First successful trial of preimplantation genetic diagnosis for pantothenate kinase-associated neurodegeneration

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Abstract

Purpose We aim to present a case of a healthy infant born after intracytoplasmic sperm injection-in vitro fertilization (ICSI-IVF) with a preimplantation genetic diagnosis (PGD) for pantothenate kinase-associated neurodegeneration (PKAN) due to *PANK2* mutation.

Methods ICSI-IVF was performed on a Thai couple, 34-year-old female and 33-year-old male, with a family history of PKAN in their first child. Following fertilization, each of the embryos were biopsied in the cleavage stage and subsequently

processed for whole-genome amplification. Genetic status of the embryos was diagnosed by linkage analysis and direct mutation testing using primer extension-based mini-sequencing. Comprehensive chromosomal aneuploidy screening was performed using a next-generation sequencing-based strategy. **Results** Only a single cycle of ICSI-IVF was processed. There were seven embryos from this couple—two were likely affected, three were likely carriers, one was likely unaffected, and one failed in target genome amplification. Aneuploidy screening was performed before making a decision on embryo transfer, and only one unaffected embryo passed the screening. That embryo was transferred in a frozen thawed cycle, and the pregnancy was successful. The diagnosis was confirmed by amniocentesis, which presented with a result consistent with PGD. At 38 weeks of gestational age, a healthy male baby was born. Postnatal genetic confirmation was also consistent with PGD and the prenatal results. At the age of 24 months, the baby presented with normal growth and development lacking any neurological symptoms.

Conclusions We report the first successful trial of PGD for PKAN in a developing country using linkage analysis and mini-sequencing in cleavage stage embryos.

Keywords Preimplantation genetic diagnosis · PGD · Pantothenate kinase · Neurodegeneration · *PANK2*

Capsule Pantothenate kinase-associated neurodegeneration is a life-threatening and incurable autosomal recessive disorder characterized by progressive extrapyramidal dysfunction. Here we present the first successful trial to utilize preimplantation genetic diagnosis technology for a couple who had an experience on the loss of their first child affected by this particular inherited neurological condition.

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Introduction

Pantothenate kinase-associated neurodegeneration (PKAN; MIM no. 234200) is a rare neurodegenerative disorder in a group of disorders characterized by progressive extrapyramidal dysfunction, i.e., dystonia, rigidity, and choreoathetosis. Additional manifestations include spasticity, extensor toe sign, retinal degeneration, and acanthocytosis. This specific

disorder is a form of neurodegeneration with brain iron accumulation (NBIA) and is caused by a mutation in *PANK2*, which is inherited in an autosomal recessive manner. The classic form usually presents in the first decade of life, and the affected child often loses ambulation within 10 to 15 years of onset. Brain MRI is the standard diagnostic evaluation, and the hallmark of the disorder is a central region of hyperintensity surrounded by a rim of hypointensity on coronal or transverse T2-weighted images of the globus pallidus, the so-called “eye-of-the-tiger” sign. The disease is incurable, and premature death often occurs due to a lung infection or nutrition-related immunodeficiency [1–4].

Since this particular disorder is inherited in an autosomal recessive manner, the chance for a couple having children experiencing this disease is 25%. Primarily, reproductive technology for the couple at risk includes prenatal and preimplantation genetic diagnosis. However, prenatal diagnosis cannot reduce the risk before the pregnancy. Nevertheless, the methods will assist in identifying the genetic status of the fetus. If the fetus does have the disorder, the couple will have to consider the options to either continue or terminate the pregnancy after obtaining comprehensive genetic counseling. Currently, preimplantation genetic diagnosis (PGD) is a technology used to identify the genetic status of embryos, created through in vitro fertilization (IVF) technology. Thus, PGD is considered as an option for risk reduction of a genetic disorder inherited in the family if the causative mutation is known [5–7].

Even though PGD has been practiced for more than 25 years, it has just recently been introduced in Thailand. However, assisted reproduction for couples at risk for a single gene disorder is not popular in developing countries, due to the expensive cost and limited number of institutes that can offer the technology. To the best of our knowledge, PGD for PKAN has not been reported elsewhere. Here, we report a couple with a previous child affected by PKAN and initiated the first PGD for a single gene disorder at our institute.

