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ORIGINAL RESEARCH

# Successful Pregnancy Following Preimplantation Genetic Diagnosis of Adrenoleukodystrophy by Detection of Mutation on the *ABCD1* Gene



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**Background:** Adrenoleukodystrophy (ALD) is a rare sex-linked recessive disorder that disrupts adrenal gland function and the white matter of the nervous system. According to recent epidemiological statistics, up to this moment, the disease is the most recorded peroxisomal disorder. *ABCD1* is a gene related to ALD, with more than 850 unique mutations have been reported. Early diagnosis of the disease would help to consult families with ALD to plan for interventions to prevent passing along the pathogenic mutations to their children.

**Material and Methods:** A heterozygous *ABCD1* gene mutation related to ALD found in a Vietnamese woman was used to design primers for the polymerase chain reaction (PCR) to amplify the segment spanning the mutation. Then, combining sequencing methods for the PCR products, especially Sanger sequencing and next-generation sequencing (NGS), a protocol was developed to detect mutations on the *ABCD1* gene to apply for the DNA samples of in-vitro fertilization (IVF) embryos biopsied at the blastocyst stage to screen for pathogenic alleles.

**Results:** The established protocol for PGD of ALD detected mutant alleles in 5/8 embryos (62.5%), while the remaining 3 embryos (37.5%) did not carry any mutation. One of the 3 embryos was transferred, and a healthy female baby was born after a full-term pregnancy.

**Conclusion:** The developed protocol was helpful for the preimplantation genetic diagnosis process to help families with the monogenic disease of ALD but wish to have healthy children.

**Keywords:** adrenoleukodystrophy, ALD, peroxisome disorder, *ABCD1* gene mutation, preimplantation genetic diagnosis, PGD

## Introduction

ALD is a rare sex-linked hereditary disease with the incidence dropped between 1:20,000 and 1:30,000 male newborns, without significant differences among countries and ethnic groups globally; however, this figure is increasing prior to the widespread application of newborn screening. Mutations in the *ABCD1* gene are the pathogenic factors leading to the impaired β-oxidation process in peroxisomes. All patients with ALD carry the mutations; up to date, more than 850 non-recurrent mutations of this disease have been cataloged without correlation to phenotypes.[1–3] The gene *ABCD1* is located near the end of the X chromosome's long arm: at Xq28 and has a length of 19.9 kb with 10 exons.[4,5] *ABCD1* encodes a transmembrane protein made up of 745 amino acids called adrenoleukodystrophy protein, or ALDP.

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This protein is located across the peroxisome membrane, transporting VLCFA (very long chain fatty acids) from the cytoplasm through the membrane into the organelle to participate in the β-oxidation process.[6–8] Mutations in the *ABCD1* gene will directly affect the ALD protein, result in a change in the protein's structure and quantity, making it unable to carry out its transport function, thereby causing the accumulation of VLCFA in the cytoplasm. This accumulation has negative effects on cells, especially on nervous system cells.[1,9,10]

There are three main phenotypes of ALD that can be listed as follows: cerebral ALD, adrenomyeloneuropathy (AMN), and primary adrenal insufficiency. ALD patients are often asymptomatic at birth; however, clinical symptoms will potentially develop during life, which will eventually cause severe disabilities, especially in males. As the disease progresses, patients might develop primary adrenal insufficiency symptoms and neurologic disease. Hence, ALD is a rapidly progressive disorder that may cause devastating situations for affected patients since an effective treatment such as stem cell transplant can only prevent disease development yet leave them at risk for AMN. In order to find the appropriate therapy, patients must have regular screening to timely detect and diminish the possibility for lethal disease.[11–15]

ALD is a sex-linked recessive genetic disorder with 95% of reported cases received the *ABCD1* pathogenic variant from their parents; only about 5% of reported cases are caused by de novo mutations.[1,4,16,17] Heterozygote detection for at-risk females leading to prenatal testing or preimplantation genetic testing for at-risk pregnancies is necessary if the familial pathogenic variant is known.[18] In the last decades, preimplantation genetic diagnosis plays a valuable tool to avoid inherited diseases by transferring unaffected in-vitro fertilization (IVF) embryos and having alternative potentiality to prenatal diagnosis. Indeed, PGD first concluded the selection usually embryos for patients at risk of transmitting X-linked recessive disorders.[19,20] Furthermore, the widespread application of advances in molecular biology fosters the advent of specific diagnoses for monogenic defects. The fifth report of the European Society for Human Reproduction and Embryology (ESHRE) PGD Consortium recommended over 40 monogenic diseases with PGD indication.[21] However, setting up and testing embryonic molecular diagnosis is work-intensive, precise, and costly because the main reason, which is the DNA sample, is limiting. Much effort has been spent to obtain genomic DNA of adequate and quality for genetic analysis. Consequently, whole genome amplification (WGA)[20] is a standard solution to solve the existing problem, but its main drawback is the generation of nonspecific amplification artifacts.[22,23] As a result, we described a PGD protocol using WGA followed by conventional PCR, then applying Sanger sequencing for detecting the mutation. This work aimed to expand the dependability of PGD for ALD and increase the accuracy and economic effect of the test.

