

# X-linked adrenoleukodystrophy caused by maternal ABCD1 mutation and paternal X chromosome inactivation

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Received April 2, 2022; Accepted June 13, 2022

DOI: 10.3892/etm.2022.11502

**Abstract.** X-linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder. It is caused by defects in the ATP-binding cassette subfamily D member 1 (ABCD1) gene, resulting in impaired peroxisomal  $\beta$ -oxidation of very-long-chain fatty acids (VLCFAs). As an X-linked recessive disease, female X-ALD carriers are typically asymptomatic. In the present study, a 7-year-old girl was diagnosed with cerebral ALD. Brain magnetic resonance imaging revealed asymmetric demyelination of bilateral white matter. Plasma VLCFAs level showed a substantial increase. Whole exome and Sanger sequencing revealed an ABCD1 c.919C>T (p.Q307X) heterozygous pathogenic mutation, which was inherited from the asymptomatic mother. X chromosome inactivation (XCI) analysis revealed that the normal paternal X chromosome was almost completely inactivated. Thus, the maternal ABCD1 mutation and paternal XCI were responsible for causing the disease in the patient. XCI may be one reason female X-ALD carriers can be symptomatic.

## Introduction

X-linked adrenoleukodystrophy (X-ALD), caused by mutations in the ATP-binding cassette subfamily D member 1 (ABCD1) gene, is the most common peroxisomal disorder. ABCD1 is located on Xq28 and encodes the peroxisomal transporter of very-long-chain fatty acids (VLCFAs) (1). Defects in ABCD1 protein prevent  $\beta$ -oxidation of VLCFAs, leading to its accumulation in tissue and plasma (1). According to age of onset, affected site and progression rate, multiple phenotypes of X-ALD are recognized, including cerebral ALD, adrenomyeloneuropathy, Addison's and asymptomatic (2).

As in the case of numerous types of X-linked recessive disease, female carriers are assumed to remain asymptomatic; however, ~20% of X-ALD female carriers develop symptoms, usually in their thirties, and the incidence rate is up to 88% in those aged >60 years (3,4). In general, female X-ALD carriers display adrenomyeloneuropathy-like phenotype. The primary clinical manifestations of this phenotype are progressive spastic gait, sensory deficit and bladder dysfunction, commonly without adrenal insufficiency (4,5).

The present report describes the case of a 7-year-old girl with cerebral ALD. We aimed to discover the possible pathogenesis of this patient in the present study.

## Materials and methods

**Sample collection.** Peripheral blood (3 ml) was collected from a female patient with X-ALD (age, 8 years in 2021), as well as from members of their family (Fig. 1A). The ages of family members I<sub>1</sub>, I<sub>2</sub>, II<sub>1</sub> and II<sub>2</sub> were 62, 60, 36 and 35 years, respectively. The present study was approved by the Ethics Committee of the Shengjing Hospital of China Medical University (approval no. 2021PS209K). Written informed consent was obtained from the patient's parents and the other adults in the family.

**Biochemical measurements.** Serum or plasma (0.5 ml) was collected after centrifugation of blood samples at 1,200 x g for 5 min at room temperature. Plasma adrenocorticotrophic hormone (ACTH) and serum cortisol levels were measured according to a chemiluminescence method (6), and serum VLCFA was measured by gas chromatography and mass spectrometry (7) at Jinyu Medical Laboratory Center.

