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Successful Pregnancy Following Preimplantation Genetic Diagnosis of Neonatal Diabetes Mellitus by Detection of Mutation on the *ABCC8* Gene

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Abbreviations: NDM: Neonatal Diabetes Mellitus; MODY: Maturity-Onset Diabetes of The Young; PGD: Preimplantation Genetic Diagnosis; PGT-M: Preimplantation Genetic Testing for Monogenic Defects; ESHRE: European Society Reproduction and Embryology; MICEH: Military Institute of Clinical Embryology and Histology; NGS: Next-Generation Sequencing

ABSTRACT

Background: Neonatal Diabetes Mellitus (NDM) is a scarce form of diabetes defined by hyperglycemia happening principally before 6 months old that affects from 1:100,000 to 1:400,000 live births across the globe. That is a monogenic disease related to mutations on the *ABCC8* gene which encodes the sulfonylurea receptor 1 (SUR1) at the surface of the pancreatic beta-cell and rectifies potassium channel proteins [1-3]. Early diagnosis with appropriate preimplantation genetic testing for monogenic defects (PGT-M) can help avoid passing along the pathogenic mutations to the next generation.

Materials and methods: Designing specific primers based on a heterozygous *ABCC8* gene mutation of the father for Polymerase Chain Reaction (PCR) the mutant DNA segment which came from blastomere obtained after embryo biopsy. Sanger sequencing was applied to detect the mutation on the *ABCC8* gene of all blastocyst stage embryonic cells.

Result: Only two out of five embryos were heterozygotes with the mutation on *ABCC8* gene while the other three were found not to carry the screening mutation. Then, one of the three wildtype embryos was transferred and achieved a successful pregnancy and birth.

Conclusion: The established protocol could help in preimplantation genetic testing for monogenic defects so that families with the monogenic disease of NDM could have healthy children that did not carrying any mutant alleles.

Keywords: Neonatal Diabetes Mellitus; NMD; Monogenic Disorder; *ABCC8* Gene Mutation; Preimplantation Genetic Diagnosis; PGD

Introduction

Diabetes mellitus is a group of metabolic disorders caused by hyperglycemia resulting from problems in production and/or use of insulin, furthermore considered as one of the largest global health emergencies in the last decades with 463 million people affected [4,5]. The disease consists of three common types are diabetes type 1, type 2, and gestational diabetes, which are generally polygenic diseases. As monogenic diabetes also exist, accounting for 1-2% of all cases, there are two main types including Maturity-Onset Diabetes of the Young (MODY) and Neonatal Diabetes Mellitus (NDM) differing in the age of onset. They are often misdiagnosed into type 1 or type 2 diabetes, therefore, leading to ineffective treatments and familial genetic counseling [6,7]. Neonatal diabetes mellitus, typically onsetting before 6 months of age, may be categorized into transient and permanent depending on the disease course, albeit at a diminished prevalence [8-10]. Transient NDM (TNDM) makes up a relatively significant proportion of the total affected cases, approximately 60%. With initial insulin treatment, TNDM resolves after around 12 weeks of age but eventually relapses years later. While permanent NDM (PNDM) is less prevalent, neonates must be treated lifelong [11]. The incidence of NDM dropped from 1:100,000 to 1:400,000 live births across the globe. Up to date, it is determined that more than 20 mutant genes and abnormal methylation at the 6q24 locus may lead to NDM with correlation to clinical phenotypes [12-14].

One of the most common causes of NDM is the active mutations of the gene *ABCC8* located in the 11p15.1 region, which has a length of 84kb with 39 exons [14]. This gene encodes the sulfonylurea receptor 1 (SUR1) at the surface of the pancreatic beta-cell and rectifies potassium channel proteins [1-3]. When serum glucose levels rise, it is rapidly taken up to intracellular β-cells and metabolized to ATP causing to close the potassium ATP channel. Consequently, the pancreatitis β-cell depolarizes, leading to activation of calcium channels and an increase of the calcium concentration that triggers insulin release. Conversely, activation *ABCC8* gene mutation causes the potassium ATP channel to remain open, therefore preventing insulin release even in hyperglycemia circumstances. To date, a total of 748 *ABCC8* pathogenic or likely pathogenic variants with NDM have been identified [15,16]. Early genetic diagnosis is important for selecting suitable and cost-effective treatment because oral sulfonylurea is a safe and effective therapy in most cases having *ABCC8* mutation, and may potentially replace insulin injection which is the first-line treatment in type 1 diabetes mellitus. Especially, if the familial pathogenic variant is known, preimplantation genetic testing for monogenic defects (PGT-M) or preimplantation genetic diagnosis (PGD) can help avoid this inherited disease. The most essential outcome is to identify the disease-causing loci then transfer unaffected embryos made by IVF technology for Human embryos. PGT-M refers to testing pathogenic variants of nuclear or mitochondrial DNA causing monogenic disorders, with autosomal dominant, autosomal recessive, X-linked defects [17]. The indications are currently recommended in over 40 monogenic diseases by the European Society Reproduction and Embryology (ESHRE) PGD Consortium [18].