# Materials and Methods

## Patients Description

A 26-year-old Vietnamese female partner whose family has a background of ALD was enrolled in the study. Her healthy husband was 28 years old and did not present any clinical or genetic alteration of interest. By studying her genetic information with Next-Generation Sequencing (NGS), it was reported that the wife carried a heterozygous mutation c.854G>C (p.Arg285Pro) on exon 1 of *ABCD1*. According to up-to-date statistics related to ALD (https://adrenoleukodystrophy.info/, accessed April 2021), the mutation c.854G>C has only been reported as pathogenic twice in the past, none of which was found in Vietnam, for that reason, the mutation identified in this female carrier can be considered as the third time recorded in the history of research on ALD and the first report in Vietnam.

All people described in this research were signed written informed consent for the publication of the case details, and the protocol was approved by the Ethical Review Committee of Vietnam Military Medical University (No.1068/2019/VMMU-IRB). This study was also conducted using good clinical practice following the Declaration of Helsinki and its later amendments or comparable ethical standards.

## DNA Extraction from Whole Blood

DNA was extracted from the collected blood samples by following the protocol of the G-spin™ Total DNA Extraction Kit (Lot.No. 105260653; Exp. Oct. 2022). DNA went through a quality check process with a SpectraMax QuickDrop to measure optical density (OD) and $A_{260}/A_{280}$ index. Hence, the DNA collected from the sample was qualified to be used in the next steps of the research. DNA samples were stored at −20°C.

## Blastocyst Embryo Biopsy

There were seven embryos (TR1 – TR7) cultured to the fifth day and one embryo (TR8) cultured to the sixth day of the described couple who had done IVF at the Military Institute of Clinical Embryology and Histology (MICEH). Then, the embryos were biopsied (3–5 cells) and washed with PBS 1X and 1% PVP solution. After that, the washed embryo cells were contained in the 0.2 mL PCR tube. The embryo cells were stored at −20°C.

## Whole Genome Amplification for Embryos' Genome

The DNA from the biopsied embryos was amplified with REPLI-g® Single Cell Kit (Lot.No. 166013027; Exp. Sep. 2021) and diluted by nuclease-free water to reach a concentration of around 20 ng/μL. The concentration and purification were calculated with a SpectraMax QuickDrop. Therefore, the amplified DNA collected from the embryonic cells was qualified to be used in the research. DNA samples were stored at −20°C.

## Polymerase Chain Reaction (PCR) Analysis

First, primers were designed to amplify the segment spanning the detected mutation c.854G>C. Afterward, a PCR was performed by using the designed primers and DNA collected from the mother – the mutated allele carrier and DNA amplified from biopsied embryonic cells. PCR products were then electrophoresed on 2% agarose gel on multiSUB Choice, Wide Midi Horizontal Electrophoresis System (Cleaver Scientific, SKU: MSCHOICE10) to check for the appropriate desired products. The detailed information on the primers and PCR reaction was presented in Supplementary 1.

## Sanger Sequencing and the Next-Generation Sequencing Analysis

The amplified PCR products showing the accurate bands on electrophoresis results would be sequenced by Sanger sequencing to scan for the c.854G>C mutation. Next, any embryos that did not have any mutated allele would be screened for chromosomal abnormalities with Next-Generation Sequencing system. The flowchart of the study protocol was shown in Figure 1.

# Results

## Preimplantation Genetic Diagnosis Program for ALD

The PCR reaction using the described component and the thermal cycle was performed in triplicate. After that, the PCR products were electrophoresed on 2% agarose gel and observed under UV light, and the results obtained were consistent in all reactions. By annotating the gel electrophoresis (Supplementary 2), it could be stated that the PCR reaction successfully amplified the desired gene segment of all embryos in comparison with the positive control that was the heterozygous woman's DNA. The product bands appeared bright and clear at the position corresponding to the standard scale size of about 200 bp, proving that the amplified segment had the size of 199 bp, consistent with the initial expectation. From here, the PCR products would be purified and sequenced via the Sanger method to detect the c.854G>C mutation in these cells.

## Sanger Sequencing and NGS Results

Sanger sequencing was carried out using only one primer to amplify the forward strand so that the interpretation of the results would be more homogeneous. The obtained electropherograms were analyzed using BioEdit software (Supplementary 3) and the annotated results are shown in Table 1.