**Whole-exome and Sanger sequencing.** Genomic DNA was extracted using QIAamp DNA Mini kit (Qiagen China Co., Ltd.). The concentration of extracted DNA was quantified with Nanodrop 2000 (NanoDrop; Thermo Fisher Scientific, Inc.). Genomic DNA was fragmented to an average size of 150 bp using a S220 Focusedultrasonicator (Covaris, LLC). The DNA library was prepared using the GenCap NGS Fast DNA Library Prep Set for Illumina (cat. no. MG16001M; MyGenostics Technologies Inc.) according to the manufacturer's instructions. The DNA library was captured using a whole-exome capture kit (GenCap Human Gene Enrichment

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**Key words:** X-linked adrenoleukodystrophy, ATP-binding cassette subfamily D member 1, X chromosome inactivation, whole exome sequencing, very-long-chain fatty acid

Kit; cat. no. MG16101M, MyGenostics Technologies Inc.). The captured DNA was eluted with buffer, and amplified for 13 cycles using the following program: 95°C for 4 min (1 cycle); 98°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec (13 cycles); 72°C for 5 min (1 cycle). The PCR product was purified using SPRI beads (Beckman Coulter, Inc.) according to the manufacturer's protocol. The enriched libraries were sequenced using 150-bp paired-end reads on the Illumina HiSeq X Ten platform (Illumina, Inc.). Raw data were stored in the FASTQ format. Illumina sequencing adapters and low-quality reads (<80 bp) were filtered. Clean reads were aligned to the UCSC hg19 human reference genome using the Burrows-Wheeler Alignment tool 0.7.17 (<http://maq.sourceforge.net>). Duplicated reads were removed using Picard ([broadinstitute.github.io/picard](http://broadinstitute.github.io/picard)). Insertion, deletion and single nucleotide polymorphism (SNP) variants were detected and filtered using the Genome Analysis Toolkit 4.0.8.1 (<https://www.broadinstitute.org/gatk>). The identified variants were annotated using ANNOVAR (<http://annovar.openbioinformatics.org/en/latest>) and assessed via the following databases: 1000 Genomes (<https://www.internationalgenome.org>), Exome Aggregation Consortium (<http://gnomad.broadinstitute.org>), Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>), Mutation Taster (<https://www.mutationtaster.org>), Sorting Intolerant From Tolerant (<http://provean.jcvi.org/index.php>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2>) and Genomic Evolutionary Rate Profiling (<http://mendel.stanford.edu/SidowLab/downloads/ger>). Variation sites were identified by comparing DNA sequences with the corresponding GenBank reference sequences using Mutation Surveyor software (SoftGenetics, Inc.). The pathogenicity of mutations was assessed in accordance with the American College of Medical Genetics and Genomics Guidelines (ACMG) (8). The mutation was also confirmed via Sanger sequencing on ABI Prism 3730 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following primers: Forward, 5'-TGTGTGAGTGGCACTGGGAGA-3' and reverse, 5'-GCTCCAGCATAACATACCACA-3'. SNP haplotype analysis was performed using Infinium Asian Screening Array-24 v1.0 kit (Illumina, Inc.). In brief, DNA samples were incubated with corresponding reagents in the kit at 37°C overnight for PCR amplification. The amplified DNA was then fragmented in the heat block, precipitated with isopropyl alcohol, resuspended after centrifugation and hybridized with the chip at 48°C overnight, followed by washing, extension and chip dying. The prepared chip was scanned in Iscan chip scanner (Illumina, Inc.). Data were uploaded to ChromGo (Yikon Genomics) for analysis.

**X chromosome inactivation (XCI) analysis in peripheral blood.** Peripheral blood (1 ml) was used for genomic DNA extraction (Qiagen China Co., Ltd.). The XCI pattern was determined by quantifying the CAG repeat in exon 1 of the androgen receptor gene using a commercial kit according to the manufacturer's instructions (XCI analysis kit, MyGenostics GenCap Enrichment Technologies). In brief, when the X chromosome is inactivated, the binding site of the methylation-sensitive restriction endonuclease *HpaII*, which is located near the CAG repeat, is methylated and cannot be cleaved, whereas