Case presentation

A Thai boy, born from non-consanguineous parents, developed gait dystonia, dysarthria, pyramidal signs and mental impairment at the age of 12 months. Neurological symptoms deteriorated progressively, and the patient died within 1 year of clinical diagnosis, due to a lung infection. DNA sequencing of *PANK2* in the patient's leukocytes revealed a pathogenic splicing variant at position IVS2-1G>C or mRNA c.982-1G>C (NM_153638.2). The patient was confirmed to be homozygous at that position, which indicates that both parents were carriers. Six months following the family's loss, the couple, a 34-year-old female and 33-year-old male, revisited the genetics clinic to discuss reproductive options for a subsequent pregnancy. Genetic counseling for PGD began, and ethical clearance was obtained.

Materials and methods

Assisted reproductive technology, embryo biopsy, and embryo culture

The antagonist protocol began on day 2 of menstruation using hMG 225 units/day (IVF-M, LG Life Sciences, Iksan-si, Korea). The GnRH antagonist (Orgalutran, MSD, Ravensburg, Germany) was injected between stimulations from day 7 to day 9. Oocyte maturation was induced by hCG treatment (IVF-C, LG Life Sciences). Oocytes were collected 36 h later. Intracytoplasmic sperm injection (ICSI) was performed 6 h after oocyte selection. Due to the limited number of embryologists qualified for blastocyst biopsy at our institute, all embryos in this study were biopsied at the cleavage stage. Two blastomeres per embryo were used for whole-genome amplification. The embryos were cultured to the blastocyst stage and frozen using the vitrification method.

Whole-genome amplification

Whole-genome amplification (WGA) was performed using the GenomePlex® complete WGA kit according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). The WGA product was subsequently processed for linkage analysis and mini-sequencing.

Linkage analysis

Linkage analysis was performed using short tandem repeat (STR) markers focused around *PANK2* (Table 1; nos. 1–5). The samples used for the analysis included genomic DNA from the parents and the son previously affected by PKAN. The fragment size of each amplicon was analyzed by capillary electrophoresis on an ABI Prism® 3500 automatic DNA sequencer (Applied Biosystems, Foster City, CA) using the GeneScan® analysis software (Applied Biosystems). A minimum of two informative STR markers is preferred for PGD analysis.

Mini-sequencing

Mini-sequencing was performed using a primer extension-based method using the SNaPshot® multiplex system (Life Technologies, Carlsbad, CA). The reaction contained three oligonucleotide primers (Table 1; no. 6) for subsequent analysis by capillary electrophoresis on an ABI Prism® 3500 automatic DNA sequencer (Applied Biosystems). To visualize the electrophoresis data, the peak signal was analyzed using the GeneScan® analysis software (Applied Biosystems).

Table 1 Summary of the primers used in the experiment

Primer's name	Nucleotide sequence (5' to 3')	T _m (°C)
1.D20S113	F-(FAM)-TAACAGTGGTTGACTCTCAGAGG	55
	R-AAGAGGTGCTGTACATATTATTC	53
2.D20S473	F-(FAM)-TCATGAGCTAAATATTACTCAGTGC	52
	R-CTTATAGCTTTTTCAAATGATCTG	48
3.D20S116	F-(FAM)-TGTCAGTGTGAAACGGTACA	53
	R-CCACACAGATTCCTCCAGG	55
4.D20S482	F-(FAM)-AGCCTCCATATCCACATGAA	53
	R-GAACCTAAACTCTAAGGAAGCG	53
5.D20S895	F-(FAM)-CCCAGGGAGGTAAAGGTT	53
	R-GTCAGGTACATCAGCAA	52
6. <i>PANK2</i> mini-sequencing	F-GCACTTTCATGGTTGTTTCACG	55
	R-CACTGTGACCGTCCATTGAA	55
	Mini-sequencing-AAAAAAAAAATGAGTACATTCTTATTCAT TACA	53
7. <i>PANK2</i> c.982-1	F-CACTGTGTCCCTAGGTTTGC	55
	R-CAAAGGCTACAATGAGTCTGC	53