Sanger sequencing results showed 3 embryos (37.5%) that did not carry any pathogenic mutant alleles. In comparison, 4 embryos (50%) had the allele with the pathogenic mutation in the heterozygous form, which had the same mutation peak as the mother or the positive control, and 1 embryo (12.5%) carried the pathogenic allele in the hemizygote form. The father was known to carry no ALD mutation proves that embryos carrying pathogenic alleles during meiosis to form gametes have received the mutated alleles from the mother. These embryos will be assessed as unsuitable for use in embryo transfer, and those that do not carry the mutant allele will continue to be subjected to preimplantation genetic screening tests for evaluating the quality of embryos before transferring. For DNA samples of TR1, TR5, and TR7 embryos with normal results which do not carry any pathogenic mutant allele will be sequenced according to the next-generation sequencing method to screen for chromosomal abnormalities. NGS screening results for these 3 embryos without ALD are shown in Supplementary 4.

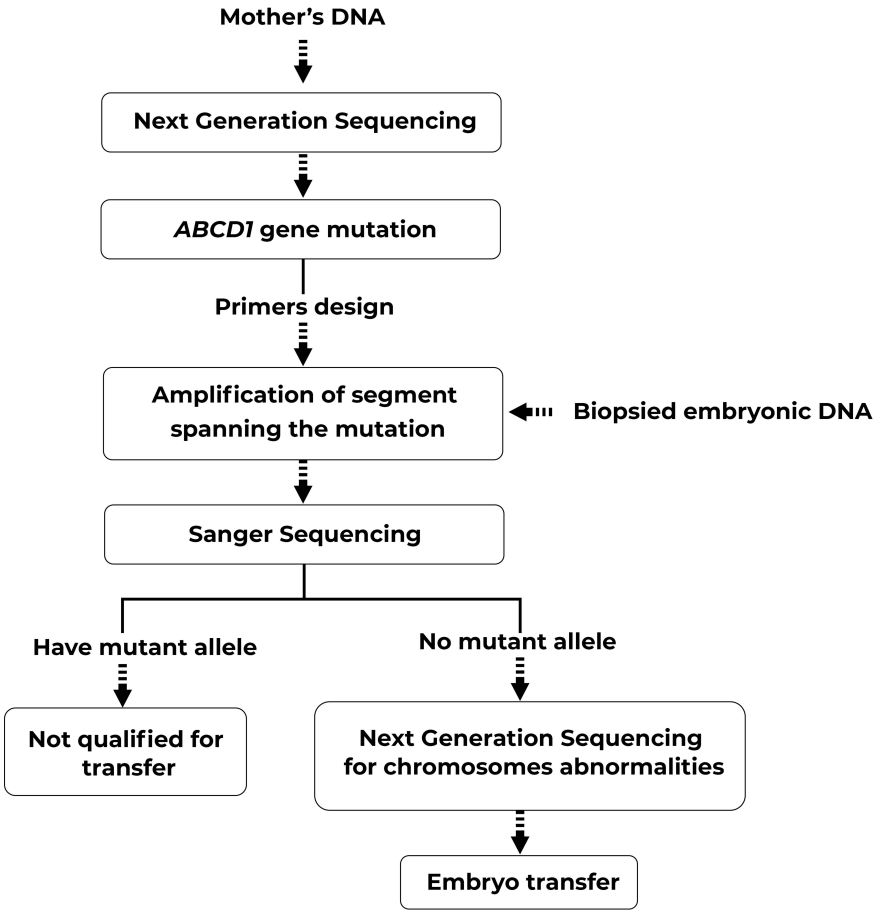


**Figure 1** Flowchart of PGD protocol for ALD.

Both TR1 and TR7 samples had abnormalities in the chromosomes; in detail, the TR1 sample had abnormalities in Chromosome 11 when TR7 has abnormalities in Chromosome 2, 4, 6, 8, 11, 17, 18, whereas the sample TR5 had none of these. Therefore, we concluded that the TR5 embryo's quality was acceptable and should be transferred into the mother's uterus.

## Discussion

As in many X-linked recessive disorders, it can be assumed that females remain asymptomatic carriers; they often have an association with their extended family having reported ALD patients. However, Engelen et al suggested that heterozygous women with ALD did show milder symptoms of neurologic diseases compared to men at a later stage of their lives, especially in their 40s and 60s.[24] Consequently, a genetic diagnosis of ALD is necessary to detect heterozygous women who could be offered prenatal or preimplantation diagnosis for pregnancies in the future.[2,25] Prenatal diagnosis of ALD was first performed in 1982 when Moser et al carried out amniocentesis in heterozygous women for the VLCFA level determination.[26] Later on, Imamura et al documented the mutation by genome analysis. Hence, prenatal diagnosis was a crucial measure for families with ALD history by analyzing the fetal DNA from amniotic fluid or placenta source.[27,28] Nevertheless, amniocentesis is an invasive measure that may have a drawback of insufficient fetal DNA level collected for diagnosis and adversely affects the mother's health.

