Original article

Eight novel mutations in the *ABCD1* gene and clinical characteristics of 25 Chinese patients with X-linked adrenoleukodystrophy

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Background: **X-linked adrenoleukodystrophy (X-ALD) is a fatal neurodegenerative disease caused by mutations in the adenosine triphosphate-binding cassette D1 (*ABCD1*) gene. This study aimed to retrospectively investigate the clinical characteristics of 25 patients with X-ALD including members of large pedigrees, to analyze *ABCD1* gene mutations, the effect of gene novel variants on ALD protein (ALDP) structure and function, and to expand gene mutation spectrum of Chinese patients.**

Methods: **Twenty-five male patients diagnosed with X-ALD were enrolled in this study. The clinical characteristics of the patients were retrospectively summarized by reviewing medical records or telephone consultation. *ABCD1* gene mutations were analyzed. The pathogenicity of novel missense variants was analyzed using cobalt constraint-based multiple protein alignment tool, polymorphism phenotyping, sorting intolerant from tolerant, Align-Grantham variation and Grantham deviation, and Swiss-Program Database Viewer 4.04 software, respectively.**

Results: **Childhood cerebral form ALD (CCALD) is the most common phenotype (64%) in the 25 patients with X-ALD. The progressive deterioration of neurological and cognitive functions is the main clinical feature. The demyelination of the brain white matter and elevated plasma very long chain fatty acids (VLCFAs) were found in all patients. Different phenotypes were also presented within family members of the patients. Twenty-two different mutations including 8 novel mutations in the *ABCD1* gene were identified in the 25 patients. Of the mutations, 63.6% were missense mutations and 34.8% located in exon 1. The amino acid residues of three novel missense mutations in eight species were highly conserved, and were predicted to be "probably" damaging to ALDP function. The other five novel mutations were splice, nonsense, deletion or duplication mutations.**

Conclusions: **CCALD is the most common phenotype (64%) in our patients with X-ALD. Eight novel mutations in the *ABCD1* gene identified are disease-causing mutations. Brain magnetic resonance imaging and plasma VLCFA determination should be performed for the patients who present with progressive deterioration of neurological development.**

World J Pediatr 2015;11(4):366-373

Key words: **ABCD1 protein;**
adrenoleukodystrophy;
adrenoleukodystrophy protein;
missense variant;
mutation

Introduction

X-linked adrenoleukodystrophy (X-ALD), a fatal neurodegenerative disorder, is caused by a defect in the adenosine triphosphate-binding cassette D1 (*ABCD1*) gene, which is involved in the peroxisomal oxidation of very long chain fatty acids (VLCFAs).[1] It is the most common monogenic leukodystrophy and peroxisomal disorder with a minimum incidence of 1/17 000 males.[2] The *ABCD1* gene located within the Xq28 region that belongs to the ATP-binding cassette superfamily of transmembrane transporters and encodes the ALD protein (ALDP) which is located in the peroxisomal membrane. The mutation in the *ABCD1* gene results in dysfunction of the ALDP, which participates in the peroxisomal

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doi: 10.1007/s12519-015-0044-0

degradation of VLCFAs. The accumulation of VLCFAs primarily affects the central nervous system, adrenal cortex and testis; but the clinical presentation of X-ALD varies greatly from the rapidly progressive cerebral form ALD (CALD) to milder adrenomyeloneuropathy (AMN), pure Addison's disease or asymptomatic form.[1,3] The CALD is mainly presented with cognitive deterioration associated with neurologic deficits such as hemiplegia or quadriparesis, cerebellar ataxia, impaired central auditory discrimination, visual field defects and seizures. Finally, a vegetative state ensues within a few years leading to death at varying intervals. AMN is a slowly progressive noninflammatory axonopathy affecting sensory ascending and motor descending spinal cord tracts.[4] Female heterozygotes symptoms may not appear in their life; however, approximately half of them present with mild or moderate neurological symptoms.[5] The increase in VLCFA levels and demyelination of the brain white matter provide reliable diagnostic clues. The mutation analysis of *ABCD1* is still the best approach to confirm the diagnosis and provide the genetic counseling to the families.[6]

There are few reports about X-ALD in China;[7-9] however, early recognition, genetic diagnosis and investigation of large pedigrees are still limited in China. Identification of more novel mutations in the *ABCD1* gene can expand the gene spectrum in the Chinese population with X-ALD. Herein, we present 25 Chinese patients with X-ALD diagnosed between 2009 and 2014. This study aimed to retrospectively investigate the clinical characteristics of the patients including large pedigrees, to analyze mutations in the *ABCD1* gene and the effect of novel variants on ALDP structure and function, and to expand gene mutation spectrum of Chinese patients.

Methods

Subjects

Twenty-five male patients referred to our clinic for cognitive and motor dysfunction between 2009 and 2014. Varying changes in the white matter were shown by cranial magnetic resonance imaging (MRI), and globoid cell leukodystrophy (Krabbe disease), metachromatic leukodystrophy and other genetic metabolic diseases had been ruled out by biochemical investigation including tandem mass spectrometry and enzymatic detection in these patients. All the patients had elevated levels of plasma VLCFAs [tetracosanoic acid (C24:0), hexacosanoic acid (C26:0), C26:0/docosanoic acid (C22:0) and C24:0/C22:0] and were finally diagnosed with X-ALD.

