Genetic, Prenatal, and Preimplantation Genetic Diagnosis of Glutaric Aciduria Type I

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Abstract

Purpose: Glutaric Acid Type I (GA-I) is an inherited metabolic disorder. Although the treatment guidelines for GA-I were established a decade ago, they cannot block the vertical heredity. We aim to apply genetic methods to block the inheritance of GA-I and verifies the efficiency of Next-Generation Sequencing (NGS)-based Preimplantation Genetic Testing for Monogenic disease (PGT-M) of GA-I.

Materials and methods: A non-consanguineous Chinese family was diagnosed with GA-I by Sanger sequencing. PGT-M and prenatal diagnosis (PND) were performed for the carrier. 5 blastocysts were used for the trophectoderm biopsy. After Whole-Genome Amplification (WGA), the WGA products were used for Sanger sequencing, NGS-based PGT-M and PGT-A. Sanger sequencing-based PND was performed in second trimester to confirm the results of PGT-M.

Results: A compound heterozygous mutation was diagnosed in the GCDH gene with co-segregation. One is [c.533G>A (p.G178E)] and another is [c.914C>T (p.S305L)]. 2 blastocysts were diagnosed as normal and one of them was transferred into the mother’s uterus. Finally, a healthy female was born 39 weeks after transplantation.

Conclusion: Our study successfully applied NGS-based PGT-M to avoid GA-I and highlights the efficiency of genetic diagnoses. It has significant implications on genetic counseling and genetic diagnosis for GA-I.

Keywords: Preimplantation genetic testing for monogenic disease, Glutaric acidemia type I, next-generation sequencing, Haplotyping, Prenatal diagnosis

Abbreviations: GA-I: Glutaric Acidemia type I; GCDH: Glutaryl-CoA Dehydrogenase; GA: Glutaric Acid; 3-OH-GA: 3-Hydroxyglutaric Acid; PGT-M: Preimplantation Genetic Testing for Monogenic disease; PGD: Preimplantation Genetic Diagnosis; NBS: Newborn Screening; IVF: In Vitro Fertilization; PND: Prenatal Diagnosis; WGA: Whole Genome Amplification; ADO: Allele Dropout; SNP: Single Nucleotide Polymorphism; NGS: Next-Generation Sequencing; MS/MS: Tandem Mass Spectrometry; C5DC: Glutaryl Carnitine; C6OH: 3-Hydroxyacetyl Carnitine; GC/MS: Gas Chromatography/Mass Spectrometry; ICSI: Intracytoplasmic Sperm Injection; TE: Trophectoderm; DOP-PCR: Degenerate Oligonucleotide Primed Polymerase Chain Reaction; PGT: A Preimplantation Genetic Testing for Aneuploidy; FAD: Flavin Adenine Dinucleotide; HPLC: High Performance Liquid Chromatography; PCR: Polymerase Chain Reaction
Introduction

Glutaric acidemia type I (GA-I, OMIM 231670) is an autosomal recessive metabolic disorder caused by glutaryl-CoA dehydrogenase (GCDH) deficiency due to mutations in GCDH (NM_000159) gene [1,2]. Deficiency of GCDH causes elevations of glutaric acid (GA) and 3-hydroxyglutaric acid (3-OH-GA) in tissues and body fluids of affected patients. Accumulation of GA and 3-OH-GA were speculated to degenerate neurons and impair the function of mitochondria, especially in the brain [3,4]. As newborn screening (NBS) is only conducted in limited number of cities in China, most GA-I patients have been diagnosed through clinical characterization at notably clinical stage [5]. Delayed diagnosis and treatment would significantly increase the risk of acute encephalopathic crises and death. To date, the guidelines for basic metabolic treatment of GA-I involves two major principles: one is diet with restrictive lysine to reduce the level of glutaryl-CoA, GA, and 3-OH-GA. The other is a supplement of carnitine and riboflavin to avoid secondary carnitine depletion [6,7]. To prevent malnutrition and secondary complications, important biochemical parameters should be frequently monitored. Furthermore, complex therapeutic strategies should be executed in different periods. Otherwise, the efficacy of dietary treatment may fail [8]. Although these treatments can reduce the risk of acute encephalopathic crises, they can neither block the vertical heredity of the pathogenic mutations nor guarantee the quality of life of patients.