However, setting up an embryonic molecular diagnosis for a new monogenic defect is work-intensive, costly, and requires high precision because of several challenges including an insufficient amount of obtained DNA sample by embryo biopsy. Therefore, DNA amplification techniques are necessary for ensuring the quantity and quality of input DNA. In detail, one or more embryonic cells in different stages of embryonic proliferation experienced a whole genome amplification (WGA) followed by targeted conventional PCR, then appropriate sequencing method, such as Sanger sequencing or next-generation sequencing is applied for detecting pathogenic variants [19,20]. Each method has several advantages and limitations, separately, thus we have spent much effort to expand the reliability of PGT-M for NDM and enlarge the accuracy and commercial effect of the test.

Materials and Methods

Patients Description

A Vietnamese nuclear family enrolled in the study including a 29 years old mother, a 30 years old father, and three years old sons. The son was diagnosed with NDM at 8 weeks old and the father was diagnosed with type 2 diabetes at age 28 while the mother had no clinical or genetic alteration of interest. By examining the affected's genetic information using Next-Generation Sequencing (NGS), both the father and the son were reported to carry a heterozygous mutation c.3422A>G (p.Glu1141Gly) at exon 28 of the *ABCC8* gene. This is a protein-level missense mutation that has not been reported in Vietnam. Therefore, the mutation identified in this patient can be considered to be the first time recorded in Vietnam. All people described in this study 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 with the G-spin™ Total DNA Extraction Kit (Lot.No. 105260653; Exp. Oct. 2022) then was quantified to ensure the quality. The DNA collected from the samples was stored at −20°C for future purposes.

Blastocyst Embryo Biopsy

Five embryos (T1-T5) of the described couple who had done ICSI at the Military Institute of Clinical Embryology and Histology (MICEH) were cultured to the blastocyst stage. These embryos were biopsied by removing 5-10 cells from the TE layer (a portion of the embryo that is destined to become placental tissue) herniating out through the hole created during ZP breaching on day 3. The cells that had been biopsied were washed with PBS 1X and 1% PVP solution then contained in the 0.2 mL PCR tube. They were stored at −20°C.

Whole Genome Amplification for Embryos' Genome

The DNA from the biopsied embryonic cells was amplified with REPLI-g® Single Cell Kit (Lot.No. 169023130; Exp. May 2022) and diluted to a desired concentration. The amplified DNA from the samples was stored at −20°C.

Polymerase Chain Reaction (PCR) Analysis

Primers were designed targeting the segment spanning the detected mutation c.3422A>G. Based on the obtained primers, a PCR was performed on DNA samples that were both collected from whole blood and embryonic cells. Then, PCR products were electrophoresed on agarose gel to check for the appropriate desired products.

Sanger Sequencing Analysis

Sanger sequencing procedures were applied on the amplified PCR products showing the accurate bands on electrophoresis results to scan for the c.3422A>G mutation. This process was carried out on the ABI 3500 Genetic Analyzer system.

Results

Preimplantation Genetic Diagnosis Program for Neonatal Diabetes Mellitus

The PCR reactions were performed in triplicate showing overall consistent electrophoresis results. The annotation of gel electrophoresis indicated that the PCR reaction successfully amplified the segment targeting exon 28 of the gene *ABCC8* in all samples. The products had a size consistent with the initial expectation in theory corresponding to the standard 100 bp ladder. As a result, the PCR products would be sequenced to scan for the c.3422A>G mutation in the biopsied embryonic cells, hence leading to the final consultations for preimplantation genetic diagnosis for NDM.

Sanger Sequencing Results

Sanger sequencing was performed using two primers including both forward and reverse for a better interpretation of the results from parallel ends. The obtained electropherograms were analyzed and compared using SnapGene software. The Sanger sequencing results of the father and the son, who were found to be carrying c.3422A>G mutation on exon 28, were consistent with those of next-generation sequencing. Reading the electropherograms, at the mutated position, the diabetes patients illustrated two peaks of different colors (red and blue) regarding the heterozygotes of the *ABCC8* gene. The partial pedigree of the family carrying the mutation of *ABCC8* is demonstrated in Figures 1 & 2. Squares represent male family members, the circle represents a female family member; diagonally-lined pattern symbols family member with neonatal diabetes mellitus, black symbols family member with type 2 diabetes occurring later in life. The first line demonstrates the genotype of each family member: wt denotes wild-type. The second line displays the age of each family member and the third line shows the onset age of diabetes: the hyphen denotes unidentified data. The arrow indicates the proband. In addition, the annotated sequencing results of five *in vitro* fertilized embryos are presented in Table 1. Sanger sequencing results showed 60% of the biopsied embryos were not affected with the pathogenic allele while the remaining embryos had one allele with c.3422A>G. As neonatal diabetes in the family was formed with the heterozygous *ABCC8* mutation, the embryonic heterozygotes were also determined to be affected with NDM. Therefore, it could be concluded that only three embryos (code T1, T2, and T3) reached the expected quality and should be transferred into the mother's uterus.

