

Non-Invasive Prenatal Detecting of Achondroplasia In Earlier Stage of Pregnancy By Droplet-Digital PCR

Yipeng Wang

Beijing Obstetrics and Gynecology Hospital, Capital Medical University

Wei Wang

Beijing Obstetrics and Gynecology Hospital, Capital Medical University

Chengrong Wang

Beijing Obstetrics and Gynecology Hospital, Capital Medical University

Shanshan Li

Beijing Obstetrics and Gynecology Hospital, Capital Medical University

Meng Zhang

Beijing Obstetrics and Gynecology Hospital, Capital Medical University

Chenghong Yin (✉ yinchh@ccmu.edu.cn)

Beijing Obstetrics and Gynecology Hospital, Capital Medical University

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Abstract

Background: Achondroplasia (ACH) is generally detected by abnormal prenatal ultrasound findings in the late stage of pregnancy and then confirmed by molecular genetic testing of fetal genomic DNA obtained invasively. Most ACH cases appear to be *de novo* mutations with FGFR3 gene, so it is a challenge to screen ACH fetus out in the early stage of pregnancy.

Objective: The aim of this study was to validate the possibility of detect fetus ACH along with non-invasive prenatal screening(NIPS) routinely in the early stage of pregnancy.

Methods: 5927 cases of pregnant women undergoing NIPS were enrolled in this study. An additional 5ml of blood was collected together with NIPS blood sampling. Cell free DNA was extracted for the detecting of fetus ACH. Droplet-digital PCR(ddPCR) method based on the amplification of the two possible mutant alleles (c. 1138G>A and c. 1138G>C) of FGFR3 gene was performed to screen fetus ACH. Prenatal ultrasound and amniocentesis were then performed to confirm the positive screening result of ACH cases. The mutation sites of fetus were identified via Sanger sequencing by using amniotic fluid cells. For the screen negative cases of pregnant women, we followed up the results of prenatal diagnosis or the general conditions of the newborns.

Results: One pregnant woman with fetus ACH were screened out at 22 weeks by Non-invasive prenatal detecting. Later prenatal ultrasound confirmed fetal skeletal dysplasia. Sanger sequencing confirmed *de novo* FGFR3 1138G>A mutant of the fetus. No ACH fetus or newborns were found in the rest detected negative cases of enrolled pregnant women.

Conclusion: ddPCR technology could effectively identify *de novo* mutation like ACH of fetus noninvasively. We prospect the clinical application of ddPCR can expand the range of prenatal screen in the future.

Introduction

Achondroplasia (ACH, MIM 100800), characterized by disproportionate short stature, is a common type of skeletal dysplasia with the prevalence of 5-15/100,000 ^[1]. The clinical features of achondroplasia include rhizomelic shortening of the limbs, macrocephaly, and characteristic facial features with frontal bossing and midface retrusion ^[2]. Though intelligence and life span are usually near normal, craniocervical junction compression increases the risk of death in infancy ^[2].

ACH is an autosomal dominant disorder, resulting from mutation in the fibroblast growth factor receptor 3 gene (FGFR3) with 100% penetrance. More than 99% of ACH is caused by the heterozygous glycine-to-arginine substitution at codon 380 in the transmembrane domain of FGFR3, of which about 98 % of cases are the G to-A transition at nucleotide 1138 of coding sequence (c. 1138G>A) and about 1 % of cases are G-to-C transversion at this position (c. 1138G>C) ^[3]. Most cases appear to be *de novo* mutations with no prior familial history of skeletal dysplasia ^[4].

Usually, ACH is detected by abnormal prenatal ultrasound findings in the third trimester of pregnancy, and then confirmed by molecular genetic testing of fetal genomic DNA obtained by percutaneous umbilical blood or amniotic fluid sampling [2]. The postponed diagnose of fetal ACH increases risk of clinical decision and anxiety of the family. To screen fetal ACH noninvasively in earlier stage of pregnancy should be a better solution for the clinic and the family.