Because of solving the matters mentioned above, the preimplantation diagnosis was developed with molecular biology advances. In the beginning, PGD was mainly the selection of female embryos for patients having a background of X-linked recessive diseases.[19] However, half of the discarded male embryos which are not transferred are unaffected by the disease. In this way, not only does the pregnancy outcome decrease, but it also brings up many ethical problems. Otherwise, fifty percent of selected female embryos carried the mutant gene that would

**Table 1** Genotypes of Eight in vitro Fertilization Embryos by Annotating Sanger Sequencing Results

| Samples | Diagnosis | Sex |
|---|---|---|
| TR1 | Wild-type homozygous | Female |
| TR2 | ALD heterozygous | Female |
| TR3 | ALD heterozygous | Female |
| TR4 | ALD heterozygous | Female |
| TR5 | Wild-type homozygous | Female |
| TR6 | ALD hemizygous | Male |
| TR7 | Wild-type homozygous | Female |
| TR8 | ALD heterozygous | Female |

transmit the mutated allele to the next generation, so the inheritance of the disease to the offspring is not permanently stopped. There are prior reports of sex selection using different techniques to identify affected Xq28 loci. They had the same procedure and outcome: to use multiple displacement amplification technologies for adequate input DNA materials and, in the end, to determine the sex of the embryos. Only one prior study reported by Miriam Iglesias et al in 2008 that made use of PGD to prevent ALD indicated a successful pregnancy.[29–32] Up to date, molecular biology becoming more and more essential with many advances has led to the fact that, once the mutation is identified, the couples would undergo specific genetic diagnosis instead of simple sex selection. Hence, our study with the successful live birth after a full-term pregnancy is more suitable with the modern trend and avoids ethical concerns by not choosing the sex but applying sequencing technology to determine the pathogenic gene.[33–35]

Setting up the new PGD protocol for monogenic diseases is always time-consuming and demanding much of the labor's effort to improve the technique. Firstly, multiple biopsy methods are now available with their advantages. Among them, 5–10 trophectoderm (TE) cells biopsied at the blastocyst stage provide more genetic material as well as make the diagnosis more reliable and have fewer errors. Even though the chosen biopsy method generates the highest DNA yields, WGA is still necessary to increase the amount of template DNA available up to $10^6$ times for diagnostic reactions, then template DNA is further amplified in a targeted fashion using conventional PCR.[36–38] PCR primers must be explicitly designed to amplify the mutation and optimize other PCR conditions to diminish the amplification failure and ADO rate.

The Sanger sequencing method was first developed in 1977. Though spreading current NGS circulation, improvements in the Sanger sequencing methodology, commercialization, and automation have enabled it to remain the most suitable sequencing method for many current applications. Sanger sequencing technology remains vastly advantageous for applications where high throughput is not required.[39] Our PGD procedure was carried out on the embryos of one family and owing to its rapidity and efficiency. However, the embryonic aneuploidy prevalence was high even in young patients with a monogenic disorder background. The majority of the patient population (53.2%) had at least one blastocyst with unaffected single gene defect were aneuploid, approximately 26.5% of normal or non-pathogenic blastocysts diagnosed based on monogenic disease PGD were aneuploid which would have been transferred, resulting in negative impacts in pregnancy outcome unless PGS had performed.[40] Conversely, concurrent screening demonstrably aided in embryo selection, as evidenced by the significant improvement in single embryo transfer rates, so 24-chromosome aneuploidies screening became more and more popular to apply in embryos biopsied for PGD to reduce the risk of miscarriage in the gestational period.[41–43] Indeed, PGD combined with PGS allowed opting for an embryo that did not carry the mutated allele and euploid embryos to transfer into the mother's uterus then, fortunately, achieved pregnancy with a baby girl. Later on, her peripheral blood sample was collected and applied to the developed protocol to confirm the accuracy. The baby girl was a wild-type homozygote and completely free of ALD.

## Conclusion

The established protocol was helpful for the preimplantation genetic diagnosis process to help families with the monogenic disease of ALD but wish to have healthy children. This process not only has the potential to identify embryos with pathogenic mutations but also prevent their inheritance among generations at an early stage.

## Data Sharing Statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request. If you have concerns about sharing the data, please contact vunhatdinh@vmmu.edu.vn.

## Ethical Statements

All people described in this research were signed written informed consent for the publication of the case details, and the protocol was approved by the Ethical Review Committee of Vietnam Military Medical University (No.1068/2019/VMMU-IRB). This study was conducted following the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## Consent for Publication

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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