ORIGINAL ARTICLE

Genetic analysis and prenatal diagnosis of 76 Chinese families with X-linked adrenoleukodystrophy

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Abstract

Background: Variants in the ATP binding cassette protein subfamily D member 1 (*ABCD1*) gene are known to cause X-linked adrenoleukodystrophy (X-ALD). This study focused on the characteristics of *ABCD1* variants in Chinese X-ALD families and elucidated the value of genetic approaches for X-ALD.

Methods: 68 male probands diagnosed as X-ALD were screened for *ABCD1* variants by the Sanger sequencing of polymerase chain reaction (PCR) products and multiplex ligation-dependent probe amplification (MLPA) combined with long-range PCR. Prenatal diagnosis was performed in 20 foetuses of 17 probands' mothers. Descriptive statistics were used to summarise the gene variants and prenatal diagnosis characteristics and outcomes.

Results: This study allowed the identification of 61 variants occurring in 68 families, including 58 single nucleotide variants or small deletion/insertion variants and 3 large deletions. Three probands with no variants detected by next-generation sequencing were found to have variants by PCR-sequencing. Prenatal diagnosis found that 10 of the 20 foetuses had no variants in *ABCD1*.

Conclusion: PCR primers that do not amplify the pseudogenes must be used for PCR-sequencing. MLPA combined with long-range PCR can detect large deletions and insertions, which are usually undetectable by PCR-sequencing. Prenatal diagnosis could help to prevent the birth of infants with X-ALD.

KEYWORDS
adrenoleukodystrophy, ATP binding cassette transporter-subfamily D and member 1, Chinese cohort, prenatal diagnosis

1 | INTRODUCTION

X-linked adrenoleukodystrophy (X-ALD, OMIM#300100) is an X-linked disorder of peroxisomal metabolism, which typically presents with the impaired degradation of saturated very long-chain fatty acids (VLCFA) (Moser, 1997; van de Beek et al., 2016). The average incidence of X-ALD in males is between 1:20,000 and 1:30,000 (Wiesinger et al., 2015). The

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major forms of X-ALD are childhood cerebral adrenoleukodystrophy, adrenomyeloneuropathy, and Addison's disease (De Beer et al., 2014; Huffnagel et al., 2017; Kemp et al., 2016; Liberato et al., 2019). By the age of 60, about 80% of female heterozygotes develop neurological manifestations (Marc et al., 2014; Moser & Ali, 2018). Age and the duration of symptoms seem to be positively associated with the rate of progression (Horn et al., 2015; Huffnagel, Dijkgraaf, et al., 2019; Huffnagel, Laheji, et al., 2019). The accumulation of VLCFA in plasma is present in all X-ALD males, including asymptomatic individuals, which is highly diagnostic for affected males and some females with X-ALD. Approaching 20% of females with X-ALD have normal plasma VLCFA levels (Jangouk et al., 2012; Kemp et al., 2012). In the absence of elevated VLCFA, the diagnosis of X-ALD can be made by identifying variants in the gene associated with X-ALD (Schackmann et al., 2016). The prior study has noted that the analysis of C26:0-lysophosphatidylcholine (C26:0-LPC) has superior diagnostic performance compared to VLCFA analysis for X-ALD but not yet used (Huffnagel et al., 2017; Jaspers et al., 2020). Variants in the ATP binding cassette protein subfamily D member 1 (*ABCD1*) gene are known to cause X-ALD. Currently, next-generation sequencing (NGS) is an efficient method for the detection of monogenic disorders. However, there are pseudogenes in the regions of human chromosomes 2p11, 10p11, 16p11, and 22q11, which are highly similar to the exons 7–10 of *ABCD1* (similarity of 92–96%) (Eichler et al., 1997). The presence of pseudogenes may interfere with variant detection especially when using NGS. Variants in pseudogenes may be misidentified as variants in the *ABCD1* gene, resulting in misdiagnosis of the disease (Lupski et al., 2010). Therefore, NGS seems to be unsuitable for variant detection in the *ABCD1* gene. In this study, Sanger sequencing of polymerase chain reaction (PCR) products, multiplex ligation-dependent probe amplification (MLPA), and long-range PCR were used to perform variant detection in the *ABCD1* gene for X-ALD probands and their family members, and the value of the combined method for the accurate diagnosis of X-ALD was evaluated.