Clinical characteristics

The clinical characteristics of the patients were retrospectively investigated by reviewing outpatient medical records or telephone consultation about birth histories, initial symptoms and age of disease onset and progression, laboratory tests, and cranial imaging. Patients with complete pedigrees were tracked and investigated to understand the genetic pattern and clinical heterogeneity in members of their families.

Mutation analysis of *ABCD1*

Genomic DNA was extracted from peripheral blood leukocytes taken from patients and their parents as well as 50 healthy individuals using the E.Z.N.A Blood DNA Kit (Yeasen Biotech, Shanghai, China) after informed consent was obtained. The coding regions of *ABCD1* including the intron-exon boundaries of 10 exons were amplified by the polymerase chain reaction, and primers have been described (http://www.x-ald.nl). Amplification products were separated and sequenced directly using forward and reverse primers on an ABI 3700 sequencer. Analyzed sequences were compared with cDNA and genomic DNA sequences in GenBank (accession number *ALDP*_NM_000033). Novel variants were confirmed after ruling out polymorphism by directly sequencing for their parents and 50 healthy individuals, and were identified after searaching gene websites (http://www.hgmd.org/ and http://www.x-ald.nl/) and reading the published articles.

Prediction of the pathogenic effect of novel missense variants

Multiple sequence alignments were performed by Cobalt Constraint-based Multiple Protein Alignment Tool for novel missense variants to investigate evolutionary conservation of amino acid residues across orthologous genes. Polymorphism phenotyping (PolyPhen), sorting intolerant from tolerant (SIFT) and Align-Grantham variation and Grantham deviation (GVGD) program were used to predict the impact of missense variants on protein function.[10,11] PolyPhen scores close to 1.000 are categorized as probably damaging. A SIFT score of less than 0.05 indicates a deleterious amino acid substitution. Class C65 represents that the substitution is most likely to interfere with function of ALDP, and class C0 suggests less likely. Human ALDP crystallographic structure and structure change which result from mutation are visualized by a Swiss-Program Database Viewer 4.04. The influence on protein was assessed through searching the website (http://www.cmbi.ru.nl/hope/home).

Results

Clinical characteristics

The 25 patients were born at term following an uneventful pregnancy from non-consanguineous Chinese couples.

The median age of disease onset was 10 years (range: 3-42 years) in all patients, 3-10 years in 16 (64%) patients with childhood CALD (CCALD), 17 years in 1 patient with adolescent CALD (AdolCALD), and 28-40 years in 7 patients with adulthood CALD (ACALD), and 1 patient with AMN. Of these patients, CALD was the most common phenotype (96%, 24/25). Initial symptoms included unsteady gait, behavioral problems (e.g., hyperactivity), memory loss, attention deficit, cognitive decline, and speech disorder. Symptoms were progressively deteriorated after several years such as walking disability, severe intellectual disability, dysphagia, impaired auditory and vision or dyspnea, eventually developed to vegetative state or paralysis. Four patients (P5, P7, P12 and P16) died at age of 9-44 years. Among them, 5 patients (P1, P9, P10, P12, and P19) also showed symptoms of adrenal insufficiency such as increased skin pigmentation in lips, gums and even the whole body. Adrenocortical insufficiency (Addison's disease) was the first manifestation in P9, P12 and P19. They began to show memory deterioration, attention deficit disorders and poor academic performance within 3-5 years later. Only one patient (P21) with AMN complained of numbness in the lower limbs when he was walking.

The positive family history was collected in detail in 4 patients (P4, P5, P12 and P16). A large pedigree of P4 is shown in Fig. 1. P4 (IV1) was the proband whose initial symptoms were changes in personality and behaviors at the age of 6 years. He would not like to play with other children, dysarthria, ataxia, and spasticity paralysis were developed at the age of 7 years. Attention deficit hyperactivity disorder was noted between 9-10 years. Subsequently he demonstrated visual disturbance, walking instability and disorientation. Brain MRI showed leukodystrophy. His heterozygous mother (III2), maternal aunt (III3) and hemizygous grandfather (II2) had high levels of VLCFAs in plasma, but all of them were asymptomatic. According to the dogma of Mendelian inheritance on X-linked disorder, we concluded that the asymptomatic I2 was heterozygous and died of a natural cause at the age of over 90 years. Symptoms at onset of the disease were leg pain in II4 and II11 with AMN, and then developed to ACALD at the age of 10-20 years. They died at the age of 70 years. Unsteady gait and cognitive dysfunction were found in II5 with ACALD at the age of 30 years. This patient presented with progressive cognitive backwards, impaired hearing, vision loss, and paraparesis and then died at the age of 40 years. The hemizygous brother of P5 was asymptomatic. He was 51 years old and had elevated levels of VLCFAs in plasma. The brother of P12 was 9 years, and his symptoms were similar to those of P12; however, his VLCFAs were not available. P16 was the proband in another family; his initial symptoms were emotional changes including depression and irritability at the age of 42 years. Then, visual impairment, gait ataxia, and paralysis were quickly developed, and he died at the age of 44 years. His hemizygous brother (42 years old) and heterozygous niece had elevated levels of VLCFAs in plasma, but they were asymptomatic.

