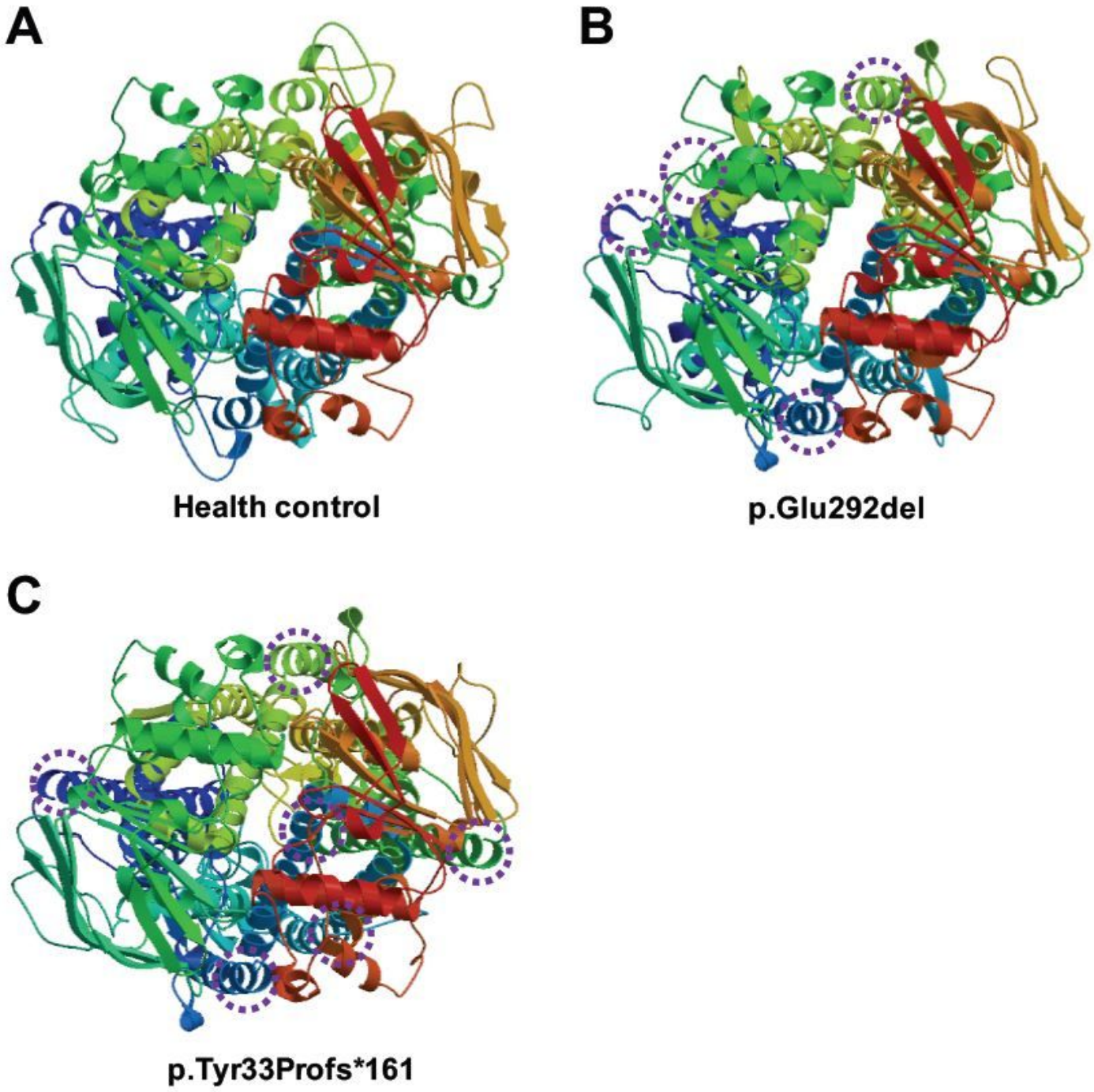

Figure 3

Three-dimensional structure prediction of adrenoleukodystrophy (ALD) protein. A. The modeled structure of health control. The visible alteration of ALD protein structure induced by mutations B. c.874_876delGAG (p.Glu292del) and C. c.96_97delCT (p.Tyr33Profs*161) were indicated in circles. It indicated that those mutations might lead to alteration of the protein structure and potential dysfunction.

