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Title

Droplet Digital PCR combined with minisequencing, a new approach to analyze fetal DNA from maternal blood: application to the non-invasive prenatal diagnosis of achondroplasia

Running Head

Non-invasive prenatal diagnosis of achondroplasia by ddPCR and minisequencing

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Bulleted statements

We developed an original strategy for the NIPD of achondroplasia from maternal blood, combining ddPCR with minisequencing. This diagnosis provides a rapid and definitive diagnosis for parents that are in a painful context of a sudden discovery of abnormal prenatal-ultrasound findings in the third trimester of pregnancy, as well as an early diagnosis for parents aware of the risk because of familial history, allowing an appropriate clinical management of pregnancy.

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Abstract

Background. Achondroplasia is generally 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 aspiration of amniotic fluid. This invasive procedure presents a small but significant risk for both the fetus and mother. Therefore, non-invasive procedures using cell-free-fetal DNA in maternal plasma have been developed for the detection of the fetal achondroplasia mutations. **Methods.** To determine whether the fetus carries the *de novo* nonsense genetic mutation at nucleotide 1138 in *FGFR3* gene involved in >99% of achondroplasia cases, we developed two independent methods: digital-droplet PCR combined with minisequencing, which are very sensitive methods allowing detection of rare alleles. **Results**. We collected 26 plasmatic samples from women carrying fetus at risk of achondroplasia, and diagnosed to date a total of five affected fetuses in maternal blood. The sensitivity and specificity of our test are respectively 100% [95% confidence interval, 56.6 to 100%] and 100% [95% confidence interval, 84.5 to 100%]. **Conclusions**. This novel, original strategy for non-invasive prenatal diagnosis of achondroplasia is suitable for implementation in routine clinical testing and allows considering extending the applications of these technologies in non-invasive prenatal diagnosis of many other monogenic diseases.

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Introduction

Achondroplasia (MIM: 100800) is one of the chondrodysplasia which can be diagnosed prenatally. This autosomal dominant genetic disease, also known as chondrodystrophic dwarfism[1,2], is the most common nonlethal form of skeletal dysplasia with an estimated incidence rate of one in 20,000 live births.

Achondroplasia is caused by mutations of the transmembrane receptor fibroblast growth factor receptor 3 gene *FGFR3* (MIM: 134934), which encodes an important regulator of linear bone growth[3]. In normal development FGFR3 has a negative regulatory effect on bone growth. In achondroplasia, the mutated form of the receptor is constitutively active and this leads to severely shortened bones. Approximately 98% of patients with achondroplasia have a *de novo* nonsense genetic mutation in *FGFR3* gene with a guanine to adenine transition at nucleotide 1138 resulting in a glycine to arginine substitution at position 380 (c.1138G>A, p.Gly380Arg), while around 1% present a guanine to cytosine transversion at the same position (c.1138G>C, p.Gly380Arg mutation)[4].

Achondroplasia is generally detected either on family history of achondroplasia or during routine ultrasound in the third trimester of pregnancy, on the basis of characteristic skeletal features such as shortened long bones with bowed femora, macrocephaly, frontal bossing and trident hand. Molecular prenatal genetic testing offers an early, rapid and reliable diagnosis, through the testing of fetal genomic DNA obtained by amniocentesis. These invasive procedures present a small but significant risk for both the fetus and mother. Therefore, non-invasive procedures using cell-free fetal DNA (cffDNA) in maternal plasma have been developed for the detection of the fetal achondroplasia mutation.

In 1997, inspired by the presence of tumor DNA in the plasma and serum of patients suffering from cancer[5], Lo et al. showed the presence of cell-free fetal DNA in the plasma

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and serum of pregnant women[6]. Therefore, the discovery of cffDNA has opened the field for many applications of non-invasive prenatal diagnosis, the vast majority being developed from maternal plasma. The DNA extracted from maternal plasma, also known as cell-free DNA (cfDNA) or circulating DNA, is formed by a mixture of cf DNA of maternal origin (~ 90%) and cfDNA of fetal origin (~ 10%)[7]. The development in 2011 of a droplet digital PCR system (ddPCR) allowed to optimize sensitivity, specificity and accuracy for the detection and analysis of nucleic acids of low abundance, such as cfDNA. Its principle is based on the partitioning of biological sample in tens of thousands of microdroplets of 1 nanoliter, wherein the plasmatic DNA fragments are distributed. When compared with the traditional technique of real time quantitative PCR, the partitioning step of ddPCR allows to reduce the effects of competition due to the presence in plasma of a large excess of maternal DNA, and thus increases the specificity and sensitivity of the detection of cfDNA of fetal origin.