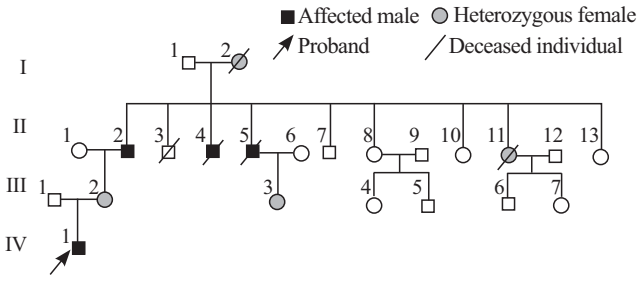

Fig. 1. Pedigree diagram in Patient 4.

Plasma VLCFAs levels were elevated in all patients. Median (range) C22:0 was 42.41 (14.68-75.69) μmol/L (normal: 31.88-71.49 μmol/L), C24:0 was 67.43 (18.78-128.50) μmol/L (normal: 24.97-61.04 μmol/L), C26:0 was 2.65 (1.50-6.57) μmol/L (normal: 0.22-0.77 μmol/L), the ratio of C26:0/C22:0 was 0.063 (0.02-0.27) (normal: 0.005-0.015) and C24:0/C22:0 was 1.667 (0.75-2.07) (normal: 0.697-0.963). Five patients with adrenocortical insufficiency (lower plasma cortisol and higher adrenocorticotropic hormone) had received hydrocortisone therapy previously. The demyelination of the brain white matter was found in 24 patients with cerebral type. The initial lesions usually involve the splenium of the corpus callosum and then extend into the adjacent white matter of the parieto-occipital lobes. Clinical information of the patients, including age of onset, age of death, presenting symptoms, family history, VLCFAs, MRI, adrenal functions, and phenotype are summarized in Table 1.

Mutation analysis of *ABCD1*

Twenty-two mutations (88%) including 14 missense mutations (63.6%), two splice site mutations, three deletion mutations, one duplication mutation and two nonsense mutations were identified in the 25 patients (Table 2). Eight of the 22 mutations [c.1017G>T (p.W339C), c.892G>C (p.G289R), c.532C>T (p.Q178*), c.1544C>A (p.S515Y), c.1428C>A (p.C476*), c.1182delG (p.A395Lfs*15), c.424delC (p.L142Sfs*56), c.1759_1761dup (p.I588H)] were novel (Fig. 2). The remaining 14 mutations had been reported previously. Of these mutations 34.8% were located in exon 1. Mutation analysis was made for 5 members (II2, III2, III3, III5, and IV1) of the P4 family, 2 members of the P5 family, and 3 members of

the P16 family. The novel variants were from patients' mothers and not detected in 50 healthy controls. Thus polymorphisms of the novel variants were excluded.

Prediction of the potential pathogenic effect of novel missense variants

The conservativeness of amino acid residues and effect of mutations on protein function were analyzed for three novel missense mutations (Table 3). These mutations were predicted to be "probably" damaging to protein function with a score of 1.00 by PolyPhen program. The SIFT score of all missense mutations were 0.00, that indicates the substitution of deleterious amino acids. Align-GVGD predicted that the three substitutions are most likely to interfere with the function of ALDP with score of class C65, class C65, and class C55 (Table 3).

Human ALDP crystallographic structure and altered ALDP structure caused by the three missense mutations are shown in Fig. 3. These mutations changed the size of amino acids and introduced amino acids with different properties, thereby disturbed this domain and abolished its function (Table 3).

Disccusion

The incidence of X-ALD is different in countries, with a minimum incidence of 1/17 000 males in European