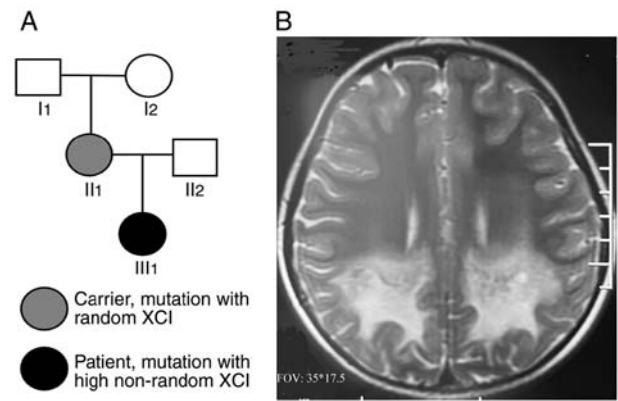


Figure 1. Pedigree and brain MRI of the patient. (A) Pedigree of the X-linked adrenoleukodystrophy family. III<sub>1</sub> indicates the patient; square, male; circle, female. (B) Brain MRI of the patient revealed asymmetric demyelination of bilateral white matter. MRI, magnetic resonance imaging; XCI, X chromosome inactivation.

the non-methylated site of the active X chromosome can be cleaved by *HpaII*. Therefore, if the genomic DNA is digested with *HpaII* and then amplified via PCR, only the inactivated X chromosome produces the expected bands (9).

## Results

**Patient clinical information description.** At the age of 7 years, from April 2020 the patient (III<sub>1</sub>; Fig. 1A) began to experience occasional confusion and needed to be called several times before answering. She was found to have a poor sense of direction and failed to find her way home when she went out. Her eyesight declined and she had 30° strabismus. She was afraid of moving to higher positions and her ability to balance worsened. Her electroencephalogram revealed slow waves. Brain magnetic resonance imaging (MRI) revealed asymmetric demyelination of bilateral white matter, suggesting the acute phase of ALD (Fig. 1B). The patient did not receive any treatment. Eight months later (December 2020), she was unable to hear, see or speak and became paralyzed. In May 2021, the patient's mother (II<sub>1</sub>) visited the obstetric clinic (Shengjing Hospital of China Medical University, Shenyang, China) and wished to have a healthy child via assisted reproductive technology. The clinical information, particularly the MRI, demonstrated that she was affected with ALD.

**Patient exhibits high plasma VLCFA levels.** Biochemical measurement analysis demonstrated that the C26:0 and C26:0/C22:0 levels in III<sub>1</sub> were notably increased (Table I). Moreover, C24:0/C22:0 and ACTH and cortisol levels in III<sub>1</sub> were slightly elevated. By contrast, VLCFAs, ACTH and cortisol levels were close to normal in II<sub>1</sub> and I<sub>2</sub>, with C26:0 level and C26:0/C22:0 slightly increased in II<sub>1</sub>. The results showed the patient exhibited high plasma VLCFA levels.

**Patient harbors a heterozygous ABCD1 mutation.** Whole-exome sequencing performed on II<sub>1</sub>, II<sub>2</sub> and III<sub>1</sub> (Fig. 2A). II<sub>1</sub> and III<sub>1</sub> harbored the ABCD1 c.919C>T (p.Q307X) heterozygous mutation, which was confirmed using Sanger sequencing (Fig. 2B). Based on ACMG guidelines,

Table I. Results of biochemical measurement.

Type	Sample ID	III <sub>1</sub>	II <sub>1</sub>	I <sub>2</sub>	Reference value
ACTH		73.560↑	18.240	10.760	7.200-63.400 pg/ml
Cortisol		26.180↑	8.390	9.790	4.260-24.850 μg/dl
VLCFA	C22:0	50.800	53.000	62.000	≤96.300 nmol/ml
	C24:0	91.000	63.400	57.100	≤91.400 nmol/ml
	C26:0	3.850 ↑↑	1.320 ↑	0.780	≤1.300 nmol/ml
	C24:0/C22:0	1.790 ↑	1.200	0.920	≤1.390
	C26:0/C22:0	0.076 ↑↑	0.025 ↑	0.013	≤0.023

ACTH, adrenocorticotrophic hormone; VLCFA, very-long-chain fatty acid.