Prenatal diagnosis (PND) by chorionic villus sampling or amniocentesis is the traditional method to identify whether the fetus has inherited disease-causing mutations from the parents. Preimplantation genetic testing for monogenic disease (PGT-M), also known as preimplantation genetic diagnosis (PGD), has become well-established technique to prevent the birth of a child with genetic disorder. PGT-M is performed as part of the in vitro fertilization (IVF) process and has been used to select unaffected embryos from patients suffering from known monogenic diseases, such as autosomal recessive, autosomal dominant, and X-linked disorders [9]. The whole-genome amplification (WGA) products of few cells biopsied from the blastocysts were used to PGT-M. Due to amplification bias during WGA, the allele dropout (ADO) may occur. The ADO has perplexed clinical application until the single nucleotide polymorphism (SNP) haplotyping was used for PGT-M to determine ADO [10]. We can identify ADO and avoid misdiagnosis through the haplotyping analysis of SNP markers flanking the mutation site via next-generation sequencing (NGS).

In this study, we clinically diagnosed GA-I caused by compound heterozygous mutations of GCDH in a Chinese family through NBS of the proband and the genetic diagnosis of her family. Furthermore, to prevent pathogenic mutations from passing down to the next generation, the patient was advised to receive PGT-M and PND. A normal blastocyst was transferred and a healthy baby girl was born at 39 weeks.

Materials and Methods

This case involves a non-consanguineous Han Chinese couple in good health. A 33-year-old female and her 35-year-old husband came to our Assisted Reproductive Technology Center for diagnosis to have healthy baby. The mother’s first delivery was at the age of 26 in 2011. The first girl suffered cerebral palsy and died of viral encephalitis few weeks after birth. Because of NBS was unavailable in our center at that time, it did not tested on the first girl. When the mother was 30 years old, she had delivery for the second time. The second girl was hospitalized for cerebral palsy, seizures, and dystonia after birth. Through NBS by tandem mass spectrometry (MS/MS), we detected that the infant had significantly elevated blood glutaryl carnitine (values in μmol/L; C5DC+C6OH: 1.95 vs. normal controls 0.03–0.26) and suspected that she would be GA-1. Then, the urinary organic acid profiles of the girl showed that GA and 3-OH-GA were significantly increased (values in μmol/mmol creatinine; GA: 264.8 vs. normal controls 0.0–4.0, 3-OH-GA: 7.5 vs. normal controls 0.0–0.0). Although treated with L-carnitine supplement and low lysine diet, the girl died three months later. Molecular diagnosis was performed for the family, and the results confirmed that the father had heterozygous mutation in GCDH c.914C>T, the mother had another heterozygous mutation in GCDH c.533G>A, and the second girl (II-2) had compound heterozygous mutations inherited from her parents (Figure 1). Considering the histories of past two abnormal pregnancy and the risk of pregnancy termination, this couple requested to receive IVF+PGT-M and was counseled contraception. However, during the PGT-M procedure in 2017, the mother was found to have an unplanned pregnancy and came to our Medical Genetic Center at 11 weeks gestation. Full informed consent was obtained from the couple, and they decided to perform PND as soon as possible. After preoperative examination, transabdominal chorionic villus sampling was performed to the mother when she was 12+4 weeks gestation. The results of PND showed that the fetus (II-3) carried both GCDH c.533G>A and c.914C>T.
Pregnancy termination was requested by the couple at 15 weeks gestation. After 6 months, a normal blastocyst was implanted into the mother's uterus and PND was performed by amniocentesis at 18 weeks gestation. The results of PND were consistent with those obtained from PGT-M. A healthy girl (II-4) was born at 39 weeks. NBS results for this newborn indicated that the level of blood glutarylcarnitine was normal.

