

# Birth of healthy twins after PGT-M for achondroplasia - first reported case in India and second in the world

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## ABSTRACT

**Aim:** To perform pre-implantation genetic testing for a monogenic (PGT-M) condition due to *FGFR3* gene mutation (c.1620C>C/G (p.N540K) in order to transfer unaffected embryos after ICSI.

**Methods:** Oocytes were fertilized by means of intracytoplasmic sperm injection. The resulting embryos were biopsied 5 days at the blastocyst stage. Trophectoderm cells were taken and whole-genome amplification was performed. Amplification of the mutation site was achieved by polymerase chain reaction (PCR) and capillary electrophoresis was performed for identification of the mutation. Two mutation free embryos were transferred, leading to the birth of healthy twins.

**Result(s):** A c.1620C>C/G (p.N540K) mutation was identified in a dwarf man with Achondroplasia / Hypochondroplasia. Female partner had no features of dwarfism. Embryos with and without mutation were identified by means of PGT-M PCR, followed by successful transfer of two mutation free embryos, resulting in a successful pregnancy and delivery of health twins.

**Conclusion(s):** We report the first case of successful PGT-M for achondroplasia in India.

**KEYWORDS:** PGT-M, PCR, *FGFR3*, Achondroplasia

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## INTRODUCTION

Achondroplasia (ACH) is the commonest form of short limbed dwarfism (birth incidence estimated at between 1:10 000 and 1:70 000) and is transmitted as an autosomal dominant trait. Fibroblast growth factor receptor 3 gene (*FGFR3*) mutations in the germ-line are well-known causes of skeletal syndromes. The lack of ossification (a process of the formation of bone achondroplasia. Phenotypic features include disproportionate short stature megalencephaly, a prominent forehead (frontal bossing), midface hypoplasia, a normal trunk length, rhizomelic (proximal) shortening of the arms and legs, prominent lumbar lordosis, genu varum (bowed legs), and a trident-hand configuration. About 80% of these cases are as a result of spontaneous mutations, remaining 20% are due to inheritance from a parent. Germline mosaicism has been reported due to paternal transmission; some studies also contradict maternal germline- somatic mosaicism contributing to the ACH (Kyung Eui Park et al., 2013).

The gene responsible for ACH is a fibroblast growth factor receptor 3 (*FGFR3*) located telomerically on the short arm of chromosome 4. In 97% of cases, ACH is due to a unique amino acid substitution of glycine to arginine at position 380 (G380R, G1138A) in the transmembrane domain of *FGFR3*. Only 3% of ACH cases are due to a G to C mutation in the same gene, at the same position (G1138C). (GAltarescu et al,2008 , Wen-Bin He et al,2018). Hypochondroplasia under the similar category of achondropalsia (ACH) a skeletal dwarfism condition also follows the dominant mode of inheritance and is milder than ACH. There is evidence of genetic linkage between hypochondroplasia and achondroplasia as they map to the same locus. A systematic screening of *FGFR3* mutations in hypochondroplasia reports a mutation which causes a C to A transversion at nucleotide 1620 resulting in an Asn540Lys substitution in the proximal tyrosine kinase domain.

More than 5000 Mendelian disorders have been identified in humans, including approximately 1300 X linked and autosomal recessive disorders. Some of them account for infant mortality, congenital abnormalities with life-threatening conditions requiring treatments and management. The most unfortunate thing with any genetic condition or a disorder is it has no cure but can always be prevented through prenatal and preimplantation genetic testing.

Genetic testing of pre-implantation embryos promises to prevent monogenic diseases in children born to at risk couples. With the advancements of technology, it is now possible to provide genetic testing for any disease and prevent it through pre-implantation genetic testing (PGT).

Genetic counselling is very important perspective in IVF with PGT. Though Providing PGT services is now a reality for most clinics, the most challenging part is finding out the disease-causing mutation if no previous work-up of couple is available. Preconception genetic screening for couples is a must in order to provide reliable PGT results.

## MATERIALS AND METHODS

### Case Report

In this report, we present a successful pregnancy and birth of healthy twins at our hospital using PCR and sequencing for PGT-M in an HCH patient for the first time in India.

In this case report, the couple comprised a 33-year-old woman and a 37-year-old man, who attended genetic counseling with primary infertility. Male partner had dwarf-like features, such as short stature consistent with ACH and the average height of 160 cm and had undergone skeletal extension. Sperm parameters were in

normal range. Male had premature ejaculation issues. Female partner had PCOS (poly cystic ovaries). The couple reported no affected members in their families. Genetic counselling was provided for a targeted mutation screening for *FGFR3* gene for the male. Male tested heterozygous for the mutation c.1620C>C/G (p.N540K) in exon 12 of the *FGFR3* gene. The couple was willing for PGT-M and signed the informed consent for the same.