Comprehensive chromosomal aneuploidy screening

Comprehensive chromosomal aneuploidy screening was performed using next-generation sequencing (NGS)-based technology. WGA, as previously described, was quantified using the Qubit dsDNA HS assay kit (Life Technologies). End-repair and purified DNA for Ion Torrent 200 bp chemistry were processed before adapter ligation according to the Ion Xpress plus gDNA fragment library preparation protocol (Life Technologies). Electrophoresis was performed, and the sample fraction at 330 bp was excised using E-Gel SizeSelect Gels (Life Technologies). At least 5 cycles of adapter-mediated amplification were required to generate quantifiable sequence-ready libraries. All libraries were evaluated using the Bioanalyzer High Sensitivity Chip (Agilent Technologies, Santa Clara, CA) before clonal amplification using the Ion OneTouch system (Life Technologies). Prior to sequencing, sample enrichment was completed using the Ion OneTouch ES module. The single-end sequencing was performed following the Ion PGM Hi-Q sequencing workflow on the Ion PGM System with the use of Ion PGM sequencing 200 kit v2 (Life Technologies). Data were analyzed using low-pass whole-genome aneuploidy analysis workflow based on the hg19 genome database utilizing Ion Reporter Cloud v.5.2 (Life Technologies). This method has a sensitivity of 100% and a specificity of 100% [8].

Embryo transfer

The endometrium preparation was started at day 3 of menstruation with estradiol valerate (Bayer Schering Pharma AG, Austria) at 8 mg/day followed by micronized progesterone

(Basin Healthcare, Belgium) at 400 mg/day. Only one unaffected embryo was thawed and transferred to the uterus using a TDT catheter (Laboratoire c.c.d., Paris, France). Afterward, the patient was treated with estradiol valerate and micronized progesterone until the serum β -hCG level was checked. The medication would be discontinued if the patient were not pregnant. However, if the patient were pregnant, the medication would continue until the gestational age of 8 weeks.

Prenatal and postnatal genetic confirmation

To validate the PGD result, genetic confirmation was performed during the prenatal and postnatal stages. Prenatal confirmation was performed at the 16th week of gestational age by amniocentesis. A postnatal specimen was collected from peripheral blood following delivery of the baby. The biological samples were analyzed by PCR and direct genomic sequencing using two oligonucleotide primers to cover the target mutation (Table 1; no. 7). Genomic sequencing was performed on ABI Prism® 3500 automated DNA sequencer (Applied Biosystems), using fluorescently labeled dideoxy terminators (Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) according to the manufacturer's protocol.

Results

In vitro fertilization and intracytoplasmic sperm injection

Eight oocytes were collected; however, only seven were mature at the MII stage. The fertilization was performed

by ICSI. The success rate for fertilization cleavage and blastocyst development was 100% (7/7).

Preimplantation genetic testing

PGD was performed both by direct genomic testing using mini-sequencing and by indirect testing using linkage analysis in WGA obtained from 3-day-old embryos. WGA from six of seven embryos was included in the study since WGA from E#3 failed in amplification (Fig. 1). The allele dropout (ADO) rate in the linkage study was approximately 30%. The results of the mini-sequencing and linkage analysis were consistent. The PGD results revealed that E#1 and E#2 were likely disease affected; E#4, E#5, and E#6 were likely PKAN carriers; and E#7 was likely wild type. Aneuploidy screening showed aneuploidy in E#4, E#5, and E#6, whereas E#1, E#2, and E#7 were euploid (Table 2).

Embryo transfer and successful pregnancy

E#7 had a normal wild-type genotype and passed the screening for aneuploidy. It was transferred on day 17 of the cycle. Afterward, the beta hCG level was measured at day 12 following the transfer. The value obtained was 669 mIU/ml, consistent with a successful pregnancy. Antenatal care revealed no complications. A healthy male baby was born by cesarean section at the 38th week of gestational age.

Prenatal and postnatal genetic confirmation

Genomic DNA sequencing of c.982-1G>C of *PANK2* was performed on DNA extracted from amniocentesis and peripheral blood after birth. No mutation was found, consistent with the PGD results (Fig. 2).

Fig. 1 Preimplantation genetic testing results. Linkage analysis using STR markers (base pair) around *PANK2* showed that embryos no. 1 and no. 2 are consistent with the haplotype of the previous child affected by

PKAN in the family. No. 4 and no. 5 are predicted to be carriers, and no. 7 is predicted to have a normal genotype. Embryo no. 3 failed in amplification, while no. 6 was inconclusive due to lack of sufficient linkage data

Table 2 Summary of PGD results for pantothenate kinase-associated neurodegeneration

Embryo ID	Linkage analysis	Mini-sequencing of c.982-1G>C	NGS karyotype	Interpretation
No. 1	Inherited both paternal and maternal disease-causing alleles	CC	46, XY	Likely affected
No. 2	Inherited both paternal and maternal disease-causing alleles	CC	46, XY	Likely affected
No. 3	N/A	N/A	N/A	N/A
No. 4	Inherited maternal disease-causing allele and paternal non-disease-causing allele	GC	48, XY, +19, +21	Likely carrier
No. 5	Inherited maternal disease-causing allele and paternal non-disease-causing allele	GC	44, XY, -9, -10, -19, +22	Likely carrier
No. 6	Inconclusive	GC	47, XY, +21	Likely carrier
No. 7	Inherited both paternal and maternal non-disease-causing alleles	GG	46, XY	Likely normal genotype

N/A not applicable, NGS next-generation sequencing

Longitudinal follow-up of the baby

The baby was monitored clinically by the pediatric neurologist until 24 months of age and presented with normal growth and development. He was able to walk at the age of 11 months and communicated recognizable words at 12 months.