Since its discovery in maternal blood, cell-free fetal DNA (cfDNA) originated from placenta trophoblasts was soon used for fetal disease testing, and rapidly adopted in clinical practice known as the noninvasive prenatal screening (NIPS) [5]. With higher sensitivity and specificity than conventional biochemical approaches, NIPS is broadly used to screen for fetal trisomy 21 (T21), trisomy 18 (T18), and trisomy 13 (T13) as early as 12 weeks of pregnancy [6-8]. In recent years, cfDNA was used to screen for sex chromosome aneuploidy and chromosome copy number variants (CNVs) [9, 10]. Studies have demonstrated the utility of NIPS in fetal sex determination, fetal Rhesus D genotyping in D-negative mothers, and in some monogenic disorders [11-13]. Achondroplasia is of the first monogenic disorders that can be noninvasively prenatal diagnosed through cfDNA, because genetic absence of *de novo* mutation from the maternal and the high prevalence of FGFR3 single point hot spot mutations makes the detection easier to get high specificity and high coverage. Since fetal FGFR3 c.1138G>A was first detected through mass spectrometry (MS) in 2000, several approaches have been demonstrated capable of detecting FGFR3 mutations from cfDNA, including MS, qPCR, droplet digital PCR (ddPCR), and Next generation sequencing [14-18]. All the reported tests aimed at non-invasive prenatal diagnosis of ACH after abnormal ultrasound findings in the third trimester. In this work, we developed a ddPCR method of detecting FGFR3 p.G380R (c.1138G>A and c.1138G>C), and screened pregnant women in earlier stage of pregnant women in parallel of NIPS for T21/T18/T13. After tested 5927 pregnant women, we detected a 22-week pregnant woman positive for c.1138G>A, and further Sanger sequencing confirmed *de novo* mutation.

Methods

Subjects

Pregnant women who visited Beijing Obstetrics and Gynecology Hospital for NIPS could choose to have ACH detection simultaneously to NIPS for chromosome aneuploidy screening after informed consent. A total of 5927 pregnant women who voluntarily had ACH detection from November 2019 to October 2021 were included in the study. The study was carried out under the approval by the Hospital Ethics Committee of the Beijing Obstetrics and Gynecology Hospital.

Exclusion criteria

Pregnant women who were ACH patients were excluded. Besides, because ACH detection was conducted synchronously with NIPS, exclusion criteria of NIPS were also followed [9]. And the recommended sampling time was at gestational age 12⁺⁰ to 22⁺⁶ weeks. In order to prevent false negative results due to

low fetal fraction, in case of the fetal fraction of cfDNA <4%, a new analysis is recommended on a second sample 2 weeks later [8].

Collection and treatment of blood samples

Maternal peripheral blood samples (5 ml) were collected in EDTA tubes, fully mixed, stored temporarily in 4 °C refrigerator. Samples were excluded if hemolysis or storage beyond 8 hours before plasma separation. The blood samples were treated as follows: centrifuged at 4 °C, 1600 g for 10min and the plasma was collected carefully and dispensed into 2.0 ml Eppendorf tubes (0030108078, Eppendorf, Germany). The plasma was centrifuged again at 4 °C, 16,000 g for another 10 min. The upper plasma was carefully divided into new 2.0 ml Eppendorf tubes, and each contained approximately 600 µl plasma, -80 °C refrigerator to save. The plasma should avoid repeated freezing and thawing before experiment.

DNA extraction

The cfDNA was extracted by QIAamp Circulating Nucleic Acid(55114,Qiagen) according to the manufacturer's introduction. cfDNA was concentrated by Agencourt AMPure XP paramagnetic bead (A63881 Beckman, USA) and stored in Nuclease-Free water(P1195,Promega,USA).

Droplet-digital PCR

ddPCR method based on the amplification of the two possible mutant alleles A allele or C allele and the wildtype G allele was developed. Primer sequences are described in Table 1. The presence or absence of amplification for the mutant allele signs the presence or absence of mutant FGFR3 sequences of fetal origin in the maternal plasma.