### Ovarian stimulation and Oocyte accumulation

Controlled ovarian stimulation (COS) was done with antagonist protocol using gonadotropins with a dosage between 150 – 300IU depending on age and body mass index. Oocytes were aspirated under local anesthesia after 36 h of agonist trigger. Denudation was done after 1h of oocyte retrieval. ICSI was performed. After ICSI, fertilization check was done the next day, followed by day 3 embryo quality check. Good quality day 3 cleavage stage embryos (embryos with 6-8 cells, equal size of blastomere and cytoplasmic fragmentation less than 10%) were allowed to grow till blastocyst stage. Zygotes were cultured in VITROMED culture medium for 5-6 days. Blastocysts quality was graded accordingly.

### Embryo/Blastocyst Biopsy and vitrification/freezing down (of blastocyst)

Trophectoderm biopsy was done on day 5 embryos (blastocyst stage) as described previously. Briefly, laser-assisted hatching was performed using LYKOS Laser (Hamilton Thorne; MA, USA) on day 3 embryo. On day 5, herniating blastocyst were selected, and 5-10 trophectoderm cells were removed by suction followed by laser pulsation. The trophectoderm cells were collected in phosphate buffer saline (PBS) and stored at -80 degree celcius maintained freezer until further processing. Vitrification method was used to

freeze down the blastocyst. The blastocysts were equilibrated in equilibration solution for 12 to 15 minutes, followed by transfer to the vitrification solution. This was finally transferred to cryolock containing liquid nitrogen.

### Molecular investigations

Genetic testing for the c.1620C>C/G (p.N540K) mutation was performed to check primer specificity on the male partner's DNA extracted from the peripheral blood. Primers used for the PGT-M were reported in a previous publication for the same mutation (Bellus GA et al,1995). The couple were counselled and informed about it. On the sixth day, 5 blastocysts were biopsied and TE (Trophectoderm cells) cells were collected in respective tubes. One tube was labelled as negative control, which contained only the washing drop to rule out contamination. Whole-genome amplification was carried out using PicoPlex Single Cell Whole Genome Amplification (WGA) kit for all the tubes separately, including the negative control. First-round PCR was performed using the first set of primers, ready to use PCR mix in a 25 ul reaction and same PCR program as per the kit protocol for 25 cycles. Second-round PCR was performed using the first-round PCR product with the same PCR conditions. The second-round PCR products were analyzed by direct sequencing using an ABI Prism 3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

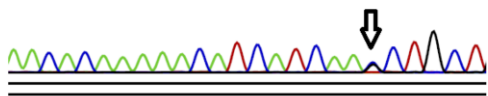
### RESULTS

Through sequencing, embryos were evaluated for the c.1620C>C/G (p.N540K), homozygote or heterozygote mutation. Among six embryonic TE cells given, amplification for both the first and second-round PCR was successful in all of them and negative control did not give any band on the gel. Two embryos were affected and were heterozygote (CG) for the mutation. One embryo showed GG genotype which could be due to allele drop out, rest of the 4 embryos showed the wild type (CC) genotype. Healthy pregnancy with the transfer of two normal embryos was achieved.

Viability scan confirmed the presence of two fetuses with the cardiac activity recorded. Pregnancy was continuously followed up. Amniocentesis was performed at 16 weeks of gestation and the results were in concordance with the PGT-M results. No growth defects or a skeletal

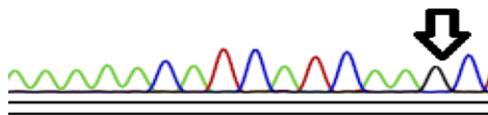
defect was seen in a follow-up scan for both the fetuses. Karyotype also revealed normal chromosome complement. The pregnancy resulted in a full-term delivery of one baby boy and one baby girl by C-section without any maternal complications.

AACACAAAAACATCATCAACCTGCT  
220 230 240



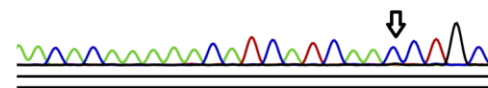
Father –heterozygous genotype

AAAAACATCATCAAGC  
230 240



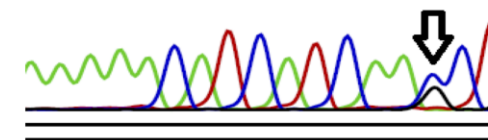
Embryo 1 –homozygous mutant

AACACAAAAACATCATCAACCTGCT  
220 230 240



Embryo 2,4,5 –homozygous wild type

AAAAACATCATCAACC  
210 220



Embryo 3 –heterozygous genotype

Fig 1. Sequencing peaks showing the wild and mutant allele.