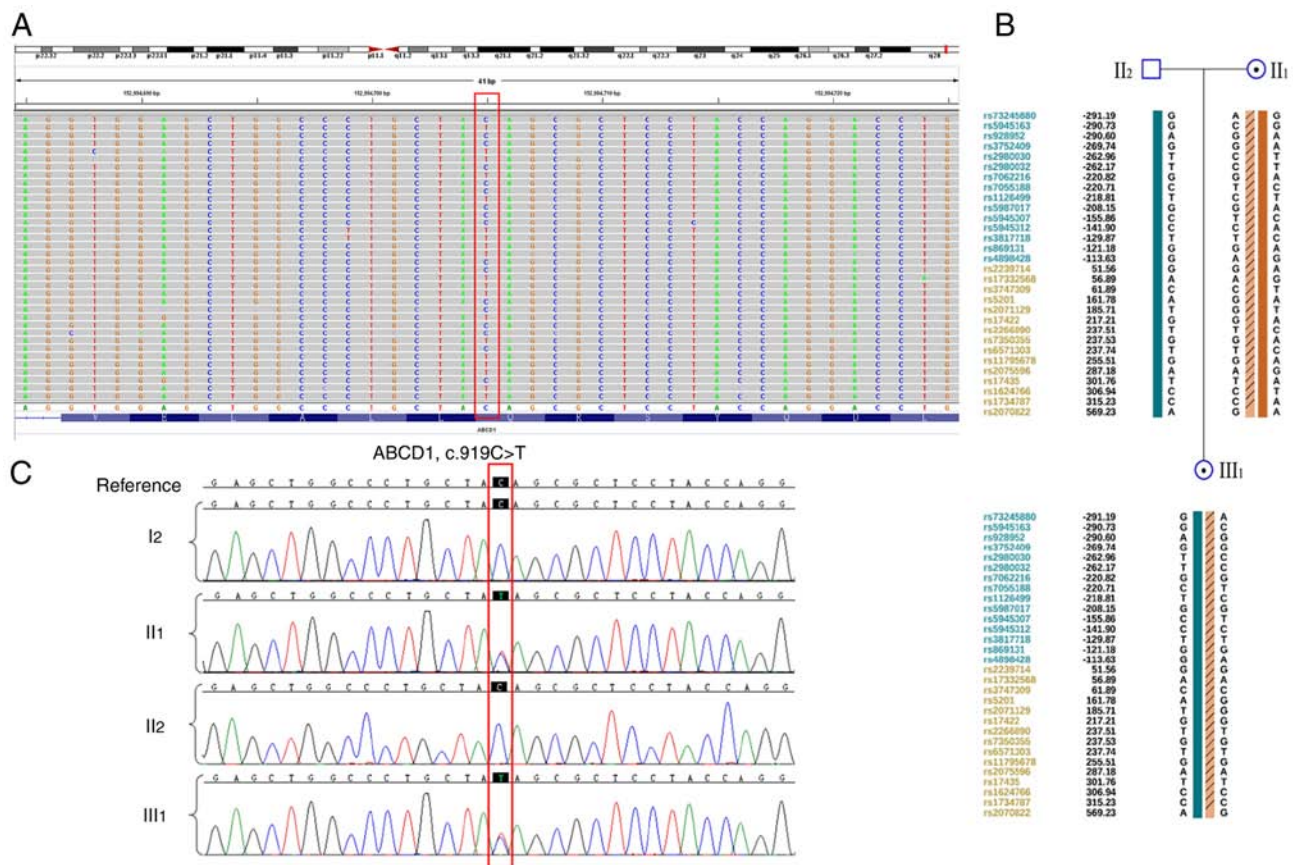


Figure 2. Sequencing analysis. (A) Mutation was identified by whole exome sequencing, suggested a heterozygous c.919C>T (p.Q307X) variation of ATP-binding cassette subfamily D member 1 (red box). (B) SNP haplotype linkage analysis of the family. The numbers represent the relative distance in kb up-(blue) and downstream (orange) of gene. (C) Mutation was positive in the patient III<sub>1</sub> and mother II<sub>1</sub>, but not in I<sub>2</sub> or II<sub>2</sub>, as shown by Sanger sequencing.