Table 1. Demographic data and clinical characteristics of 25 patients with X-ALD

Patient number	Sex	Age at onset (y)	Age of death (y)	Clinical features	Family history	VLCFAs C22:0 (μmol/L)	VLCFAs C24:0 (μmol/L)	VLCFAs C26:0 (μmol/L)	VLCFAs C26:0/C22:0	VLCFAs C24:0/C22:0	MRI abnormal	Adrenal dysfunction	Phenotype
P1	Male	10	-	Visual impairment, exercise intolerance, intellectual impairment	-	45.73	62.05	1.68	0.037	1.357	+	+	CCALD
P2	Male	11	-	Ataxia, speech delay	-	28.06	42.91	1.89	0.067	1.529	+	-	CCALD
P3	Male	3	-	Ataxia, speech delay	NA	75.69	56.85	1.65	0.022	0.751	+	-	CCALD
P4	Male	6	-	Exercise intolerance, intellectual impairment	+	46.17	73.06	2.64	0.057	1.582	+	-	CCALD
P5	Male	37	42	Depression impaired vision, encephalopathy, ataxia	+	60.80	117.15	3.67	0.060	1.927	+	-	ACALD
P6	Male	7	NA	Exercise intolerance	-	33.28	64.34	3.19	0.096	1.933	+	NA	CCALD
P7	Male	5	7	Apathy, unsteady gait	-	29.33	39.82	1.68	0.057	1.358	+	-	CCALD
P8	Male	31	-	Visual impairment, unsteady gait	NA	38.88	69.81	2.84	0.073	1.795	+	-	ACALD
P9	Male	17	-	Exercise intolerance, intellectual impairment	NA	41.15	68.34	3.39	0.082	1.661	+	+	AdolCALD
P10	Male	10	-	Attention-deficit, impaired language skills	-	46.96	70.93	2.85	0.061	1.510	+	+	CCALD
P11	Male	40	NA	Exercise intolerance, intellectual impairment	-	36.58	66.52	2.50	0.068	1.819	+	NA	ACALD
P12	Male	28	30	Speech delay, progressive paralysis	+	66.97	117.21	4.07	0.061	1.750	+	+	ACALD
P13	Male	5	NA	Cognitive decline, visual impairment, encephalopathy	-	71.71	119.91	3.37	0.047	1.672	+	-	CCALD
P14	Male	8	NA	Cognitive decline, exercise intolerance	NA	24.78	51.36	3.34	0.135	2.073	+	NA	CCALD
P15	Male	10	NA	Intellectual impairment	NA				0.266	2.266*	+	NA	CCALD
P16	Male	42	44	Depression, encephalopathy, cognitive decline, paralysis	+	50.18	72.27	2.37	0.047	1.440	+	NA	ACALD
P17	Male	40	NA	Cognitive decline	NA	63.28	128.50	6.57	0.104	2.014	+	NA	ACALD
P18	Male	9	-	Dizziness	NA	43.68	71.70	2.22	0.051	1.641	+	-	CCALD
P19	Male	10	-	Attention-deficit, skin pigmentation, memory decline	NA	34.78	59.97	2.36	0.063	1.725	+	+	CCALD
P20	Male	6	-	Walking instability	NA	39.38	60.04	2.26	0.057	1.524	+	NA	CCALD
P21	Male	24	-	Weakness in the left lower extremity, hair rarefaction	NA	14.68	18.78	1.50	0.102	1.279	-	NA	AMN
P22	Male	6	NA	Cognitive decline	NA	46.43	85.03	2.98	0.064	1.831	+	NA	CCALD
P23	Male	42	NA	Memory and cognitive decline	NA	35.11	71.34	3.09	0.088	2.032	+	NA	ACALD
P24	Male	6.5	NA	Cognitive decline	NA	37.68	65.86	2.65	0.070	1.748	+	-	CCALD
P25	Male	8.5	NA	Cognitive decline	NA	45.42	56.72	1.50	0.033	1.248	+	NA	CCALD

X-ALD: X-linked adrenoleukodystrophy; NA: not available; VLCFAs: very long chain fatty acids; MRI: magnetic resonance imaging; AMN: milder adrenomyeloneuropathy; CALD: cerebral form ALD; CCALD: childhood CALD; ACALD: adulthood CALD; AdolCALD: adolescent CALD; C22:0: docosanoic acid; C24:0: tetracosanoic acid; C26:0: hexacosanoic acid. *: C26:0/C22:0, C24:0/C22:0 of VLCFAs available only in P15; "+": positive; "-": negative.

countries.[2] The incidence of X-ALD in males is 1/30 000-1/50 000 in Japan,[21] and the hemizygote frequency is 1/21 000 in the USA and at least 1/35 000 in South Brazil.[22,23] But epidemiological data on X-ALD are not available in China. In general, X-ALD is classified into several subtypes according to the age of onset, affected organs and progression of neurologic symptoms. The age of onset in CCALD, AdolCALD, and ACALD are less than 10 years, 10-21 years, and more than 21 years, respectively. CALD is a severe subtype which is characterized by rapidly progressive neuropsychological retrogression, white matter demyelination and early death.[24] Whereas AMN is characterized mainly by non-inflammatory "dying-back" axonopathy involving the long spinal tract, and the age of onset is usually 20-30 years, but before the fifth decade, neurologic disability is slowly progressive.[25,26] Approximately 10% of X-ALD males present initially with adrenocortical insufficiency (Addison's disease) without evidence of nervous system involvement. However, some X-ALD males remain asymptomatic and one-third of heterozygous women remain free of clinical symptoms during their