2 | METHODS

2.1 | Ethical compliance

The study was approved by the Ethics Committee of Peking University First Hospital.

2.2 | Patients

This study included 76 unrelated Chinese boys who were clinically diagnosed as X-ALD and 100 family members (60 mothers, 1 father, 2 brothers, 13 sisters, 6 maternal cousins, 12 maternal aunts, 3 maternal uncles, and 3 maternal grandmothers of probands). The main clinical manifestations of the probands were vision and hearing loss, unsteady gait, skin pigmentation, and intellectual deterioration. Laboratory tests revealed increased VLCFA and adrenal insufficiency. Diagnosis of the X-ALD probands was made on the basis of clinical findings and the characteristic elevation of VLCFA. Three (Patients X14, X18, and X67) of the 76 probands had been examined by NGS and no variant was found. Informed consent was obtained from their parents or guardians.

2.3 | DNA preparation and variant detection

Genomic DNA was extracted from peripheral blood leukocytes by QuickGene DNA whole blood kit (Kurabo, Osaka, Japan). The 10 exons comprising the coding region of the *ABCD1* were amplified by PCR. Primers were specifically selected to avoid amplification of the four pseudogenes (https://adrenoleukodystrophy.info/mutations-biochemistry/pseudogenes-mutation-analysis). The PCR protocol was according to previously described methods (Pan et al., 2005). Purified PCR products were then sequenced and compared with the human genomic *ABCD1* sequences (GenBank accession number: NM_000033.4). If no variant was found, MLPA (SALSA MLPA Probemix P049 SLC6A8-ABCD1, MRC-Holland, Amsterdam, The Netherlands) and long-range PCR were employed to detect large deletions or insertions. Pathogenicity was confirmed by searching databases including the Human Genome Variation Society (HGVS), online Mendelian inheritance in man (OMIM), the ClinVar database and the ALD database. Based on the American College of Medical Genetics (ACMG) standards and guidelines (Richards et al., 2015), variants not reported were classified into five categories: pathogenic, likely pathogenic, variants of uncertain significance, likely benign, and benign.

Prenatal diagnosis of foetuses was performed in 20 pregnancies of 17 mothers who were either women carrying a defective *ABCD1* gene or women at risk of developing X-ALD. DNA was extracted from chorionic villi or amniotic fluid using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and detected by PCR sequencing and MLPA. Linkage analysis was also performed to exclude false results due to maternal blood contamination using five short tandem repeat (STR) markers close to *ABCD1* and identify whether foetuses carry disease-causing chromosomes (Steinberg et al., 2005).

Descriptive statistics were used to summarise the gene variants and prenatal diagnosis characteristics and outcomes.

3 | RESULTS

3.1 | Variants of the *ABCD1* gene were detected by the combined method

A total of 76 male X-ALD probands (aged 1 year and 9 months to 16 years) were subjected to molecular diagnosis of the disease, and 68 (89.5%) were found to have variants in the *ABCD1* gene, including 52 pathogenic variants (85.2%, 52/61), 4 likely pathogenic variants (6.6%, 4/61) and 5 variants of uncertain significance (8.2%, 5/61); in total, there were 61 kinds of variants, of which 24 variants were not recorded in the ALD database (https://adren oleukodystrophy.info/mutations-and-variants-in-abcd1) (Figure 1). Of the 56 pathogenic and likely pathogenic variants, there were 30 missense variants (53.6%, 30/56), 5 nonsense variants (8.9%, 5/56), 15 frameshift variants (26.8%, 15/56), 3 large deletions (5.3%, 3/56), 2 splicing variants (3.6%, 2/56) and 1 insertion (1.8%, 1/56). Fifty-three variants were unique to individual families (86.9%), and eight variants were found in more than one. Three probands carried two variants in the *ABCD1* gene: proband

X17 carrying c.1909delA (pathogenic) and c.1911_1920del (pathogenic), proband X44 carrying c.1270C>G (uncertain significance) and c.1271_1275del (pathogenic), and proband X72 carrying c.910G>C (uncertain significance) and c.910_911ins21 (likely pathogenic).