Figure 1: Pedigree of the glutaric acid type I (GA-I) family. Circles, squares and triangle represent females, males and termination of pregnancy, respectively. The black symbols represent patients who suffered GA-I. II-2 represents the proband. II-3 represents the naturally conceived one and has been terminated. II-4 represents the normal female from blastocyst No. 4.

**Tandem mass spectrometry and gas chromatography/mass spectrometry**

MS/MS was used for screening of genetic metabolic diseases in newborns. Three heel-punctured blood spots were collected on S903 Whatman filter paper. Then, 3.2 mm blood-spot samples were obtained and placed in a 96-well sample plate. Those samples were detected using a NeoBase™ non-derivatized MS/MS Kit (PerkinElmer, USA) and were respectively injected into a Waters 1525µ binary HPLC pump (Waters, USA) using a Waters 2777C sample manager (Waters, USA). After separation by liquid column, each sample was quantified based on its charge/mass ratio by a Waters TQ detector (Waters, USA). The amino acid and acylcarnitine profiles of the blood spots were analyzed by MassLynx V4.1 (Waters, USA).

Gas chromatography/mass spectrometry (GC/MS) was used in screening for genetic metabolic diseases in newborns as supplement. A 2.5 cm × 3 cm urine filter paper was tested using an Agilent 7890A GC system (Agilent Technologies, USA) coupled with an Agilent 5975C (Agilent Technologies, USA) mass spectrometer. The testing process and urinary organic acid analysis was conducted at Kingmed Diagnostics laboratory (Zhuhai, China).

**Preparation of DNA samples**

The peripheral blood of the proband (II-2) and her parents that used for genetic diagnosis were collected in vacuum anticoagulant tubes respectively. The DNA was extracted with the RelaxGene Blood DNA System (Tiangen, China) according to the manufacturer's standard protocol.

The villus of II-3 and amniocytes of II-4 that used for PND were respectively collected in 15-mL centrifuge tubes (Corning, Mexico). After high-speed centrifugation, the cells were transported into 1.5-mL PCR tubes. The DNA of villus was extracted with the DNeasy® Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's Quick-Start Protocol. The DNA of amniocytes was extracted with the Puregene® Core Kit B (Qiagen, USA) according to the manufacturer's protocol.

The biopsied cells of blastocysts were respectively transferred into 0.2-mL PCR tubes with 4.5 µL of buffer for WGA. Degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) method was performed using PicoPLEX WGA Kit (Rubicon Genomics, USA) to conduct WGA of the blastocyst biopsy samples in accordance with the manufacturer's standard protocol. After assessment by agarose gel electrophoresis, the WGA products were used for PGT-A and PGT-M (including Sanger sequencing and NGS-based SNP haplotyping).

**PCR and Sanger sequencing**

Primer 5.0 software was used to design primers of the GCDH mutation sites (c.533G>A and c.914C>T). The primers were as follows: GCDH c.533 forward primer (CCGCCACAGGATAATTTTGAG), GCDH c.533 reverse primer (TACCAGGCTCTTGGTCCATTGA), GCDH c.914 forward primer (CTTCCCTGCTTCAGAGTGGT), and GCDH c.914 reverse primer (GGGAGTCACCCATGGTATAGAG). PCR was performed as described previously [13].

ABI Prism BigDye Terminator Cycle Sequencing Kit version 3.1 (Life Technologies, USA) was used in detection of mutation sites (c.533G>A and c.914C>T) for genetic diagnosis samples, PND samples and WGA products for PGT-M. Sanger sequencing was performed on 3500 Dx Genetic Analyzer (Life Technologies, USA) after PCR amplification. All procedures were performed according to the manufacturer's protocol.
PGT-A

The WGA products were used to form sequencing libraries using the Gene Sequencing Library Kit (Peking Jabrehoo Med Tech Co., China) and the libraries were sequenced on Illumina Miseq platform (Illumina, USA) using MiseqDx Universal Kit V3 (Illumina, USA). PGT-A was performed as follows. After removal of low-quality bases and adaptors, clean and high-quality reads were compared with the hg19 reference genome (University of California, Santa Cruz Genome Browser; genome.ucsc.edu/) using Burrows–Wheeler Alignment Tool, version 0.7.12-r1039 [14]. The exclusive mapped reads were calculated and a reference dataset was obtained to represent the relative deletions and duplications.