Table 2. *ABCD1* gene mutations identified in 23 patients with X-ALD

Patient number	Exon	Nucleotide change	Amino acid change	Protein localization	References
P1	2	c.1017G>T	p.Trp339Cys	TMD	Novel
P2	8	c.1850G>A	p.Arg617His	NBD	Fanen et al, 1994[12]
P4	1	c.892G>C	p.Gly298Arg	TMD	Novel
P5, P6	5	c.1415_16delAG	p.Gln472Argfs*83	TMD to NBD	Barcelo et al, 1994[13]
P7	1	c.532C>T	p.Gln178*	TMD	Novel
P8	1	c.473T>C	p.Leu158Pro	TMD	The peroxisomal diseases laboratory (unpublished)
P10	6	c.1552C>T	p.Arg518Trp	NBD	Fanen et al, 1994[12]
P11	3	c.1202G>A	p.Arg401Gln	TMD to NBD	Fuchs et al, 1994[14]
P12	1	c.887A>G	p.Tyr296Cys	TMD	Takano et al, 1999[15]
P13	1	c.893G>A	p.Gly298Asp	TMD	Lachtermacher et al, 2000[16]
P14	1	c.310C>T	p.Arg104Cys	TMD	Kok et al, 1995[17]
P15	IVS 8	c.1866-10G>A	p.Pro623fs*	NBD	Kemp et al, 1995[18]
P16	5	c.1428C>A	p.Cys476*	NBD	Novel
P17	5	c.1421T>C	p.Ile474Thr	NBD	Shimozawa et al, 2011[19]
P18	6	c.1538A>G	p.Lys513Arg	NBD	Pitié-Salpétrière Hospital (unpublished)
P19	1	c.310C>T	p.Arg104Cys	TMD	Kok et al, 1995[17]
P20	6	c.1544C>A	p.Ser515Tyr	NBD	Novel
P21	2	c.901-1G>A	p.Val301fs*	TMD	Kemp et al, 2001[20]
P22	2	c.974T>C	p.Leu325Pro	TMD	The peroxisomal diseases laboratory (unpublished)
P23	3	c.1182delG	p.Ala395Leufs*15	TMD to NBD	Novel
P24	1	c.424delC	p.Leu142Serfs*56	TMD	Novel
P25	7	c.1759_1761dup	p.Ile588His	NBD	Novel

ABCD1: adenosine triphosphate-binding cassette D1; X-ALD: X-linked adrenoleukodystrophy; TMD: transmembrane domains; NBD: nucleotide-binding domains. *: termination codon.

Table 3. Prediction of the potential pathogenic effect of three novel missense mutations

Variables	P1	P4	P20
cDNA mutations	c.1017G>T	c.892G>C	c.1544C>A
Protein level	p.Trp339Cys	p.Gly298Arg	p.Ser515Tyr
Conservation	Highly conserved	Highly conserved	Highly conserved
Polyphen prediction	Probably damaging	Probably damaging	Probably damaging
SIFT prediction	Deleterious	Deleterious	Deleterious
Align-GVGD	Most likely to interfere with function	Most likely to interfere with function	Most likely to interfere with function
Amino acid location (wild-type residue)	A transmembrane domain	ABC transmembrane type-1	ABC transporter
Amino acid size change (than the wild-type residue)	Smaller	Bigger	Bigger
Amino acid physicochemical property change	Aromatic amino acids to neutral polar amino acids (hydrophilicity)	Non polar aliphatic amino acids to alkaline amino acids	Neutral polar amino acids to aromatic amino acids (hydrophilic reduced)
Charge characteristic change	No	Neutral to positive	No
Form a hydrogen bond with the neighbors	No	No	Yes
Influence from the mutant residue	Disturb either the contacts with the other transmembrane domains or with the lipid-membrane	Change the flexibility of wild-type residue which might abolish protein function	The size difference makes that the new residue is not in the correct position and interferes with hydrogen bond

SIFT: sorting intolerant from tolerant; GVGD: Grantham variation and Grantham deviation; ABC: ATP-binding cassette.