Discussion

Genetic counseling issues concerning preimplantation genetic diagnosis for pantothenate kinase-associated neurodegeneration

To the best of our knowledge, this is the first case of a PGD protocol established for PKAN to be reported in the literature. PKAN is a neurological disorder that results in serious and critical outcomes. Specific treatments, nor pharmacotherapy, to prevent or relieve the symptoms of the disease, are available at the current time. Most patients die before the age of 20 due to respiratory tract infections [1, 4]. This situation is similar to other inherited neurological disorders that require a solution for the parents of the proband, in order to prevent the burden in subsequent pregnancies. Examples of other neurological disorders, either common or rare, that have had successful outcomes from PGD include spinal muscular atrophy, Duchenne muscular dystrophy, fragile X syndrome, Huntington disease, and myotonic dystrophy among others [9].

Genetic counseling is crucial before beginning the PGD process. Since PKAN is inherited in an autosomal recessive manner, couples are required to understand that there is a 25% chance of having a baby with this particular disorder, a 50% chance of having a carrier, and a 25% chance of having a baby with a normal genotype. The couples have a right to know that

prenatal diagnosis can be offered in this situation, but there is a risk of both physical and mental disturbance if the mutation is present and termination of the pregnancy is requested [1]. IVF-PGD is the best option for avoiding the termination concern; however, the couples must also understand the risks associated with the IVF process. The couples should be aware that the accuracy for PGD is approximately 95%, and prenatal diagnosis is necessary to confirm the PGD result. PGD results are usually reported as predictions, such as “likely affected” or “likely disease free.” It is also common to report “failed amplification” or “inconclusive,” which the couples must also understand before signing the informed consent for PGD [5–7]. In addition, couples will need to understand that the successful pregnancy rate after the IVF cycle is approximately 40–60%, if the embryos are screened for 24-chromosome aneuploidy [10–12]. A point that couples have to understand is that the chance of having a baby affected by other congenital malformations following IVF treatment is slightly increased, compared with natural conception [13, 14].

Preimplantation genetic diagnosis protocol

In our study, we performed PGD in 3-day-old embryos, which gave rise to a high rate of ADO. We required two cells from each embryo, aiming to pool the WGA for performing both monogenic disease testing and comprehensive chromosomal screening. In our experiment, WGA was achieved from both analyzed cells from only three of seven embryos (embryos no. 1, no. 4, and no. 7). Two-cell biopsy may disturb the growth and differentiation of the embryos [15, 16]. However, we attempted to perform *PANK2* PGD the first time and utilized the excess specimen for chromosomal screening. Fortunately, we were able to complete both studies in six of the seven embryos (only embryo no. 2 failed in the

Fig. 2 Chromatogram showing the successful preimplantation genetic diagnosis for pantothenate kinase-associated neurodegeneration. Family cosegregation analysis at the nucleotide position c.982-1G>C of *PANK2* revealed that the previously affected son had a homozygous C/C genotype, whereas the parents were heterozygous G/C. The successful use of PGD is shown in the DNA from the prenatal and postnatal stages in which the baby carries a homozygous G/G genotype, consistent with the prediction of the PGD result

study). Several publications suggest that day 5 trophectoderm biopsy has a lower ADO rate and yields increased amounts of DNA. The WGA method actually contributes to ADO since the entire genomic DNA cannot be recovered by the amplification; therefore, an increased amount of DNA in the blastocyst allows the chance for greater detection of multiple biological markers [15, 17]. An additional method for increasing the target genome coverage is WGA preparation technique using non-PCR-based multiple displacement amplification (MDA). MDA has been reported

to generate 1–2 μ g of DNA from single cell with genome coverage of up to 99%. This technique also generates larger-sized products with a lower error frequency [18, 19]. The other strategy for reducing the ADO rate reported is a qPCR-based method. Previous studies demonstrate that qPCR-based PGD utilizing TaqMan assay revealed 0–0.3% ADO with 99.7–100% success rate for providing the diagnosis of monogenic disorders in the embryos [20]. Establishing a qPCR-based PGD is also intriguing for future clinical use.