ddPCR was performed using the BioRad QX200™ droplet digital PCR System (BioRad, USA) according to the manufacturer's instructions. In each well, 10µL of cfDNA solution were mixed with 11 µL of ddPCR master mix (BioRad, dd PCR supermix for probe, 1863028, Biorad, USA) and 1µL of each duplex PCR primers/probes. The reaction mix was dispensed into the 96-well PCR plate (0030128575, Eppendorf, USA). The PCR plate was then placed in QX200 Droplet Generator (BioRad, USA) to generated droplets. The PCR plate was then heat-sealed with a foil seal by PX1 heat sealer (BioRad, USA), and the sealed plate was placed in the PCR thermocycler. Thermal conditions were: 1 cycle of 95 °C for 5 min, 40 cycles of 94 °C for 30s, 57 °C for 60s, and 1 cycle of 98 °C for 10 min. After the reaction, the droplets were read using the Droplet Reader, and QUANTASOFT software (BioRad, USA) converted the data into concentrations using Poisson distribution statistical analysis. Briefly, the wildtype allele (G) and the mutant alleles (C and A) were indicated with different fluorescences, and detecting a fluorescence indicates the DNA target was initially present in the droplet and negative of fluorescence indicates no DNA target was initially present in the droplet. The concentrations of DNA targets could be calculated when >10,000 droplets were generated in each well. Result was considered positive when two replicates showed more than five positive droplets for the mutant allele each. The absence of amplification of A-

allele and C-allele, together with the positive amplification of the G-allele indicated a fetus free of the hotspot mutation of nucleotide 1138 of FGFR3 gene.

Confirmation of FGFR3 gene mutation of the fetus

Prenatal ultrasound and amniocentesis were then performed to confirm the positive screening result of ACH cases. Fetal DNA was extracted by using amniotic fluid cells. The mutation sites of the fetus were then identified via Sanger sequencing.

Clinical outcome follow-up

For the women screened negative of FGFR3 mutation, length of the limb and finger length, shape of the hand and face, muscular tension of the newborns were recorded to exclude fetal achondroplasia.

Results

5927 pregnant women, most of whom were 12-22⁺⁶ weeks pregnant, were screened for ACH by this method along with NIPS. One woman was screened positive for FGFR3 c.1138G>A in cfDNA[figure 1].

The 36-year-old woman had already given birth to a healthy child, and had NIPS and ACH test at 22 weeks of pregnancy. Sanger sequencing of the fetal amniotic fluid sample and blood samples of the parents confirmed *de novo* FGFR3 c.1138G>A mutation of the fetus [figure 2]. Ultrasound finding at 29 weeks of pregnancy indicated the features of fetal ACH[figure 3].

Ultrasound examination in the third trimester and pregnancy outcome follow-up of 2950 pregnant women negative in the screen confirmed true negative of ACH. The sensitivity and specificity of the test was 100%(5.5%-100%) and 100%(99.8%-100%), respectively.

Discussion

cfDNA is becoming a crucial tool for diagnosis and management in various clinical disorders. In the field of noninvasive prenatal testing, cfDNA has been applied for fetal gender determination, RHD genotyping, and detecting of chromosomal aneuploidies, chromosomal aneuploidies, and increasing number of monogenic disorders [5-13]. NIPS is becoming a routine clinical management screening more and more genetic disorders [19].

In this work, we developed a high-efficient and low-cost method for noninvasive prenatal ACH detecting, and first applied noninvasive ACH detection in screening of earlier stage pregnant women. 5927 pregnant women voluntarily had this additional test at the time they were having NIPS for chromosomal aneuploidies screening. One woman was diagnosed carrying a FGFR3 c.1138G>A mutant. Following up of 1 positive and 2950 negative results showed the sensitivity and specificity of the test was high, 100% (5.5%-100%) and 100%(99.8%-100%) respectively.

Interestingly, in consistence with the conclusion of previous studies, that advanced father age increases the risk of disease caused by *de novo* mutations, the husband the ACH positive pregnant woman was 49 years old, suggesting that pregnant women with advanced-age husband are recommended to have this screen [4,20,21].

Diagnosis of ACH in earlier stage of pregnancy before the abnormal ultrasound findings will give the patients more time to make decision and to get prepared for an ACH baby. Besides, there are less ethical concerns and less physical and phycological harm to the family if they choose termination of pregnancy in earlier stage of pregnancy compared to doing that in the third trimester of pregnancy.

In addition to ACH, there are other severe monogenic disorders caused by *de novo* mutations, for example four types of osteogenesis imperfecta caused by mutations in COL1A1/2, Pfeiffer syndrome caused by mutations in FGFR1/2, and Noonan Syndrome caused by mutations in one of several Ras/MAPK pathway genes. In the future, the screen can be expanded to cover more disorders caused *de novo* mutations.