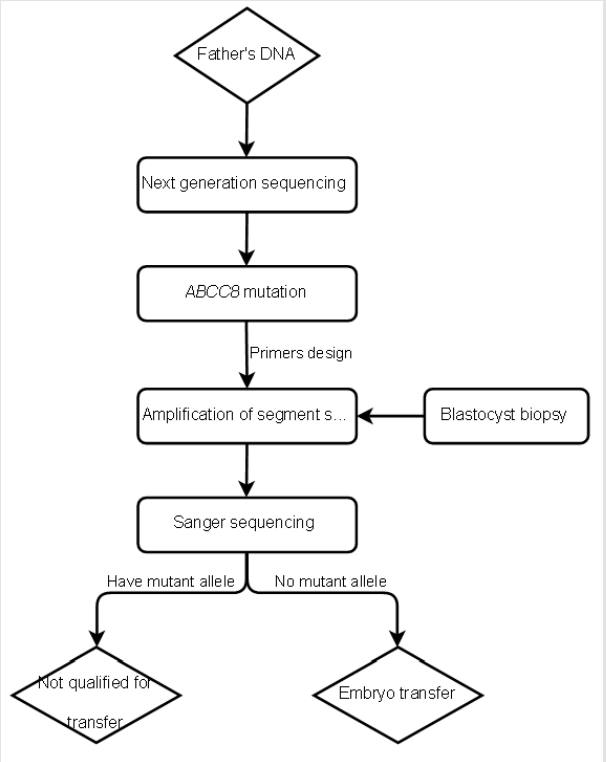

Figure 1: Flowchart of PGD protocol for NDM.

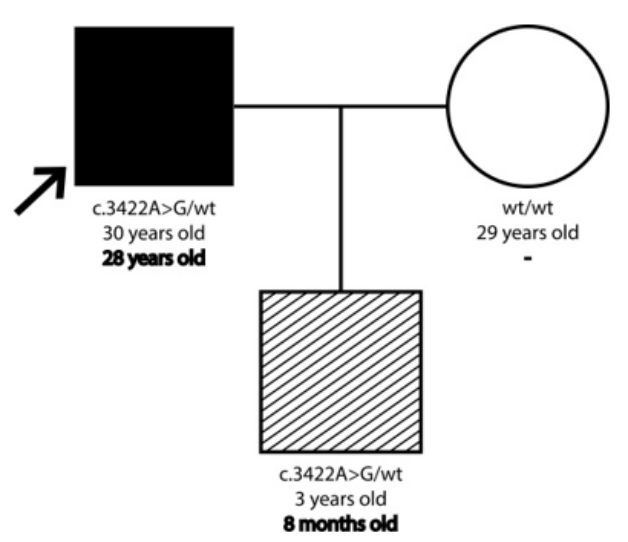

Figure 2: Diabetes and *ABCC8* mutation in the family enrolled in the study.

Table 1: PGD results of Five *in vitro* Fertilization Embryos by Annotating Sanger Sequencing Results.

Samples	Genotype	Diagnosis
T1	wt/wt	Normal
T2	wt/wt	Normal
T3	wt/wt	Normal
T4	c.3422A>G/wt	NDM affected
T5	c.3422A>G/wt	NDM affected

Discussion

Neonatal diabetes mellitus (NDM) is a scarce form of diabetes defined by hyperglycemia happening principally before 6 months old that affects all races and ethnic groups. It can be categorized into transient NDM (TNDM), which resolves by a few months, and permanent NDM (PNDM), which continues throughout life. TNDM, accounting for approximately 55% of all NDM cases, requires initial insulin treatment but appears to resolve spontaneously by a median of 12 weeks of age, only to relapse years later [1-3,21]. While most TNDM cases are due to the overexpression of chromosome 6q24, the majority of PNDM cases are caused by mutations in the *KCNJ11* or *ABCC8* genes, encoding the K_{ATP} channel subunits Kir6.2 (ATP-sensitive inward rectifier potassium channel) and SUR1 (sulfonylurea receptor) respectively [15,16,22]. By using both PCR and Sanger Sequencing, we found a point mutation on exon 28 of gene *ABCC8*. In detail, this is a substitution mutation, substituted nucleotide A with nucleotide G, leading to the replacement of Glutamine by Glycine, which causes missense mutation at the protein level.