## DISCUSSION

This case report describes successful PGT-M and the birth of healthy twins in the first cycle of IVF using nested PCR and sequencing to diagnose a mutation c.1620C>C/G (p.N540K) in an HCH case.

This is the first successful case in INDIA and one of the few reported cases so far in the world as far as our knowledge goes. Prenatal diagnosis was the only option available earlier to diagnose any genetic condition. With the technological

advancements in terms of pre-implantation genetic testing, it is now possible to offer PGT-M for most of the genetic conditions. When it comes to diagnosing a single gene disorder, it must to get the index child tested for the mutation. Most of the times single-gene diseases are only diagnosed after a child is born with an affected condition. Some of them have no clue about the condition and then seek a clinician opinion in order to plan the next pregnancy. If there is no genetic testing done on the index child, no conclusion can be drawn in order to find out the genetic cause. In some cases, the index child may even pass away due to some congenital defects, making it too tricky to diagnose as the DNA also might not be available for the index child. In such cases, expanded carrier screening on a couple may give a definitive answer (Shirley Henderson et al). Carrier screening is an important component in IVF in order to find out if the parents are carriers of any genetic defects. This case report reports a mutation c.1620C>C/G (p.N540K) which was found in father. Targeted mutation analysis on NGS with special emphasis on skeletal dysplasia genes must be done rather than a PCR detection assay in order to identify uncommon mutations. Even IVF clinics involving donor programs must consider genetic screening for donors as well in order to categorize known genetic defects. In this case, we preferred to perform whole genome amplification before PCR in an effort to increase the minute amounts of genetic material, as a result of which we could get successful amplification for all the biopsied cells and also reduced contamination and allele drop out (ADO) rates to allow more accurate diagnosis. Single-cell amplification is quite challenging due to factors like allele drop out, amplification failure rate, and contamination. DNA amplification protocols including linked markers to rule out ADO remains gold standard; however, it might not be possible to design markers for every disease. In addition to this minimum, 8 to 10 linkage markers or STR's (short tandem repeats) along with the targeted mutation locus is required. Many a times, it is also noticed that these

polymorphic linked markers might not successfully amplify and may give inconclusive results and thereby we might label an embryo undiagnosed that ultimately results in the wastage of the cycle. We have adopted the direct sequencing analysis method of the second round PCR product for accuracy in detecting single nucleotide substitution within the restricted time frame. Although in our hospital, we perform freezing of the embryos and transfer later on. Furthermore, direct sequencing enabled detection of the pathogenic mutation more accurately than other indirect methods. In our case report, one of the embryos showed GG genotype, suggesting either an ADO would have occurred or as per previous literature and studies published maternal germline mosaicism might also be the case. Since we did not use polymorphic linked markers, we were unable to categorize the inheritance of other G alleles.

HCH is a rare condition, but due to ethical reasons if parents want to have PGT done then its solely their choice. In this case, the couple wanted to transfer two wild type embryos, which resulted in the birth of healthy twins.

## CONCLUSION

Very few successful cases of PGT-M are reported for HCH in literature. The purpose of this study was to show that direct sequencing can provide accurate detection of the mutation. However, polymorphic linked markers if used could have provided the explanation of the GG genotype in one of the embryos. Genome-wide linkage analysis can also be beneficial for PGT-M cases. Unavailability of DNA samples of family relatives might limit the use of genome-wide linkage studies. NGS based linkage analysis will be more efficient and accurate as the results are reads dependent. PGT-M cases can be successfully dealt in future by targeted NGS approach using linkage-based studies. The aim of this article is to provide information that PGT-M based PCR and direct sequencing approach also provides an accurate diagnosis for the detection of the mutant allele,

and this is the first successful study on blastocyst biopsy for HCH. Wherever linkage studies are not possible, PCR and sequencing-based diagnosis remains gold standard. Pre-test and pre-implantation test counseling is must and should be provided with all detailed information to the patients emphasizing the limitations of the techniques and prenatal diagnosis confirmation. This case illustrates the reliability and feasibility of PGT-M, reporting a healthy twin birth following application of the PCR and sequencing method.

### Acknowledgements

We acknowledge the entire team for this successful case.

### Conflict of interest statement

There are no conflict of interest.

### Authors' contributions

RG Performed the PGT-M procedure and genetic counseling. MA undertook the ovarian stimulation and embryo transfer.

### Declaration of originality

The authors have declared that this submission is their own work and this rare case is reported first time in India in this work.

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