CASE REPORT
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Successful birth with preimplantation genetic diagnosis using single-cell allele-specific PCR and sequencing in a woman with hypochondroplasia due to *FGFR3* mutation (c.1620C>A, p.N540K)

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Hypochondroplasia (HCH) is an autosomal dominant inherited skeletal dysplasia, usually caused by a heterozygous mutation in the fibroblast growth factor receptor 3 gene (*FGFR3*). A 27-year-old HCH woman with a history of two consecutive abortions of HCH-affected fetuses visited our clinic for preimplantation genetic diagnosis (PGD). We confirmed the mutation in the proband (*FGFR3*:c.1620C > A, p.N540K), and established a nested allele-specific PCR and sequence analysis for PGD using single lymphocyte cells. We performed this molecular genetic analysis to detect the presence of mutation among 20 blastomeres from 18 different embryos, and selected 9 embryos with the wild-type sequence (*FGFR3*:c.1620C). A successful pregnancy was achieved through a frozen-thawed cycle and resulted in the full-term birth of a normal neonate. To the best of our knowledge, this is the first report of a successful pregnancy and birth using single-cell allele-specific PCR and sequencing for PGD in an HCH patient.

Keywords: Hypochondroplasia; Preimplantation genetic diagnosis; Receptor, fibroblast growth factor, type 3

Introduction

Preimplantation genetic diagnosis (PGD) is available for many kinds of monogenic disorders, which are caused by a known single gene mutation that can be diagnosed from a single-cell. The most frequent single gene disorders for which PGD is carried out are beta-thalassemia, cystic fibrosis, myotonic dystrophy, Huntington's disease, and fragile X syndrome [1]. However, single-cell polymerase chain reaction (PCR) for PGD has been challenged by serious difficulties such as contamination, amplification failure due to the low quantity of template DNA, and allele drop-out (ADO) in heterozygous loci [2]. To overcome these problems, some advances such as multiplex PCR, fluorescent PCR, and whole genome amplification can be applied [3].

Hypochondroplasia (HCH) is an autosomal dominant inherited skeletal dysplasia characterized by a similar but milder phenotype of short limbs, short stature, and lumbar lordosis compared to achondroplasia (ACH), usually caused by a heterozygous mutation in the fibroblast growth factor receptor 3 gene (*FGFR3*) [4,5]. The gene, *FGFR3*

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is a transmembrane tyrosine kinase receptor that binds fibroblast growth factors. Mutations causing shortening of the long bones associated with HCH are gain-of-function mutations that result in ligand-independent activation of *FGFR3* [6,7]. Among several mutations in the *FGFR3* gene, p.N540K substitution accounts for about 70% of HCH cases [8].

In this report, we present a successful pregnancy and birth using single-cell allele-specific PCR and sequencing for PGD in an HCH patient for the first time in Korea.

Case report

A 27-year-old HCH woman with a history of two consecutive abortions of HCH-affected fetuses visited our clinic for PGD. The woman had her diagnosis clinically and genetically confirmed as HCH by the orthopedist when she was 9 years old, and reconfirmed by another orthopedist just before the PGD. Previously, she had undergone prenatal diagnoses during two consecutive natural pregnancies, which resulted in affected fetuses after confirming the presence of the c.1620C > A, p.N540K mutation in the *FGFR3* gene confirmed by chorionic villi sampling, which was same as her own mutation. Terminations of the pregnancies were carried out in both cases at the couple's request. Following adequate counseling, the couple decided to undergo PGD for the third pregnancy and signed informed consent.