Regarding the molecular techniques used for the detection of hereditary mutations, we combined both direct and indirect mutation testings in our experiment. Theoretically, direct mutation testing may result in errors in WGA samples, depending on the allele fraction rate. We performed mini-sequencing using a PCR extension-based method, which was reported to have the capability of detecting an allele fraction as low as 5% [21]. In the situation where there is difficulty in WGA, the allele fraction may be lower than 5%, resulting in false-negative findings. Therefore, direct mutation testing is not appropriate for stand-alone use in the PGD process. Thus, additional indirect genetic testing, such as linkage analysis, is always preferred. The main issue with linkage analysis using STR is the distance from the gene of interest and the high risk of recombination between the STR and the disease locus. Some genes have no STR in the surrounding region, posing challenges with this method. In some families, the STR haplotype is not able to distinguish between disease-causing and non-disease-causing alleles, generating problems for interpretation by researchers [6, 15]. Based on the European Society of Human Reproduction and Embryology (ESHRE) guidelines, at least two informative STR markers are preferred for PGD [22]. Our study met this guideline, as we used five STR markers and only one of them (D20S116) was lowly informative. The fragment size at D20S116 allele was not polymorphic in the previous affected son or the mother, which proved difficult to distinguish between paternal disease-causing, maternal disease-causing, and maternal non-disease-causing alleles. Another factor that has an influence on linkage data is chromosomal recombination, which occurs at a rate of 5% in vivo [6, 22]. Our study demonstrated that although there was a high ADO rate, the results from linkage analysis and direct mini-sequencing remained consistent.

Current situation of preimplantation genetic diagnosis for monogenic disorders in developing countries

Although PGD has been introduced as the reproductive option for couples carrying monogenic disorders for more than 25 years, the progression of this field in developing countries is slow. Several obstacles for this technology include (1) the cost of technology, (2) the difficulty to perform certain genetic diagnosis in the affected case and (3) limited number of

qualified human resource. Based on our experience in Thailand, the overall cost for ICSI-IVF technology with monogenic PGD and comprehensive chromosomal screening by NGS or array comparative genomic hybridization performed in our medical school is approximately 14,000 USD and 10,000 USD for ICSI-IVF with chromosome screening only. This is costly compared to the gross domestic product (GDP) per capita (5816.40 USD; data from the World Bank Organization in 2015). Therefore, a very limited number of local people can afford and have access to genetic technology. Since mutations inherited in the family are the most critical elements for performing PGD for monogenic disorders, diagnosis of other rare single gene disorder remains a barrier. Molecular technology used for diagnosis is costly, and not all of them are nationally reimbursed. The only monogenic disorder that is available for testing nationwide is thalassemia and hemoglobinopathy, which is endemic and integrated into the national recommendation to screen in all couples at pre-conception stage. Thus far, few PGD reports from Thailand have been performed with the exception of these particular disorders [23, 24]. Regarding the limited human resource problem, PGD for monogenic disorders requires a multidisciplinary team of diverse specialists such as reproductive medicine specialists, clinical geneticists, molecular biologists, embryologists, and genetic counselors. The number of geneticists, embryologists, and genetic counselors in Thailand is insufficient. In particular with our study, we lack a qualified embryologist who is capable of performing a blastocyst biopsy, which is the reason why we performed a cleavage stage embryo biopsy instead. Hence, only few medical centers can establish a PGD team to serve clinical services and the majority of them are organized by the private sectors and in a partnership with international institutes. A number of couples accessing clinical services in the private sectors are foreigners, since the overall cost is economical compared to their domestic price, although it remains high for natives. Separate from our experience, other developing countries have a negative attitude against assisted reproduction technology that may be related to culture and religion [25].

Conclusion

In conclusion, we established the first successful use of PGD for a family affected by PKAN. We demonstrated a successful pregnancy and birth of a baby avoiding the inherited *PANK2* mutation. Our protocol requires minimal revision, i.e., day 5 trophoctoderm samples and generating larger size of WGA prepared from MDA. An increased number of linkage markers are required to circumvent the lack of a diagnosis due to ADO. This report aims to inspire and encourage the initiation of PGD services for monogenic disorders in developing countries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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