this mutation was identified as pathogenic. The pathogenic mutation in the patient was inherited from her mother who did not demonstrate any symptoms. In addition, SNP haplotype analysis was constructed for the linkage analysis (Fig. 2C); a total of 15 available SNP sites upstream of the gene and 15 sites downstream were selected for the haplotype construction. These findings indicated that the patient harbors a heterozygous *ABCD1* mutation from her asymptomatic mother.

*Paternal X chromosome is inactivated in the patient.* Although both II<sub>1</sub> and III<sub>1</sub> harbored the same *ABCD1*

heterozygous mutation, III<sub>1</sub> showed obvious clinical manifestations, whereas II<sub>1</sub> was an asymptomatic carrier. As an X-linked recessive disease, female *ABCD1* heterozygous carriers are typically asymptomatic, especially in childhood (4). To identify the pathogenic cause in III<sub>1</sub>, other pathogenic *ABCD1* mutations were excluded. As XCI is involved in ALD, the polymorphism of the CAG repeat in exon 1 of the androgen receptor gene was investigated. X chromosome in III<sub>1</sub> displayed highly non-random inactivation, implying that her paternal X chromosome was almost completely inactivated (Fig. 3). This indicated that the severe

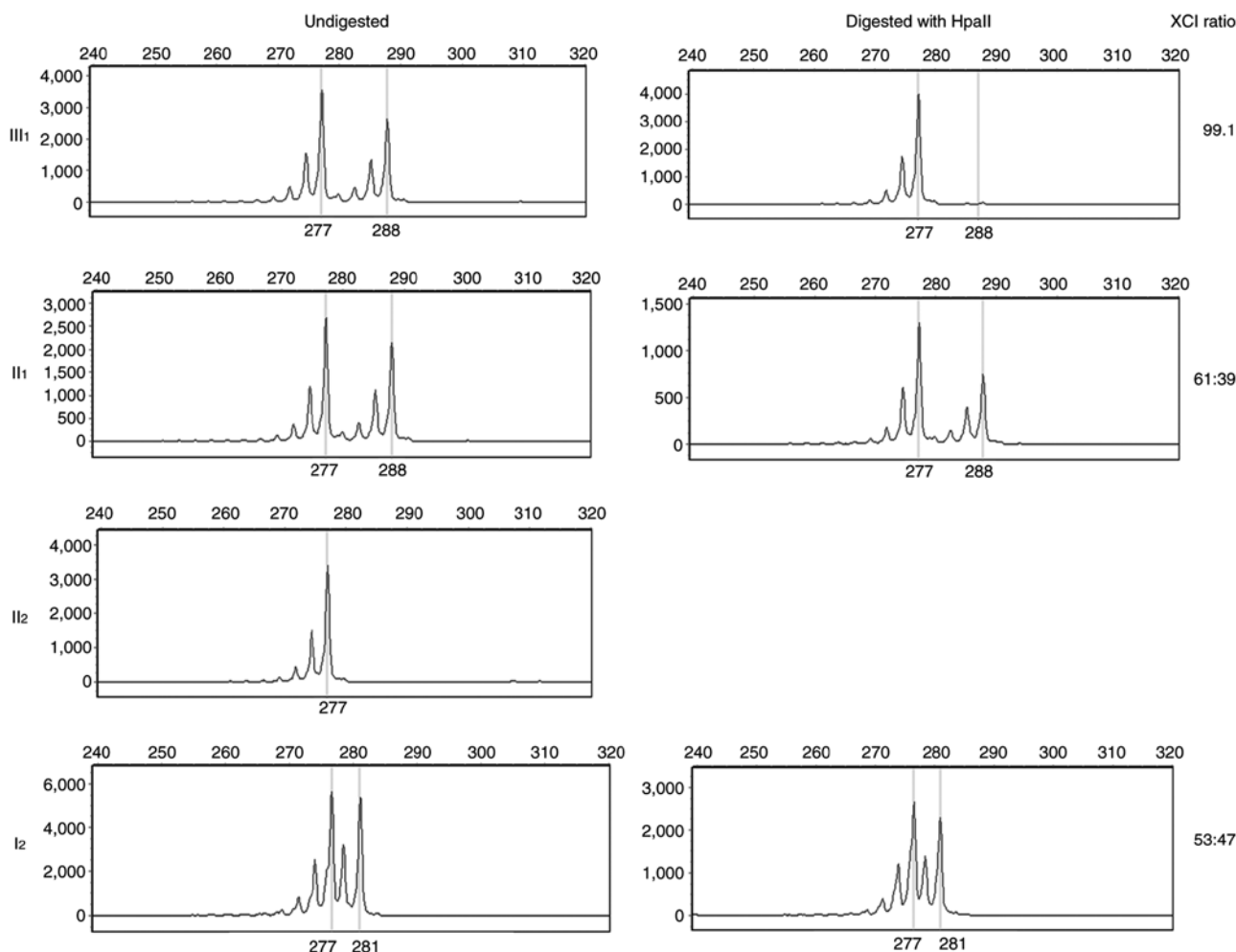


Figure 3. XCI analysis. From the undigested chart, the 277 bp fragment in patient III<sub>1</sub> was from her father II<sub>2</sub> and the 288 bp fragment was from her mother II<sub>1</sub>. From the *HpaII*-digested chart, patient demonstrated skewed XCI and paternal X chromosome was almost completely inactivated. X-axis represents the fragment size (bp) and Y-axis represents the peak value. XCI, X chromosome inactivation.

clinical symptoms in III<sub>1</sub> were caused by maternal ABCD1 mutation and paternal XCI.