1. Ovarian stimulation, oocyte retrieval and blastomere biopsy

For the GnRH agonist long protocol, 0.1 mg/day of subcutaneous triptorelin acetate (Decapeptyl, Ferring Phamaceuticals, Malmo, Sweden) was initiated on day 21 of the previous cycle. After pituitary suppression, the triptorelin dose was reduced to 0.05 mg/day and recombinant FSH (follitropin alfa, Gonal-F, Merck Serono, Geneva, Switzerland) was added. After recombinant hCG (Ovidrel, Merck Serono) triggering, 34 oocytes were retrieved and fertilized by ICSI. Twenty-two embryos with two pronuclei were cultured, and the biopsy was performed with 18 embryos that reached the six- to eight-cell stage. Partial zona dissection and biopsy were performed using a micromanipulator (Narishige, Tokyo, Japan) mounted on an inverted microscope (Nikon). The presence of a clearly visible nucleus guided the selection of the blastomere to be biopsied in G-PGD medium (Vitrolife, Goteborg, Sweden). In the end, 20 blastomeres biopsied from 18 embryos were analyzed with positive and negative controls and were loaded in a reaction tube containing 2.5 μL alkaline lysis buffer (200 mM NaOH, 50 mM dithiothreitol) [9].

2. Molecular genetic analysis

A total of 20 blastomeres with each washing drop were immediately lysed by incubation at 65°C for 10 minutes. The first round of PCR was performed in a 50 μL volume containing 2.5 μL of lysate, 5 μL of 10× PCR buffer, 10 mM tricine (pH 4.93), 200 μM dNTP, 10% dimethyl sulfoxide (DMSO), 0.4 μM of each outer primer (Table 1), and 1.25 U Taq DNA polymerase [10]. The first round PCR program was as follows: 5 minutes at 94°C, 25 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C, then 5 minutes at 72°C. Two microliters of the first-round PCR products were then re-amplified in a second round of PCR. The second round was performed in a 50 μL volume containing 2 μL of the first-round PCR products, 5 μL of 10× PCR

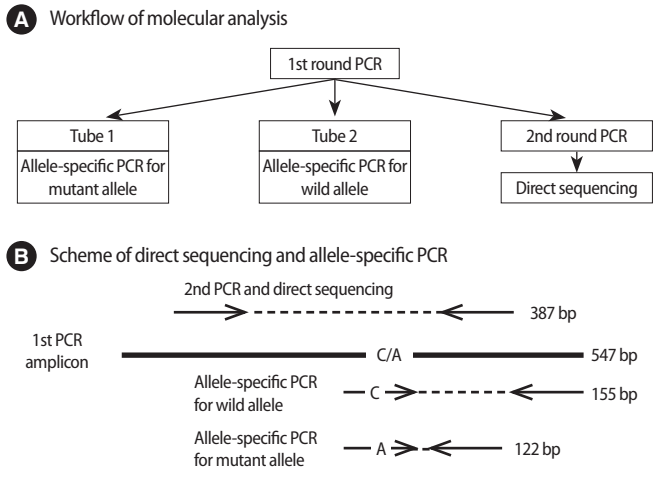

Figure 1. Workflow of molecular analysis (A) and scheme of direct sequencing and allele-specific polymerase chain reaction (PCR) (B).

Table 1. Information of PCR primers

Application	Primer	Sequence (5′ → 3′)	Amplicon size
1st PCR	Outer_F	ATGGAGGGCTTCCTGGAG	547
	Outer_R	GCGTACTCCACCAGCACGTA	
2nd PCR and sequencing	Inner_F	TGACCCTGGGCAAGCCCCTT	387
	Inner_R	ACACGGGCTCCTCAGACGGG	
Allele-specific PCR	1620C-AS-F	GAAACACAAAAACATCATCgAC	122/155
	1620A-AS-F	GAAACACAAAAACATCATCgAA	
	1620-R	GACACGGGCTCCTCAGAC	
	1620-R2	GCGTACTCCACCAGCACGTA	

PCR, polymerase chain reaction.