In three probands (Patients X14, X18, and X67), the variants had not been determined by the NGS method but were detected by PCR-sequencing method. Two variants were missense variants and one was a nonsense variant, and they were located in exon 10, 7, and 8, respectively, which were pseudogene regions of *ABCD1*.

The probands with typical clinical symptoms but without variants in the *ABCD1* gene by PCR-sequencing were subjected to MLPA combined with long-range PCR to identify any large deletions or insertions in the gene.

Patients who had been previously screened for variants in the *ABCD1* by PCR-sequencing and had failed to amplify were tested by MLPA. Two patients (X32, X76) were shown to have a deletion of exon 10 and a deletion of exons 6–10, respectively, by MLPA. One patient (X53) whose *ABCD1* gene failed to be amplified by PCR-sequencing but was amplified successfully by MLPA was screened by long-range PCR with two primers, one within the MLPA region and the other downstream of the MLPA region. These combined approaches confirmed the large deletion of patient X53 (c.254_c.900+760del1406) (Figure 2).
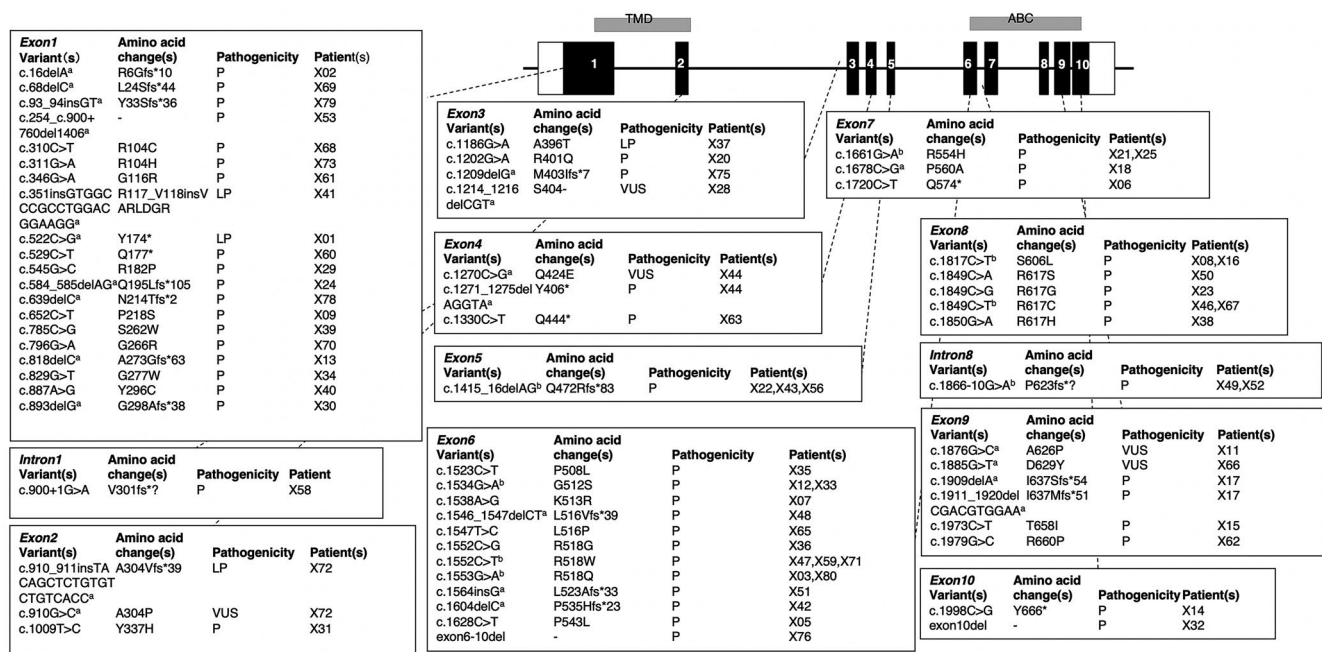