In conclusion, we developed a rapid, cheap, and convenient method for noninvasive ACH screening in earlier stage (12-22+6 weeks) of pregnancy, and earlier diagnosis of ACH benefits the affected families a lot. We prospect a broad clinical application of this method in the future.

Table 1. Oligonucleotidic sequences of primers and probes for FGFR3 mutants

Allele	Primer or probe	Sequence
FGFR3_mutant A-allele	Forward primer	CAG TGT GTA TGC AGG C
	Reverse primer	ACC ACC AGG ATG AAC AG
	MUT_A_probe	AGC TAC AGG GTG GGC
	WT_A_probe	AGC TAC GGG GTG GGC
FGFR3_mutant C-allele	Forward primer	CAG TGT GTA TGC AGG C
	Reverse primer	ACC ACC AGG ATG AAC AG
	MUT_C_probe	CTA CCG GGT GGG CTT
	WT_C_probe	CTA CGG GGT GGG CTT

The y- and x-axes correspond to the FAM and HEX intensities, for mutant and WT-alleles, respectively. Negative droplets (gray dots, 14717) and positive ones (blue dots for FAM+ only, 24; green dots for HEX+ only, 424; and brown for FAM+ and HEX+, 0) are assigned as a function of the FAM and HEX florescence amplitudes. The copies of wildtype DNA were 668/well, and the copies of mutant DNA was 38/well, and the ratio of mutant DNA in the cfDNA was $38/(38+668)=5.4\%$.

Sanger sequencing of fetal amniotic fluid sample and parents' blood samples respectively.

Conclusions

ddPCR technology could effectively identify *de novo* mutation like ACH of fetus noninvasively in our clinical practice. We prospect the clinical application of ddPCR can expand the range of prenatal screen in the future.

Declarations

Ethics declaration

Ethical Approval and Consent to participate

Ethical approval was provided by the Human Research Ethics Committee, Beijing Obstetrics and Gynecology Hospital, Capital Medical University (No.2019-KY-046-01).

All subjects authorized the using of the data for reference by signing the consent form. (No. BJOGH201810PDC3, available since 12/06/2018).

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

All patients in this report provided their consent for publication.

Availability of supporting data

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.W conceived of the study and performed the statistical analysis, and helped to draft the manuscript. C.Y participated in the design of the study, and participated in its design and coordination. W.W, C.W, S.L, M.Z carried out the molecular genetic studies and follow-ups.

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Not applicable

Authors information

Affiliations

Beijing Obstetrics and Gynecology Hospital, Capital Medical University.