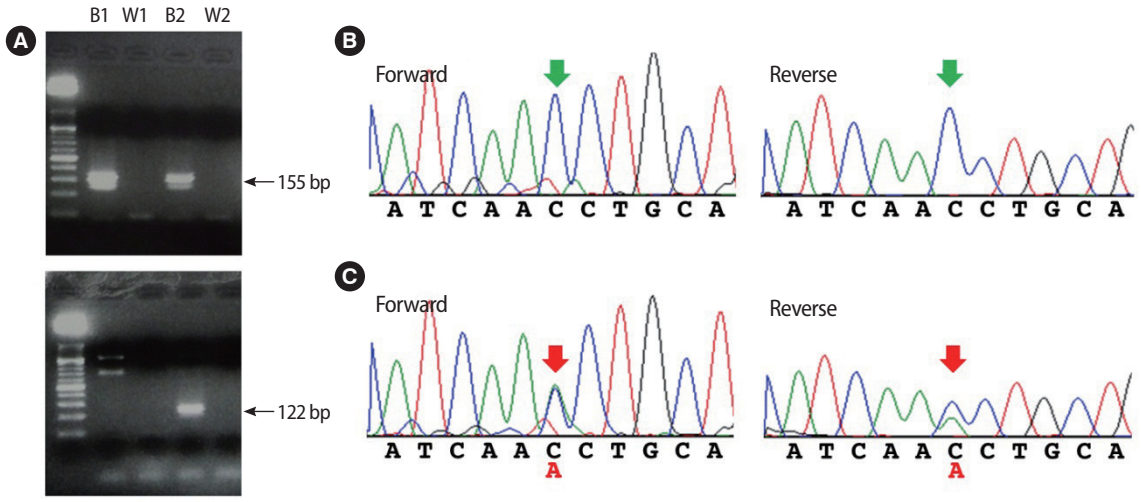

Figure 2. (A) Nested allele-specific polymerase chain reaction (PCR) which targets c.1620C (155 bp, upper column) and c.1620A (122 bp, lower column), and electrophoresis. Lanes B1 show blastomere biopsy with c.1620C, p.N540, homozygote, and lanes B2 with c.1620C > A, p.N540K, heterozygote. Lanes W1 and W2 are PCR results of washing drops of each specimen. (B) Sequence analysis for c.1620C, p.N540, homozygote. (C) Sequence analysis for c.1620C > A, p.N540K, heterozygote (location of nucleotides marked by arrows).

buffer, 200 μM dNTP, 10% DMSO, 0.4 μM of each inner primer (Table 1), and 1.25 U Taq DNA polymerase. The second round PCR program was as follows: 5 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C, then 5 minutes at 72°C.

The second-round PCR products were analyzed by direct sequencing using an ABI Prism 3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA). To confirm the result of the sequence analysis, allele-specific PCRs for the wild allele and mutant allele were performed on the first-round PCR product, separately. These processes are presented schematically in Figure 1. Primers for the mutant allele were designed to generate the 122 bp amplicon and those for the wild allele were designed to generate the 155 bp amplicon (Table 1).

3. Results

Through the allele-specific nested PCR and sequencing as described, blastomeres were classified as c.1629C, p.N540, homozygote or c.1620C > A, p.N540K, heterozygote (Figure 2). Among the 20 blastomeres, amplifications of the first-round PCR product were successful in 16 (80%) blastomeres. Ten blastomeres were negative for the mutation with sequencing and allele-specific PCR. Five blastomeres were positive for mutation with both methods. A blastomere was positive for mutation in allele-specific PCR, although negative in sequencing. None of the washing drops showed amplification in first-round PCR.

Finally, 9 embryos with the wild-type sequence (*FGFR3*:c.1620C) were selected and cryopreserved by vitrification in a fresh cycle due to the patient's high risk of ovarian hyperstimulation syndrome (OHSS). After a failure of the first frozen-thawed cycle, a successful singleton pregnancy was achieved through the second frozen-thawed cycle, in which the embryo transfer of 2 blastocysts had been attempted. An appropriate initial rise in β-hCG and fetal cardiac activity were noted sequentially. On 16 weeks of gestation, amniocentesis was carried out for verification. Genetic analysis of cultured amniotic fluid cells showed a normal karyotype, and the presence of the c.1620C > A, p.N540K mutation in the *FGFR3* gene was excluded. On second trimester ultrasound findings, no evidence of skeletal dysplasia was identified. The pregnancy resulted in a full-term delivery without any significant complications: a 3,620-g male infant was delivered at 40 weeks by Cesarean section.