FIGURE 1 61 different *ABCD1* variants in 68 X-ALD families. [a]Novel variants are reported here for the first time. [b]Variants are detected in more than one family. P, pathogenic variant; LP, likely pathogenic variant; VUS, variant of uncertain significance; TMD, transmembrane Domain; ABC, ATP Binding Cassette
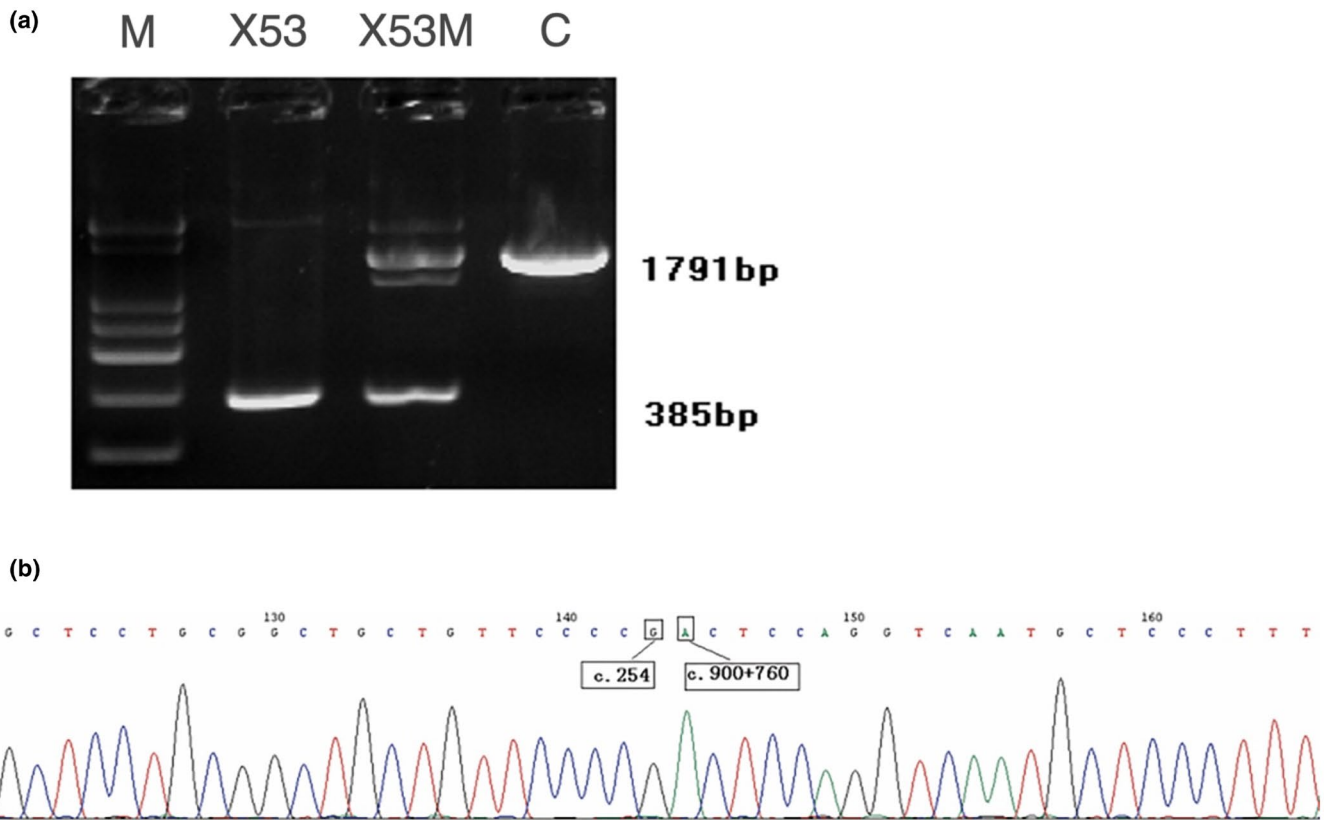
FIGURE 2 The results of long-range PCR and Sanger sequencing. (a) Long-range PCR is used in pedigree X53. The patient X53 and his mother are checked in lanes X53 and X53 M, respectively. The negative control and DNA marker are shown in lanes C and M. Lane C only has one band (1791bp). X53 only has one band (385bp) and his mother has two bands (1791bp and 385bp). (b) Sanger sequencing results of patient X53