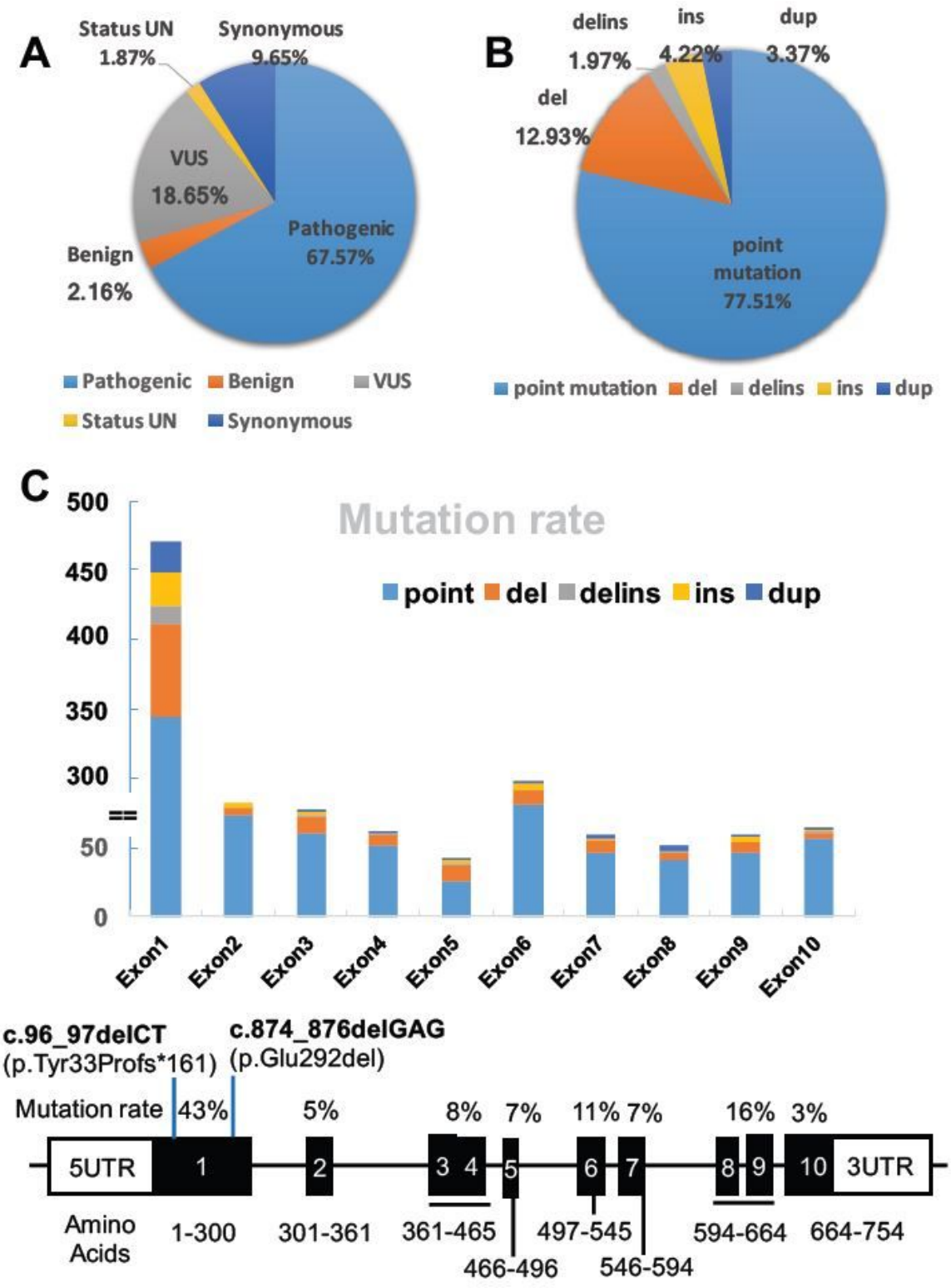

Figure 4

ABCD1 gene reported from ALD mutation database. A. Types of variants of ABCD1 gene, including pathogenic, synonymous, benign, variants of undetermined significance (VUS) and screening of newborns state of unknown (UN). B. The content of ABCD1mutations, including points mutations, deletion, delinsertion, insertion, and duplication. (http://www.x-ald.nl/). C. The distribution of mutations, including point mutation, deletion, del-insertion, insertion, and duplication. Most of the mutations

harboring in the Exon 1. The lower panel shows the mutation rate in each exon. Bold to show the location of two novel mutations indicated in this study.