## Discussion

The present study describes the case of a patient with X-ALD caused by maternal ABCD1 mutation and paternal XCI. The incidence of X-ALD is 1 in 20,000-50,000 individuals (10). According to the age of onset, affected site and progression rate, X-ALD can be classified into four major groups (2): i) Cerebral ALD, which is characterized by demyelination of white matter of the brain; ii) adrenomyeloneuropathy, with spinal cord demyelination and axonal degeneration; iii) Addison-like phenotype due to adrenocortical insufficiency and iv) asymptomatic type. Childhood cerebral ALD and adrenomyeloneuropathy are the most common types observed during childhood and adulthood, with incidences of 31-35 and 20-60%, respectively (11,12).

Onset of childhood cerebral ALD usually occurs at the age of 3-10 years, with a peak age onset of 7 years, and is characterized by progressive deterioration of cognitive, behavioral, and motor function. The patient initially presents with learning and behavioral problems, as well as attention deficit, followed

by cognitive decline, ataxia, cortical blindness, deafness and seizures within 6 months to 2 years. This can progress to a vegetative state and death within years of diagnosis (1,8). The lower the onset age, the faster the disease progression (13). Brain MRI shows demyelination in the white matter of the brain and this finding can precede symptoms (13). In the present study, the patient's clinical manifestations was typical, including hearing, vision and behavioral abnormality, which progressed to the vegetative state rapidly. The subsequent genetic analysis identified the ABCD1 heterozygous pathogenic mutation.

To date, >900 variants of ABCD1 have been reported (14). Although Campopiano *et al* (15) addressed the genotype-phenotype correlation in a large family, most studies have showed weak correlation between genotype and phenotype (1,2). Thus, ongoing clinical symptoms and prognosis cannot be determined by the patient genotype. It is accepted that other factors, including genetic, epigenetic or environmental, may affect rate of progression (2).