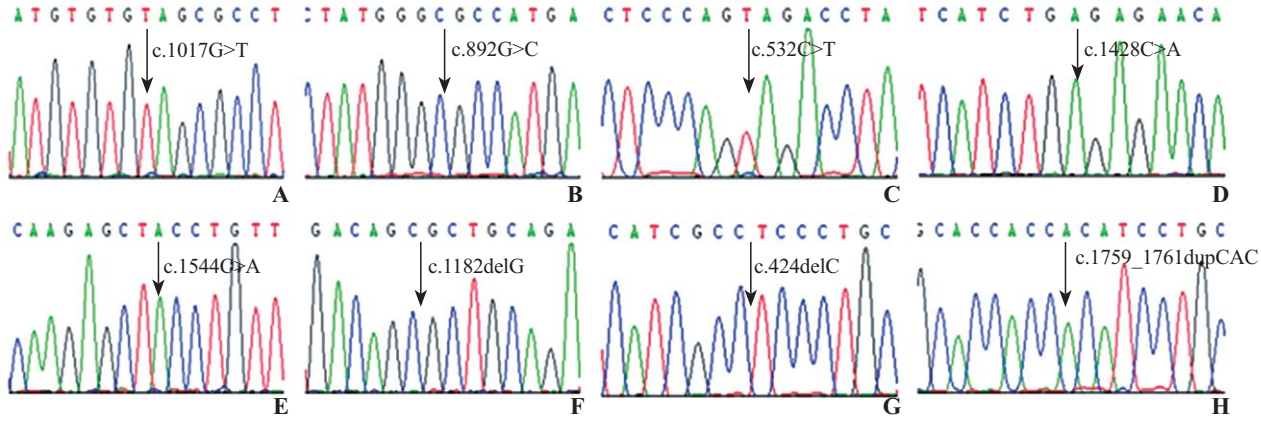

Fig. 2. The sequencing profiles of eight novel mutations. **A**: c.1017G>T (p.W339C); **B**: c.892G>C (p.G289R); **C**: c.532C>T (p.Q178*); **D**: c.1544C>A (p.S515Y); **E**: c.1428C>A (p.C476*); **F**: c.1182delG (p.A395Lfs*15); **G**: c.424delC (p.L142Sfs*56); **H**: c.1759_1761dup (p.I588H).

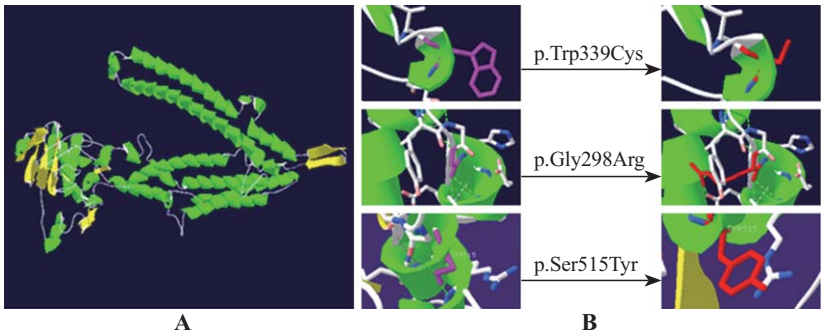

Fig. 3. A: 3D-structure of wild type ALDP. α-Helix is presented in green, β-strands is displayed in yellow and coil is marked with gray respectively; **B**: Change of ALDP structure in three patients with novel missense mutations: c.1017G>T (p.Trp339Cys) in P1, c.892G>C (p.Gly298Arg) in P4, and c.1544C>A (p.Ser515Tyr) in P20. Purple configuration: original amino acids; red configuration: mutant amino acids; green dotted line: hydrogen bond.

entire life.[1] Analysis of the phenotypes of female carriers showed that skewed X chromosome inactivation in favor of the mutant *ABCD1* allele would be associated with the manifestations of heterozygous symptoms.[7] According to clinical characteristics of our patients in this study, CCALD is the most common phenotype and accounts for 64% (16/25). It is similar to a large cohort of Chinese patients with X-ALD.[8,9] Only one patient in our study presented AMN, and adrenal insufficiency was seen in five patients. This finding is different from the reports that AMN is the most common phenotype.[25,27] Possibly ALD is not widely known by clinical physicians, and plasma VLCFAs are not routinely measured for patients with AMN or adrenal insufficiency in China. The onset of CALD may be triggered by environmental factors (such as trauma, drinking).[28] Such phenomenon was also noted in our patients. P16's brother experienced a short-term memory loss after drinking, P4-III3 had numbness and dizziness after vomiting, and P5 presented with depression and memory loss after suffering from spiritual trauma.

About 64% of the mutations identified in our patients are missense mutations, which is higher than 51% reported elsewhere. *ABCD1* mutations have been found in the entire gene. Most of missense mutations are located in the transmembrane domain (encoded by exon 1, 47%) and ATP-binding domain (encoded by exons 6 to 9, 34%), the remaining are located in other parts of the gene, and promoter mutations or complete gene deletions have not been noted in patients with X-ALD.[18,29] However, contiguous *ABCD1* and DXS1357E gene deletion have been found in contiguous *ABCD1* DXS1357 deletion syndrome, but the phenotypes are different from X-ALD.[30]

Three novel missense mutations were predicted to change ALDP structure and affect protein function by multiple software analysis. The study of ALDP functional expression for novel missense mutations is required to confirm the pathogenic effect of novel mutations. The other 5 novel mutations including splice, deletion, duplication and nonsense mutations can be pathogenically effective by frame shift and termination.

So it is speculated that the 8 novel mutations we described are disease-causing mutations, and these results expand the mutation spectrum of the *ABCD1* gene in Chinese patients with X-ALD.

However, lack of correlation between genotype and phenotype makes the precise prediction of disease in an affected individual impossible, even within familial cohorts.[31] In this study we found no correlation between genotype and phenotype. The same phenotype of CLAD may be due to diverse genomic mutations. A mutation may result in different phenotypes even within a family (P4, P5 and P16). Phenotype characteristics and gene mutation patterns in Chinese patients with X-ALD are not identical to those in patients of other countries.

To the present, no predictive markers have been used to detect X-ALD including plasma VLCFA, gene mutation, and family history. However, reports [1,32] showed that increased brain VLCFA levels are correlated with the clinical phenotype preceded by histopathological alterations and are an important factor for the development of cerebral disease. Genetic segregation analysis suggests that except for the disease causing *ABCD1* mutations and environmental factors, other genetic autosomal inherited factors are involved in the clinical manifestation of X-ALD.[33,34] And silent single nucleotide polymorphisms can affect the rate of translation from mRNA to protein.