Yipeng Wang, Wei Wang, Chengrong Wang, Shanshan Li, Meng Zhang, Chenghong Yin

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Figures

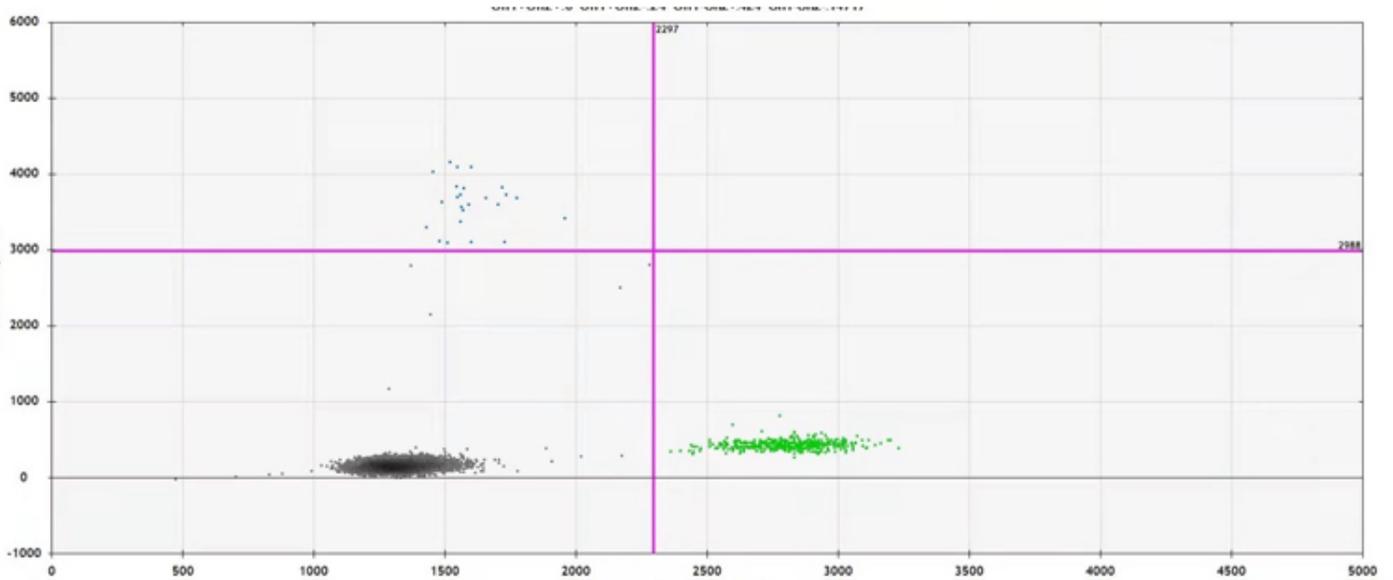


Figure 1

Two-dimensional representation of the fluorescence intensity obtained by droplet digital PCR (ddPCR) using FGFR3 1138G>A ddPCR assay.

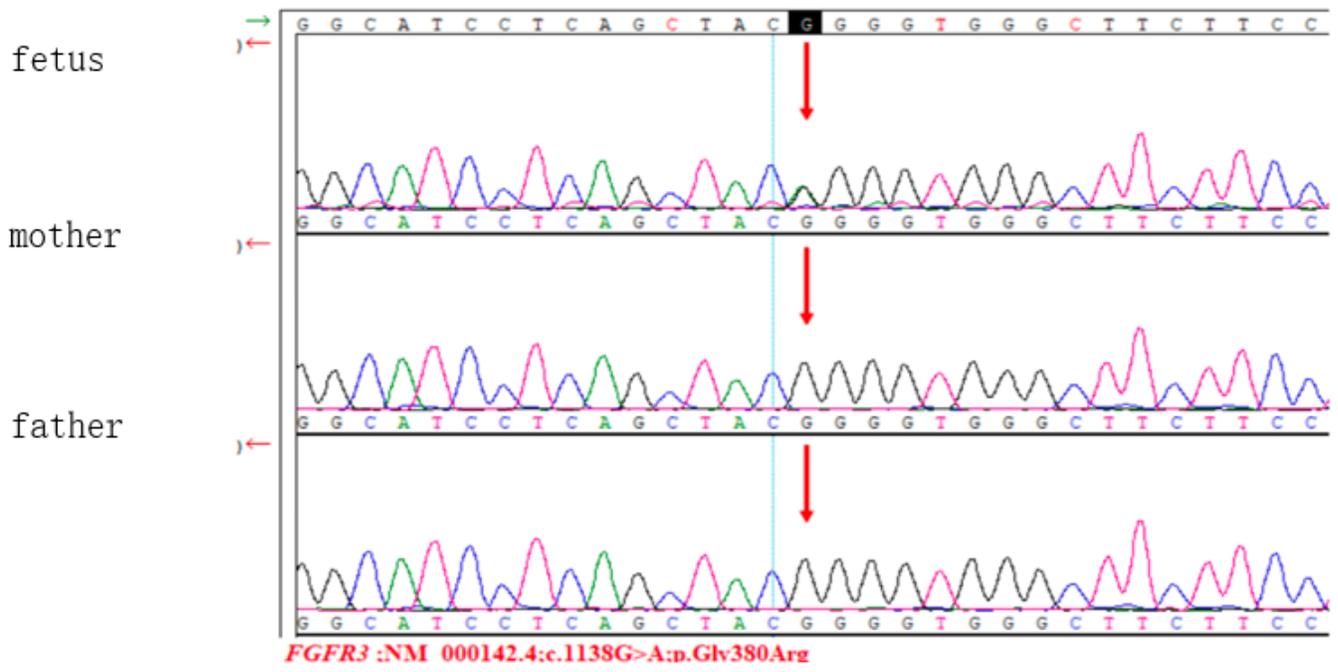


Figure 2

Sanger sequencing confirmed de novo FGFR3 1138G>A mutant of the fetus.