3.2 | Maternal carrier status and prenatal diagnosis were investigated using the same method

The carrier status of 60 mothers and 40 relatives of the probands was analysed by PCR-sequencing or the same methods as used for the probands. The results indicated that 52 mothers (52/60, 86.7%) and 19 other family members (including sisters and maternal cousins/aunts/grandmothers of the probands, 19/40, 47.5%) carried the same variants as the probands.

17 mothers of the probands, including 16 females carrying a defective *ABCD1* gene and 1 female with no variant detected in her peripheral blood, and three of which were pregnant twice during the study, asked for prenatal diagnosis of the foetuses. All pregnancies were singleton gestations. The results showed that there were 14 male and 6 female foetuses. Sixteen heterozygous mothers had ten foetuses that carried the same variants as the probands (six males and four females) and nine foetuses with no variant detected (seven males and two females); one woman with no variant detected had a male foetus with no detectable variants

in the gene. STR genotypes were consistent with the genetic diagnosis.

3.3 | The verification results were in accordance with the prenatal diagnosis

Ten foetuses with no variants detected were genetically tested post-natally and no variants were found in these newborns; this was consistent with the prenatal diagnosis.

In the ten cases where foetuses carried variants, all parents chose to terminate the pregnancies. After the induction of labour, genetic tests were performed on the foetuses, and the results were consistent with prenatal diagnosis.

4 | DISCUSSION

In 1976 (Igarashi et al., 1976), the first report appeared on the characteristic accumulation of saturated unbranched VLCFA in brain lipids and the adrenal

cortex in X-ALD. In the early 1980s, the measurement of VLCFA in the plasma provided a reliable diagnostic test (Moser et al., 1981), which biochemically confirmed the suspected diagnosis via the measurement of VLCFA (Moser et al., 1999). X-ALD was linked to the long arm of the X chromosome (Xq28) in 1981 (Migeon et al., 1981). The gene predisposing for X-ALD (*ABCD1*) was identified in 1993 (Mosser et al., 1993). In 1997 (Eichler et al., 1997), four paralogues of *ABCD1* (2p11, 10p11, 16p11 and 22q11) were shown to share 92–96% of nucleotides with *ABCD1*, which made the sequence analysis of *ABCD1* difficult. In 1999, Boehm et al. developed and validated the Xq28 *ABCD1* specific primers that provided a highly reliable means of determining *ABCD1* variants (Boehm et al., 1999). A review of *ABCD1* variants in X-ALD found that the majority of the variants were single nucleotide variants (Kemp et al., 2001).

There is no known genotype-phenotype correlation in X-ALD (Wiesinger et al., 2015). Currently, hematopoietic stem cell transplantation (HSCT) remains the only disease-modifying therapy if completed in the early stages of cerebral adrenoleukodystrophy, but it does not affect the course of adrenal insufficiency (Kemp et al., 2016). In addition, it does not reverse neurologic findings present at the time of HSCT and does not stabilize cerebral disease for 3 to 24 months after stem cell infusion (Zhu et al., 2020). No curative treatment for X-ALD is currently available. The most commonly recurring pathogenic variant is c.1415_1416delAG in exon 5 (Kemp et al., 2001), which was found in three families in this study. There were no other apparent hotspots. Although variants have been documented throughout the entire *ABCD1* gene, the variants are unevenly distributed. More variants are distributed in exon 1 (32.8%) and exon 6 (19.7%), encoding the transmembrane domain and the ATP-binding domain of ALD protein, respectively. Variants in exons 1 and 6 account for 43% and 11% of the variants in the ALD database, respectively. A previous study has also reported that exon 6 is a potential variant cluster region in Asian populations (Niu et al., 2013).