Mutation on amino acid sequence encoded for high - conservative transport protein causes protein function to be affected seriously [23-25]. Up until now, there have not been any official publishments about this protein, however, according to HGSV's report (Human Genome Variation Society) in 2019, this is an autosomal dominant that cause TNDM (transient neonatal diabetes mellitus), which was first recognized in Paris, France [15]. Mutations in the *ABCC8* gene and *KCNJ11* gene lead to two different molecular mechanisms, however, at the physiological level, they both manifest as insulin deficiency and remain indistinguishable in clinical practice. Overlapping clinical features with regular forms of diabetes makes diagnosis of monogenic diabetes more challenging [7,25]. Accordingly, the recognition of these mutations in NDM patients is critical because it not only helps providing the diagnosis but also, builds the long-term medical treatment and management strategy and genetic counseling. Once patients being diagnosed with NDM due to *KCNJ11* gene and *ABCC8* gene mutations, using oral sulfonylurea agents proves highly effective, providing better glycemic control than insulin.

Of the known genetic subtypes of NDM, only patients with activating *KCNJ11* or *ABCC8* gene mutations respond to treatment with a sulfonylurea [14,15]. Bonnefond et al. carried out WES in a patient with NDM and identified a novel de-novo mutation in the *ABCC8* gene [26]. This mutation is very uncommon and causes no prevalent symptoms in clinical practice, which makes the role in diagnose of sequencing more critical [27,28]. The combination of both NGS and Sanger Sequencing makes detecting mutation from the beginning more accurate. Then applying Sanger to detect mutation in family members more simply and commercially. This protocol might be used widely to detect other mutations relating to monogenic diabetes in specific, and another monogenic diseases [29,30]. Before starting a clinical cycle, extensive genetic and reproductive counseling is provided to the prospective parent(s). Genetic consultation for people with NDM firstly relies on their genetic etiology because the genes involved vary widely in how they are inherited.

Most cases of TNDM are caused by UPD6, which occurs sporadically, so the probability of recurrence in siblings and offspring is low. In hereditary cases of paternal duplications in the chromosome 6q24 region, males with TNDM have a chance of 50% to transfer TNDM to their offspring. On the one hand, in the situation of maternal duplication, children are not influenced, but the sons might be able to pass TNDM to their offspring [21,22]. On the other hand, all most situations of KATP channel mutations in PNDM are the result of instinctive de novo heterozygous mutations, making a different to children without family etiology. However,

ABCC8 gene activating mutation can result in autosomal dominant inheritance, with a 50% chance of inheriting the mutation in the offspring of affected individuals [23,24,28,31]. Furthermore, unlike *KCNJ11* gene mutations, *ABCC8* gene mutations have been reported to be inherited in an autosomal recessive pattern. Therefore, understanding the inheritance mode is crucial and has changed the dynamics of genetic consultation.

In our study, embryonic cells are collected by using trophectoderm (TE) or blastocyst biopsy, which is most widely used technique at present the. Usually on day 5 (or 6), a laser beam is used to open the zona pellucida. Blastocyst biopsy provides sufficent cells (ideally five to eight cells) for genetic analysis providing a better accuracy. This embryonic stage is also considered less sensitive to possible embryo damage as the inner cell mass from which the fetus originates is left intact. Another benefit of trophectoderm biopsy is the lower level of chromosomal mosaicism at this stage as compared to the cleavage stage. The problem of limited time for analysis in the case of a fresh embryo transfer at day 5 or 6 is conquered by using frozen embryo transfer method [32-34]. Each method has several advantages and limitations, separately, thus we have spent much effort to expand the reliability of PGT-M for NDM and enlarge the accuracy and commercial effect of the test. In order to provide more DNA for genetic analysis, embryonic cells in different stages of embryonic proliferation experienced a whole genome amplification (WGA) followed by targeted conventional PCR, then appropriate sequencing method, in particular, Sanger sequencing is applied for detecting pathogenic variants [19,20].

Sanger sequencing results indicated that three embryos did not carry any pathogenic mutant alleles while two embryos (40%) had the same mutation peak as the father and the son, which means they had the allele with the pathogenic mutation in the heterozygous form. Because NDM in the family was created with the heterozygous *ABCC8* mutation, the embryonic heterozygotes were also determined to be affected with NDM. Therefore, it could be concluded that only three embryos meet the expected quality and one of those have been transferred into the mother's uterus, leading to success in giving birth to a baby without NDM. This protocol might be used widely to detect other mutations relating to monogenic diabetes in specific, and other monogenic diseases [32,35-38].

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