SNP haplotyping of PGT-M

The WGA products were used to form sequencing libraries using the PGD DNA Library Kit for Illumina (Peking Jabrehoo Med Tech Co., China) and then sequenced on the Illumina Miseq platform (Illumina, USA). All of the procedures were performed in accordance with the manufacturer’s protocol. SNP haplotyping was conducted by selecting 100 SNPs within 2 Mbp regions flanking the mutation site. After sequencing the DNA of the proband and her parents, we selected 10 informative SNPs to analyze the pedigree haplotypes. NGS-based SNP haplotyping was then used to distinguish the chromosomes carrying the mutations. The NGS sequencing data were analyzed by Peking Jabrehoo Med Tech Co. Ltd (Peking, China).

Results

Newborn screening and genetic diagnosis

MS/MS was used to screen the metabolic diseases of newborns. Compared with the reference of C5DC+C5OH (C5DC+C5OH: 0.03-0.26 μmol/L), the result of proband (II-2) was markedly elevated to 1.95 μmol/L. Urinary organic acid analysis was conducted by GC/MS. The urinary organic acid profiles of II-2 showed that both GA and 3-OH-GA were significantly increased in urine. The respective value of GA was 264.8 μmol/mmol creatinine (vs. normal controls 0.0–4.0 μmol/mmol creatinine) and 3-OH-GA was 7.5 μmol/mmol creatinine (vs. normal controls 0.0–0.0 μmol/mmol creatinine).

Genetic diagnosis was conducted by Sanger sequencing. The results showed that the father (I-1) had a heterozygous mutation in GCDH c.914C>T, the mother (I-2) had another heterozygous mutation in GCDH c.533G>A, and the proband (II-2) had either mutation c.533G>A or mutation c.914C>T in the GCDH gene (Figure 2A). Based on the ACMG guidelines, the mutation c.533G>A was evaluated as “likely pathogenic” (PM2 + PM5 + PP1 + PP3 + PP4) and the mutation c.914.C>T was also evaluated as “likely pathogenic” (PM2 + PP1 + PP3 + PP4 + PP5, Supplementary Table).

Prenatal diagnosis

Based on full informed consent from the couple, the PND of II-3 was performed by chorionic villus sampling at 12+3 weeks gestation. Unfortunately, the fetus carried both GCDH mutations c.533G>A and c.914C>T (Figure 2B). The PND result indicated that this fetus (II-3) would be a GA-I patient similar to II-2. Pregnancy termination was requested by the couple at 15 weeks gestation.

Full informed consent was obtained when the mother was pregnancy after implantation, and PND of II-4 was conducted by amniocentesis at 18 weeks gestation. Sanger sequencing results showed that the fetus carried a normal type with site c.533 and site c.914 (Figure 2C). These results were consistent with those of PGT-M.

Embryo preparation and preimplantation genetic diagnosis

After long-protocol ovulation was promoted, 13 oocytes were collected and 10 of these were at metaphase II-stage (MII). Inseminated by intracytoplasmic sperm injection (ICSI), 7 of the 10 MII oocytes were developed into 6–8 cells on day 3 post-insemination. After cultivation, we finally obtained five blastocysts (1, 3, 4, 5, and 7). These blastocysts were all used for the trophectoderm (TE) biopsy on day 5 post-insemination. The biopsied cells of these five blastocysts were successfully amplified by DOP-PCR except sample 1. Those WGA products were used for PGT-M and PGT-A.

As shown in figure 3, the PGT-A result of sample 5 indicated a mosaic deletion in the chromosome 7 (p22.3–q36.3) (159.12 Mb). The rest of the samples (3, 4, and 7) were all balanced (46, XN). PGT-M used Sanger sequencing and SNP haplotyping to test together. After PCR at the mutated region of GCDH, we performed Sanger sequencing to detect the mutations firstly. The results of Sanger sequencing showed that samples 3 and 5 carried the mutation c.914C>T, whereas samples 4 and 7 did not. However, sequencing of the site c.533 in all samples were failed. Meanwhile, SNP haplotyping confirmed that samples 3 and 5 carried the mutation c.914C>T, whereas samples 4 and 7 did not and all of the samples did not carry the mutation c.533G>A (Table 1). One of the normal
blastocysts (4) was implanted into the uterus and finally born as II-4.