The treatment of X-ALD is usually not specific, and bone marrow transplantation is the only effective long-term treatment for childhood cerebral X-ALD. Lorenzo's oil (mixture of oleic and erucic acid) in combination with a diet low in VLCFA is helpful to reduce VLCFA level, but can not improve symptoms of patients with cerebral adrenoleukodystrophy.[35] In our study patient P4 received the treatment with Lorenzo's oil, but failed. The patients with adrenal insufficiency can be treated with hydrocortisone.

In conclusion, CCALD is the most common phenotype in patients with X-ALD. The progressive deterioration of neurological and mental development is a clinical characteristic. Different phenotypes are seen in patients and even in a family. In our study, 22 different disease-causing mutations were detected in the *ABCD1* gene. 34.8% of the mutations are located in exon 1. Eight novel mutations including 3 missense mutations were predicted as disease-causing mutations. Brain MRI and plasma VLCFAs determination should be done for patients with progressive deterioration of neurological development so as to make early diagnosis. Analysis of the *ABCD1* gene can provide genetic counseling and a precise prenatal diagnosis.

Acknowledgements

We are very grateful to the patients and their families who participated in this study and to the pediatricians who provided patient data and DNA samples.

Funding: This study was supported by a grant from the National Key Technology R&D Program (2012BAI09B00).
Ethical approval: This study was approved by the Ethics Committee of Xinhua Hospital (XHEC-D-2015-103).
Competing interest: All authors declared no conflict of interest.
Contributors: Chu SS did data analysis and wrote the draft. Ye J revised the manuscript and acted as the guarantor of the study. Zhang HW, Han LS, Qiu WJ and Gu XF recorded the medical histories and reviewed critically the article. Gao XL determined very long chain fatty acid in plasma. All authors approved the final version of the article for publication.

References

1 Kemp S, Berger J, Aubourg P. X-linked adrenoleukodystrophy: clinical, metabolic, genetic and pathophysiological aspects. Biochim Biophys Acta 2012;1822:1465-1474.
2 Schluter A, Espinosa L, Fourcade S, Galino J, Lopez E, Ilieva E, et al. Functional genomic analysis unravels a metabolic-inflammatory interplay in adrenoleukodystrophy. Hum Mol Genet 2012;21:1062-1077.
3 Mosser J, Douar AM, Sarde CO, Kioschis P, Feil R, Moser H, et al. Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. Nature 1993;361:726-730.
4 Berger J, Forss-Petter S, Eichler FS. Pathophysiology of X-linked adrenoleukodystrophy. Biochimie 2014;98:135-142.
5 Jack GH, Malm-Willadsen K, Frederiksen A, Glintborg D, Andersen M. Clinical manifest x-linked recessive adrenoleukodystrophy in a female. Case Rep Neurol Med 2013;2013:491790.
6 Pereira Fdos S, Matte U, Habekost CT, de Castilhos RM, El Husny AS, Lourenco CM, et al. Mutations, clinical findings and survival estimates in South American patients with X-linked adrenoleukodystrophy. PLoS One 2012;7:e34195.
7 Wang Z, Yan A, Lin Y, Xie H, Zhou C, Lan F. Familial skewed x chromosome inactivation in adrenoleukodystrophy manifesting heterozygotes from a Chinese pedigree. PLoS One 2013;8:e57977.
8 Ping LL, Bao XH, Wang AH, Pan H, Wu Y, Xiong H, et al. Clinical features and genotype-phenotype studies of 89 Chinese patients with X-linked adrenoleukodystrophy. Zhonghua Er Ke Za Zhi 2007;45:203-207. [In Chinese]
9 Ping LL, Bao XH, Wang AH, Pan H, Wu Y, Xiong H, et al. The genotype and phenotype studies of 40 Chinese patients with X-linked adrenoleukodystrophy (X-ALD). Beijing Da Xue Xue Bao 2006;38:66-70.
10 Mathe E, Olivier M, Kato S, Ishioka C, Hainaut P, Tavtigian SV. Computational approaches for predicting the biological effect of p53 missense mutations: a comparison of three sequence analysis based methods. Nucleic Acids Res 2006;34:1317-1325.
11 Ng PC, Henikoff S. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res 2003;31:3812-3814.
12 Fanen P, Guidoux S, Sarde CO, Mandel JL, Goossens M,