Combining this new, original approach of ddPCR with minisequencing, we developed a new strategy for the non-invasive prenatal diagnosis of achondroplasia. Since 2013, close to 190 plasmatic samples were collected from women at risk of transmitting monogenic disorders, such as Duchenne muscular dystrophy, hemophilia, cystic fibrosis, neurofibromatosis type 1 or achondroplasia. Among those, 26 samples were collected from women carrying fetus at risk of achondroplasia, on the basis of abnormal prenatal ultrasound findings in the third trimester of pregnancy. This allowed us to diagnose to date a total of five cases of achondroplasia in maternal blood.

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Methods

Participants

Two achondroplastic patients previously diagnosed with *FGFR3* mutations by standard methods (sequence of the DNA isolated from peripheral blood leucocytes) with c.1138G>A and c.1138G>C mutation respectively, were used to create artificial mixtures of DNA with very low concentrations and variable proportions of mutant allele to test sensitivity and specificity of our assays. A healthy pregnant woman with 22 weeks of pregnancy of a fetus with characteristic skeletal features of achondroplasia and whose husband carried the c.1138G>A mutation was studied as a positive control to determine whether studying cfDNA could be used for the diagnosis of the fetal c.1138G>A mutation. Additionally, a total of 25 pregnant women with an echographic suspicion of fetal chondrodysplasia were recruited. Two additional healthy subjects, negative for *FGFR3* mutation, were used as negative controls. Written informed consent was obtained prior to venepuncture and the study was ethically approved by the Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le domaine de la Santé (CCTIRS – ref 13.386) and the Comité de Protection des Personnes (CPP - ref 2014-janvier-13465). In all cases, confirmation of prenatal testing result was done by conventional procedures, blindly to the results of non-invasive testing.

Sample collection

For each pregnant patient, two tubes of blood were collected by venipuncture in BCT Cell-Free DNA™ Blood Collection Tube (Streck, Omaha, NE). Plasma samples were separated after double centrifugation within 4 days of collection, one at 1600g for 10 minutes and the second at 16000g for 10 minutes. The buffy coats were retained. The whole was frozen at -80 ° C in a sterile tube. In all cases, a tissue of fetal origin was taken invasively by puncture of

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the amniotic fluid (n = 26 samples). For these cases, the blood collection was performed before invasive sampling.

DNA extraction

cfDNA was extracted from three mL of plasma using the QIAamp Circulating Nucleic Acid (Qiagen, Valencia, CA) according to the protocol recommended by the manufacturer in a laboratory dedicated exclusively for this purpose. The extracted cfDNA was eluted in 100 µL of elution buffer, and stored at -20° C until use. The maternal nuclear DNA and the fetal nuclear DNA were extracted respectively from buffy coats or amniotic fluid by the automatic extractor Maxwell (Promega, Madison, USA), according to the recommendations provided by the manufacturer.

Sanger sequencing

For the conventional prenatal diagnosis of achondroplasia from fetal nuclear DNA extracted from amniotic fluid, a PCR is performed on thermal cycler Applied Biosystem 9700, generating a product of 559 bp, containing the 1138 position of the *FGFR3* gene exon 9. Primer sequences and cycling conditions are described in Table 1. PCR reactions were performed in a total volume of 50 µL with a reaction mixture containing 1 µL of eluted DNA, 1 µL each primer (10 µM), 3 µL MgCl2 (25 mM) and 5 µL 10X buffer. After a purification step, sequencing reactions were performed in a total volume of 20 µL with a reaction mixture containing 3 µL of purified PCR product, 1 µL of either forward or reverse primer (10 µM), 2 µL of 5X BigDye buffer (applied Biosystems) and 4 µL of enzyme Big Dye V1.1. The same primers were used for sequencing and cycling conditions are described in Table 1.

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Digital Droplet PCR

To determine whether the fetus carries a *de novo* nonsense genetic mutation at nucleotide 1138 in *FGFR3* gene, ddPCR method based on the amplification of the two possible mutant alleles –A-allele or C-allele – and the wild-type G-allele was developed. Primer sequences are described in Table 1. The presence or absence of amplification for the mutant allele signs therefore the presence or absence of mutant *FGFR3* sequences of fetal origin in the maternal plasma, which indicates a genotype of the fetus *FGFR3* mutant or wild-type (WT) respectively. Moreover, the amplification of the promoter region of *RASSF1A* gene differentially methylated between the mother and her fetus also ensures the presence of DNA of fetal origin[8] ((Table 1).