**Figure 2:** (A) Molecular diagnosis results of GA-I family. Compared with the reference sequence of National Center for Biotechnology Information (NCBI), father carried the GCDH mutation c.914G>T, mother carried the GCDH mutation c.533G>A and the proband inherited both mutations. (B) Prenatal diagnosis (PND) results of II-3. Compared with NCBI, the PND results showed that II-3 carried GCDH mutation c.533G>A and c.914G>T. (C) PND results of II-4. Compared with NCBI, the PND results showed that II-4 did not carry mutation in c.533 and c.914.

**Figure 3:** Preimplantation genetic testing for aneuploidy (PGT-A) results of each blastocyst. Compared with the hg19 reference genome through analysis, the sketch maps shown that sample 5 carried a mosaic deletion in the chromosome 7 (p22.3-q36.3) (159.12 Mb). The rest samples’ chromosomes were all balanced.
Discussion

GA-I results from the deficiency of glutaryl-CoA dehydrogenase caused by the mutations of GCDH gene localized on human chromosome 19p13.13 and included 12 exons. To date, more than 250 disease-causing mutations have been identified worldwide [2,15]. These mutations negatively affect the activity, stability, and oligomerization of glutaryl-CoA dehydrogenase [16]. The G178R (c.532G>A) mutation may significantly change the GCDH polypeptide and affect FAD binding due to G178 is close to the isoalloxazine ring of the FAD coenzyme [17]. Coding the same codon 178, G178E (c.533G>A) may impair FAD binding either. The mutation S305L (c.914C>T) may obstruct the binding of GCDH with substrate or impair the function of GCDH by altering its folding [18]. Besides, [19] reported that mutation c.533G>A (4/40 alleles, 10%) was more frequent in their Chinese patients, so we wondered whether the mutation c.533G>A is another common mutation in Chinese [19]. Future studies incorporating diagnosis of GA-I in Chinese are needed to reach a definitive conclusion. While, another study reported that mutation c.914C>T was a common mutation in Japanese [20]. Unlike mutation c.533G>A, the mutation c.914C>T may be the first case to be reported in a GA-I patient of Han Chinese ethnicity.

Birth defects bring serious psychological and economic problems to Chinese families and society. The prevalence of GA-I was estimated to be 1 in 100,000 newborns according to data from North America, Australia, and Germany [21]. However, the veracious incidence of GA-I in China has not been reported and may be much higher when the NBS is widely used [5]. China is the country with the largest population in the world. Furthermore, with the release of the two-child policy in 2015, a new baby boom is expected to occur, which means that more GA-I patients may be born. Although the guidelines for basic metabolic treatment of GA-I were established and had been used for a decade all over the world, the risk and benefit of dietary treatment still requires an assessment. In addition, the individual therapy strategies need to be adjusted by specialized interdisciplinary teams [7,22]. Accordingly, it is difficult to guarantee the living quality of patients with GA-I in China. In this study, even the proband (II-2) was screened 2 weeks after birth, diagnosed 3 weeks after birth, and treated timely according to the guidelines, she died 3 months caused by infection. So we really need a efficient method to avoid birth defects fundamentally.

PND has been widely used to avoid serious genetic disorders for many years. The mother who had given birth to proband with inherited disorders was advised to perform PND while preparing for another pregnancy or during the pregnancy. However, the mother could suffer extreme psychological pressure and the risk of abortion due to invasive sampling during PND. Meanwhile, PGT-M provided another way for couples to avoid the vertical heredity of the pathogenic gene. But it is expensive to most Chinese families. In addition, it is difficult for the parents to receive IVF even they are fertile and to have a risk of ovarian hyper stimulation syndrome. Therefore, it is consistent with other studies that both methods have advantages and disadvantages [23,24]. Which method should be chosen depending on the couple's physical conditions and wishes.