As in the case of many X-linked diseases, X-ALD female carriers typically remain asymptomatic; however, 18-88% of female carriers develop symptoms (4). The incidence rate of X-ALD in ABCD1 heterozygous female carriers is



associated with age; 18% of female carriers aged <40 years develop symptoms and this increases to 88% of those aged >60 years (4). Horn *et al* (16) also found that nearly all female carriers aged >50 years develop neurological symptoms. One of the earliest descriptions of an adrenomyeloneuropathy-like X-ALD phenotype was a female patient (17).

In X-ALD, XCI is an important factor. XCI refers to silencing of one of the two X chromosomes in mammalian female cells that ensures equal expression of X-linked genes between female and male. This process occurs during early development; one X chromosome is transcriptionally silenced while the other remains active (18,19). Which X chromosome is inactivated is usually random; therefore, ~50% of all cells express genes from the paternal-derived X chromosome and the remaining 50% from the maternal chromosome (5). When 80% of cells demonstrate a preferential inactivation of the same X chromosome, it is defined as skewed XCI (5). Extremely skewed XCI implies inactivation of paternal or maternal-derived X chromosome in >95% of all cells (20). To date, whether skewed XCI is associated with symptomatic X-ALD female carriers is unclear. Maier *et al* (21) reported that the manifestation of symptoms in 22 X-ALD carriers was associated with skewed XCI and that there was a significant correlation between the extent of skewing and the severity of neurological abnormality. By contrast, Watkiss *et al* (22) and Salsano *et al* (5) did not find any association between neurological manifestation and XCI pattern. The present study suggested that extremely skewed XCI served an important role in the patient's severe clinical presentation. In general, XCI skewing appears to favor the inactive mutant allele, which may explain why female carriers may be asymptomatic in severe X-linked disorders with a highly skewed XCI (18,19). However, ABCD1 mutations in X-ALD demonstrate a proliferative advantage rather than a disadvantage, caused by XCI skewing in favor of the normal X chromosome (5). The present findings were consistent with this hypothesis as the normal paternal X chromosome was almost completely inactivated. However, in the present study, the mechanism by which the paternal X chromosome was inactivated was not clarified. Further experiments and samples (from this and other families) will be needed to study the mechanism in-depth.

In addition to X-ALD, XCI serves an important role in other common X-linked recessive diseases. Yang *et al* (23) found skewed XCI in a female patient who had a heterozygous missense mutation in the FIX gene and a complete clinical manifestation of moderate hemophilia B was noted. A total of 28% of heterozygous females with FVIII or FIX variants have clinical manifestations and exhibit skewed XCI (24). In Becker muscular dystrophy, Viggiano *et al* (25) reported that skewed XCI serves a crucial role in the onset of symptoms in female carriers. Skewed XCI also appears to have a correlation with a severe phenotype in Duchenne muscular dystrophy (26). He *et al* (9) found that XCI was implicated in symptomatic female carriers of dystrophinopathies and XCI analysis of amniocytes may be useful in predicting the risk of dystrophinopathy in fetal carriers. Thus, the incidence of skewed XCI may be common and contribute to the manifestation of symptoms in carriers of recessive X-linked disorders (18,19).

In conclusion, X-AD in the present patient was caused by both maternal ABCD1 mutation and paternal XCI. This suggests that XCI may be a key factor in X-ALD.

### Acknowledgements

Not applicable.

### Funding

The present study was supported by the 345 Talent Project of Shengjing Hospital (grant no. M0298).

### Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the Sequence Read Archive database (submission number: SUB11494821, Bio Sample accession number: SAMN28555541; <https://www.ncbi.nlm.nih.gov/sra/PRJNA841819>).

### Authors' contributions

ZL provided obstetrical service, collected the samples and clinical information, performed the biochemical measurements, and wrote the paper. GL performed the sequencing and analyzed the data, provided genetic counseling, and revised the manuscript. ZL and GL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Shengjing Hospital of China Medical University (approval no. 2021PS209K). Written informed consent was obtained from the patient's parents.

### Patient consent for publication

Consent for publication was obtained from the patient's parents. Patient personal information was removed from the picture.

### Competing interests

The authors declare that they have no competing interests.

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