Discussion

Here, we present a successful pregnancy and birth using a nested allele-specific PCR and sequence analysis for PGD in an HCH patient carrying the c.1620C > A, p.N540K mutation in the *FGFR3* gene, for the first time in Korea to the best of our knowledge. Only two cases of PGD results have been reported for ACH, which is characterized as similar but more severe than HCH. One case achieved successful pregnancy and birth with a wild-type maternal allele; however, the other case failed to achieve pregnancy [11,12].

HCH is a skeletal dysplasia characterized by short limbs, short stature, and lumbar lordosis, compatible with the patient's clinical features, and she had previously suffered two consecutive abortions before PGD, due to affected fetuses that in which the presence of the c.1620C > A, pN540K mutation in the *FGFR3* gene was confirmed and proven identical to hers. The prenatal diagnosis of HCH has been

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published in several antecedent reports [6,13,14]. For the prenatal diagnosis, ultrasound findings usually provide a clue to detecting fetal skeletal dysplasia and identifying the mutation can ensure the diagnosis. The molecular diagnosis is performed on fetal DNA extracted from amniotic fluid cells or chorionic villi cells. In this PGD case, molecular genetic analysis of blastomeres with a relatively low quantity of template DNA could be accomplished based on prior experience with prenatal diagnosis.

Mutations in HCH patients are widespread in *FGFR3* gene and several kinds of pathogenic amino acid substitutions in the *FGFR3* gene have been identified, including p.N540K, pN540T, p.N540S, p.I538V, p.N328I, p.K650N, and p.K650Q [13,15-19]. This case showed the p.N540K substitution, which is known to account for about 70% of HCH cases [8].

PGD has been developed for patients who have a monogenic defect transmitted to their offspring. Single-cell amplification for PGD has been challenged by difficulties in achieving a high level of accuracy and reliability such as contamination, amplification failure, and ADO. Owing to the risk of contamination and ADO, it is recommended that DNA amplification protocols include the use of linkage markers in addition to the target mutation locus [20]. Some methods such as multiplex PCR, fluorescent PCR, and whole genome amplification have been developed to reduce the presence of contamination and ADO and allow a more accurate diagnosis [3,20]. However, we adopted the new techniques of direct sequence analysis and allele-specific PCR for accuracy in detecting single nucleotide substitution within the restricted time frame. Although we decided on freezing all of the embryos in a fresh cycle due to the patient's OHSS risk, wild-type proven embryos had been prepared in time for the transfer. Furthermore, direct sequencing enabled detection of the pathogenic mutation more accurately than other indirect methods. In our preliminary analysis with a single lymphocyte, the ADO rate was 18% in sequencing; however, there was no ADO in allele-specific PCR. In this PGD analysis with both sequencing and allele-specific PCR, we reported only one ADO in sequencing among 20 blastomeres. ADO in sequencing seems to be caused by low amplification of mutant alleles during first-round PCR. Confirmatory allele-specific PCR could be applied to produce a robust result and to compensate for the low sensitivity of direct sequencing.

HCH is a rare condition, of which the reported prevalence is approximately 1 per 50,000 births [14], but if the affected parent wants to have an unaffected baby, this brand new method using direct sequence analysis accompanied by allele-specific PCR can be successfully applied to improve the detection rate of the precise mutation site and finally to select a wild-type embryo for PGD, providing a new option applicable to many kinds of monogenic disorders for which the causative single-gene mutation is known. This case illustrates the reliability and feasibility of new single-cell analysis for PGD, reporting a healthy birth following application of the PGD method.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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