This study successful applied NGS-based haplotyping for PGT-M to avoid GA-I caused by compound heterozygous mutations of GCDH. Unfortunately, the WGA of sample 1 was failure. The reason could be the difference in each blastocyst's morphological grading.

Table 1: Results of NGS-based SNP haplotyping.

| Number of SNPs | Position | Father | Mother | Proband | Sample 3 | Sample 4 | Sample 5 | Sample 7 |
|---------------|----------|--------|--------|---------|----------|----------|----------|----------|
|               |          | F1     | M1     | M2      | F1       | F2       | M2       | F2       | M2       |
| GCDH_M_7      | 12613326 | G      | G      | G       | T        | G        | G        | T        | G        |
| GCDH_36       | 12624750 | C      | C      | C       | T        | C        | C        | T        | C        |
| GCDH_37       | 12641060 | A      | A      | A       | T        | A        | A        | T        | A        |
| GCDH_49       | 12989560 | G      | A      | G       | G        | G        | G        | G        | G        |
| GCDH_50       | 12994715 | A      | G      | A       | A        | A        | A        | A        | G        |
| GCDH_M_13     | 13010520 | G      | G      | G       | A        | G        | G        | G        | G        |
| GCDH_62       | 13291776 | A      | G      | G       | G        | A        | G        | G        | A        |
| GCDH_63       | 13323606 | G      | A      | A       | A        | G        | A        | A        | G        |
| GCDH_M_15     | 13430786 | G      | G      | A       | G        | G        | A        | G        | A        |
| GCDH_75       | 13444864 | T      | A      | T       | T        | T        | A        | T        | T        |

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Blastocyst 1 was grading 3BC, blastocyst 3 was grading 5BC, blastocyst 4 was grading 5CC, blastocyst 5 was grading 4BC, and blastocyst 7 was grading 5BC. These indicated that samples 3, 4, 5 and 7 were superior to sample 1. On the other hand, losses of cells during biopsy and transfer cannot be excluded. Due to blastocysts 4 and 7 were available for implantation, we stored blastocyst 1 in liquid nitrogen for later use. Sanger sequencing has been considered as the gold standard for directly detecting the mutation site in monogenic diseases, but ADO may occur and lead to sequencing failure in WGA products. ADO is caused by the amplification bias of two alleles. One of the two alleles is preferentially amplified, and the other allele is amplified at an undetectable level; thus, the sequencing might be failed in this condition. In this study, sequencing of site c.533 were failed in all samples, NGS-based SNP haplotyping was performed to distinguish the mutation-carrying chromosome from normal chromosomes and determine ADO. Finally, one of the normal blastocysts (4) was implanted into the uterus and born as II-4. Thus, our study highlights the efficiency of using NGS-based SNP haplotyping for PGT-M and avoiding inheritance of GA-I.

In conclusion, our study integrated genetic diagnosis, PND, and PGT-M to successfully block the vertical heredity of the pathogenic mutations of the GCDH gene in Chinese family. To the best our knowledge, our research team is the first to report an application of NGS-based SNP haplotyping for PGT-M to avoid inheritance of GA-I and screen a normal blastocyst for a healthy newborn in China.

**Declarations**

**Ethics approval**

This study was approved by the Ethics Committee of Northwest Women and Children’s Hospital (No.2020-013). All patients provided written informed consent.

**Consent for publication**

Informed consent for publication was obtained from all participants.

**Availability of data and material**

The datasets used during the current study are available from the corresponding author on reasonable request.

**Conflicts of interest**

The authors declare that they have no conflicts of interest.

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**Author contributions**

Conceived and designed the experiments: Xiaobin Wang and Rong Qiang; Performed the experiments: Bin He, Qiuhua Wu and Chengrong Song; Acquisition the data: Xingzhe Ji, Wenhao Shi and Juanzi Shi; Analyzed the data: Bin He, Lin Wang and Wei Li; Wrote the manuscript: Bin He and Lin Wang.

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