Many variants have been described since the cloning of the *ABCD1* gene in 1993 (Mosser et al., 1993). Most variants found in X-ALD are single nucleotide variants and deletion/insertion variants. We performed PCR-sequencing for variant detection in the *ABCD1* gene in 76 Chinese X-ALD probands and identified 68 probands with variants in the *ABCD1* gene. Most of our probands had single nucleotide variants or small deletion/insertion variants that can be detected by PCR-sequencing, indicating that this method is suitable for the molecular diagnosis of most X-ALD patients.

There are four *ABCD1* pseudogenes in the human genome. False-positive or -negative results seen by the

NGS method are primarily due to the interference from pseudogenes (Claes & De Leneer, 2014). Excluding any interference from pseudogenes is, therefore, necessary for the detection of *ABCD1* variants. We used the PCR primer pairs listed in the ALD database (www.adrenoleukodystrophy.info) and the PCR-sequencing method to obtain the correct molecular diagnosis of X-ALD. In this study, the variants in three probands had not been determined by NGS but were detected by the PCR-sequencing method and the variants were located in the pseudogene region of *ABCD1*, which suggested that the NGS method was not the best method for this gene.

However, PCR-sequencing may be unable to detect large insertions and deletions in females with X-ALD and some male patients. MLPA is therefore a useful approach to identify these variants. In this study, two large deletions were detected by these methods. However, sometimes MLPA is not effective. One patient whose *ABCD1* gene failed to amplify by PCR-sequencing but was amplified successfully by MLPA underwent long-range PCR with two primers, one within the MLPA region and the other downstream of that region. These combined approaches confirmed the large deletion in patient X53 (c.254_c.900+760del1406).

We also performed prenatal diagnosis in 20 foetuses (14 males and 6 females) and identified 10 normal foetuses (8 males and 2 females) without identified variants in the *ABCD1* gene, which was confirmed by postnatal examinations. Prenatal diagnosis for X-ALD provides the parents to make an informed decision whether they want to continue the pregnancy or not. The identification of X-ALD infants allows for close clinical follow-up and the earlier timing of treatment, which is also the period when the outcome of HSCT is excellent.

NGS, as an efficient method for the detection of monogenic disorders, may be negatively affected by the presence of pseudogenes. As there was a defect in NGS, we used PCR-sequencing, MLPA, and long-range PCR to identify variants in X-ALD patients, which showed a high diagnostic yield (68/76, 89.5%), indicating that the combination of the three methods had obvious advantages in the diagnosis of X-ALD. If some samples fail to amplify by PCR-sequencing, MLPA must be performed.

Large deletions accounted for 3.9% (3/76) of variants in this study. In females with heterozygous deletions, the *ABCD1* gene can be successfully amplified by the PCR-sequencing method, which highlights the limitation of using PCR-sequencing alone as a diagnostic tool in female probands. Therefore, adding MLPA to the variant analysis of *ABCD1* in female probands will provide a comprehensive and solid evaluation.

5 | CONCLUSION

Due to the presence of pseudogenes, PCR-sequencing is superior to NGS for the molecular diagnosis of X-ALD. The combined use of MLPA and long-range PCR is a useful approach with which to detect large insertions and deletions, which may be undetectable by PCR-sequencing in females with X-ALD and some male patients. There were no hotspot variants in this series of X-ALD patients and most were inherited from the patients' mothers. Prenatal diagnosis for mothers of X-ALD patients could help to prevent the birth of infants with X-ALD.

CONFLICTS OF INTEREST

All authors have no conflicts of interest relevant to this article to disclose, and the study was done with no specific support.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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