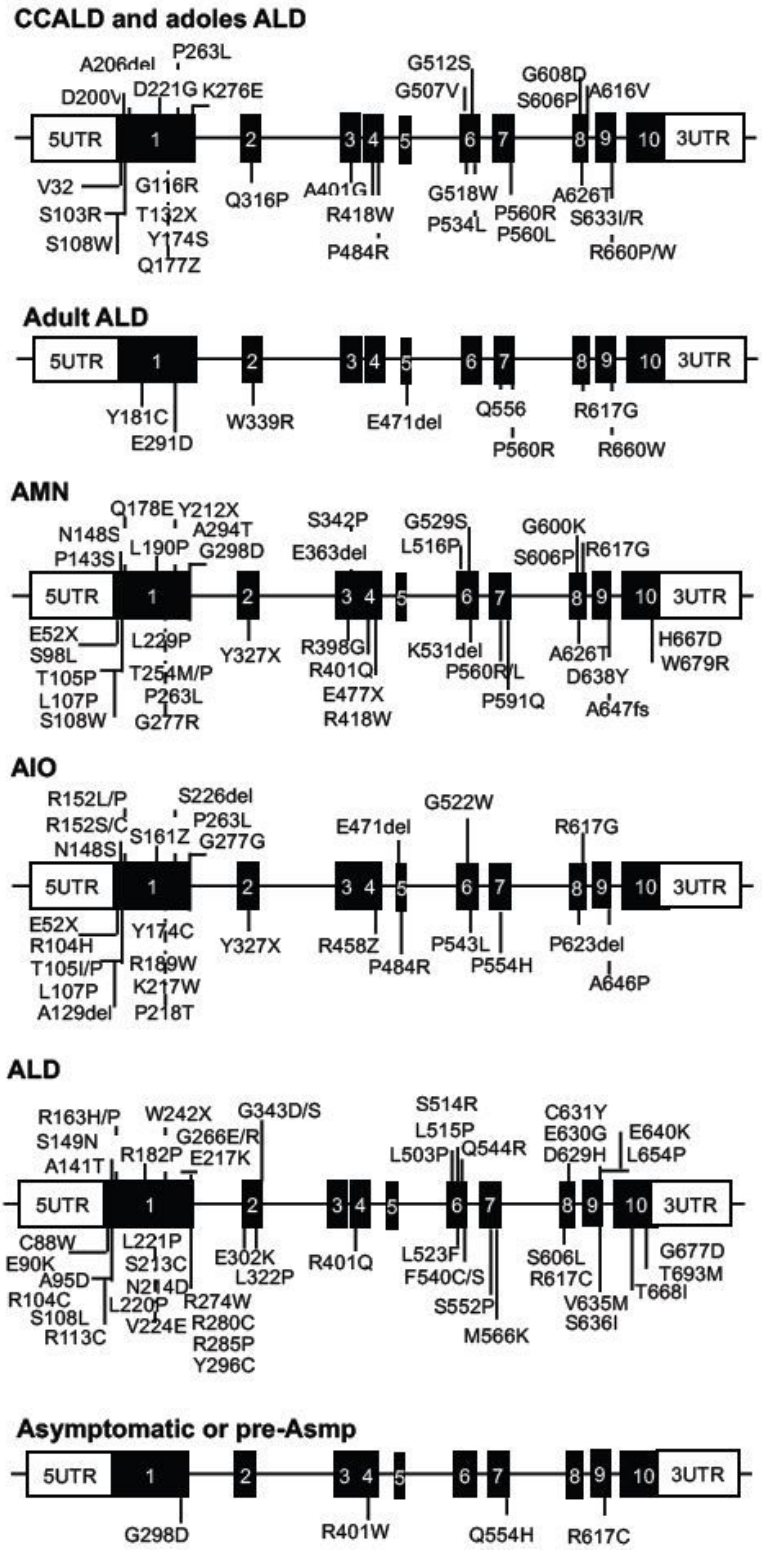

Figure 5

Summary of the reported mutation sites and clinical manifestations from the database of Uniprot (https://www.uniprot.org/uniprot/) and X-ALD Mutation (https://adrenoleukodystrophy.info/). The clinical types include childhood and adolescent cerebral ALD (CCALD and adoles ALD), adult ALD, AMN

(adrenomyeloneuropathy), adrenal insufficiency-only (AIO), complicated ALD which cannot be classified, and asymptomatic or pre-symptomatic types.