Aubourg P. Identification of mutations in the putative ATP-binding domain of the adrenoleukodystrophy gene. J Clin Invest 1994;94:516-520.
13 Barceló A, Girós M, Sarde CO, Martínez-Bermejo A, Mandel JL, Pàmpols T, et al. Identification of a new frameshift mutation (1801delAG) in the *ALD* gene. Hum Mol Genet 1994;3:1889-1890.
14 Fuchs S, Sarde CO, Wedemann H, Schwinger E, Mandel JL, Gal A. Missense mutations are frequent in the gene for X-chromosomal adrenoleukodystrophy (*ALD*). Hum Mol Genet 1994;3:1903-1905.
15 Takano H, Koike R, Onodera O, Sasaki R, Tsuji S. Mutational analysis and genotype-phenotype correlation of 29 unrelated Japanese patients with X-linked adrenoleukodystrophy. Arch Neurol 1999;56:295-300.
16 Lachtermacher MB, Seuánez HN, Moser AB, Moser HW, Smith KD. Determination of 30 X-linked adrenoleukodystrophy mutations, including 15 not previously described. Hum Mutat 2000;15:348-353.
17 Kok F, Neumann S, Sarde CO, Zheng S, Wu KH, Wei HM, et al. Mutational analysis of patients with X-linked adrenoleukodystrophy. Hum Mutat 1995;6:104-115.
18 Kemp S, Ligtenberg MJ, van Geel BM, Barth PG, Sarde CO, van Oost BA, et al. Two intronic mutations in the adrenoleukodystrophy gene. Hum Mutat 1995;6:272-273.
19 Shimozawa N, Honda A, Kajiwara N, Kozawa S, Nagase T, Takemoto Y, et al. X-linked adrenoleukodystrophy: diagnostic and follow-up system in Japan. J Hum Genet 2011;56:106-109.
20 Kemp S, Pujol A, Waterham HR, van Geel BM, Boehm CD, Raymond GV, et al. *ABCD1* mutations and the X-linked adrenoleukodystrophy mutation database: role in diagnosis and clinical correlations. Hum Mutat 2001;18:499-515.
21 Takemoto Y, Suzuki Y, Tamakoshi A, Onodera O, Tsuji S, Hashimoto T, et al. Epidemiology of X-linked adrenoleukodystrophy in Japan. J Hum Genet 2002;47:590-593.
22 Bezman L, Moser AB, Raymond GV, Rinaldo P, Watkins PA, Smith KD, et al. Adrenoleukodystrophy: incidence, new mutation rate, and results of extended family screening. Ann Neurol 2001;49:512-517.
23 Jardim LB, da Silva AC, Blank D, Villanueva MM, Renck L, Costa ML, et al. X-linked adrenoleukodystrophy: clinical course and minimal incidence in South Brazil. Brain Dev 2010;32:180-190.
24 Kumar N, Taneja KK, Kalra V, Behari M, Aneja S, Bansal SK. Genomic profiling identifies novel mutations and SNPs in *ABCD1* gene: a molecular, biochemical and clinical analysis of X-ALD cases in India. PLoS One 2011;6:e25094.
25 Bruyn RP. X-linked adrenoleukodystrophy. Neurology 1996;46:1192.
26 van Geel BM, Bezman L, Loes DJ, Moser HW, Raymond GV. Evolution of phenotypes in adult male patients with X-linked adrenoleukodystrophy. Ann Neurol 2001;49:186-194.
27 Moser HW, Mahmood A, Raymond GV. X-linked adrenoleukodystrophy. Nat Clin Pract Neurol 2007;3:140-151.
28 Raymond GV, Seidman R, Monteith TS, Kolodny E, Sathe S, Mahmood A, et al. Head trauma can initiate the onset of adrenoleukodystrophy. J Neurol Sci 2010;290:70-74.
29 Smith KD, Kemp S, Braiterman LT, Lu JF, Wei HM, Geraghty M, et al. X-linked adrenoleukodystrophy: genes, mutations, and phenotypes. Neurochem Res 1999;24:521-535.
30 Iwasa M, Yamagata T, Mizuguchi M, Itoh M, Matsumoto A, Hironaka M, et al. Contiguous *ABCD1* DXS1357E deletion syndrome: report of an autopsy case. Neuropathology 2013;33:292-298.
31 Berger J, Molzer B, Fae I, Bernheimer H. X-linked adrenoleukodystrophy (ALD): a novel mutation of the ALD gene in 6 members of a family presenting with 5 different phenotypes. Biochem Biophys Res Commun 1994;205:1638-1643.
32 Asheuer M, Bieche I, Laurendeau I, Moser A, Hainque B, Vidaud M, et al. Decreased expression of *ABCD4* and *BG1* genes early in the pathogenesis of X-linked adrenoleukodystrophy. Hum Mol Genet 2005;14:1293-1303.
33 Moser HW, Moser AB, Smith KD, Bergin A, Borel J, Shankroff J, et al. Adrenoleukodystrophy: phenotypic variability and implications for therapy. J Inherit Metab Dis 1992;15:645-664.
34 Maestri NE, Beaty TH. Predictions of a 2-locus model for disease heterogeneity: application to adrenoleukodystrophy. Am J Med Genet 1992;44:576-582.
35 Terre'Blanche G, van der Walt MM, Bergh JJ, Mienie LJ. Treatment of an adrenomyeloneuropathy patient with Lorenzo's oil and supplementation with docosahexaenoic acid--a case report. Lipids Health Dis 2011;10:152.

Received March 11, 2015
Accepted after revision June 19, 2015