Droplet digital PCR was performed using the QX100™ Droplet Digital™ PCR System from BioRad (Hercules, CA) according to the manufacturer's instructions. Briefly, three duplex PCR are carried out in two replicates: mutant FGFR3 A-allele and WT FGFR3 G-allele are amplified together as well as mutant FGFR3 C-allele / WT FGFR3 G-allele and RASSF1A / ACTIN B. In each well, 9 µL of the eluted cfDNA solution were mixed with 10 µL of ddPCR master mix (BioRad), and 1 µL of each duplex PCR primers/probes. Droplets were generated using the Droplet Generator (DG) with 70 µL DG Oil per well with a DG8 cartridge and cartridge holder, 21 µL PCR reaction mix, and DG8 gasket. Droplets were dispensed into the 96-well PCR plate by aspirating 40 µL from the DG8 cartridge into each well. The PCR plate was then heat-sealed with a foil seal and the sealed plate was placed in the PCR thermocycler (thermal conditions in table 1). After the reaction, the droplets were read using the Droplet Reader, and QuantaSoft software converted the data into concentrations using Poisson distribution statistical analysis. Detecting a fluorescence indicates that specific amplification has occurred, and thus that the genomic target was initially present in the biological sample.

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The decision algorithm is presented in Figure 1. Briefly, a concomitant amplification of either *FGFR3* mutant A-allele or mutant C-allele with the *FGFR3* WT G-allele indicates an achondroplastic fetus. After PCR amplification of cfDNA from healthy pregnant women, result was considered positive when >10,000 droplets were generated in each well, and when 2 replicates showed more than 5 positive droplets for the mutant allele each. The absence of amplification of *FGFR3* mutant A-allele and mutant C-allele, together with the positive amplification of the *FGFR3* WT G-allele and the demonstration of the presence of cffDNA (through *RASSF1A* assay) indicated a fetus free of the hotspot mutation of nucleotide 1138 of *FGFR3* gene. The fetal fraction was estimated through *RASSF1A/ B-ACTIN* assay as previously described[8]. Briefly, co-amplification of *RASSF1A* and *B-ACTIN* was performed, before and after BstUI digestion (New England Biolabs, Beverly, MA, USA). In absence of droplets positive for *B-ACTIN* after digestion, fetal fraction was calculated as follow: $[(\text{RASSF1A})_{\text{post-digestion}}/(\text{RASSF1A})_{\text{pre-digestion}}]\%$. In order to prevent false negative results due to low fetal fraction, in case of fetal fraction <4%, a new analysis is recommended on a second sample two weeks later.

Minisequencing

PCR amplification and minisequencing reaction primers were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/). PCR amplification was performed using the LightCycler 480 HRM Master (Roche Diagnostics, Penzberg, Germany). Minisequencing was performed using the SNaPshot™ kit (Applied Biosystems, Foster City, CA). All reactions were performed according to manufacturer's protocols. PCR reactions were performed in a total volume of 20 µL with a reaction mixture containing 5 µL of eluted DNA at a concentration of 2ng/µL, 1 µL each primer (5 µM), 2.4 µL MgCl2 (25 mM) and 10 µL 2X Master Mix. Primers and cycling conditions for FGFR3 amplification and minisequencing assay are described in table 1. After the Exo/sap enzymatic purification step, sequencing

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reactions were performed in a total volume of 10 µL with a reaction mixture containing 3 µL of purified PCR product, 1 µL each primer (2 µM) and 5 µL of SnapShot Multiplex Ready Reaction Mix. PCR products were sequenced on 3130xl genetic analyzer (Applied Biosystems), analyzed with GeneMapper software (Applied Biosystems) and compared to minisequencing results in two achondroplastic subjects with *FGFR3* c.1138G>A and c.1138G>C mutation respectively and in two healthy controls.

Results

Genotyping the *FGFR3* c.1138G>A and c.1138G>C mutations by ddPCR and minisequencing in DNA isolated from peripheral blood leucocytes

To test the ability of our ddPCR and minisequencing methods to detect of the *FGFR3* mutations in a small quantity of *FGFR3* mutant DNA, we used samples mimicking cfDNA from pregnant women. Peripheral bloods were obtained from a non-pregnant female carrier of the mutant A-allele, a non-pregnant female carrier of the mutant C-allele and two non-pregnant females blood donor without *FGFR3* mutation. Given that cfDNA is characterized by a low concentration of fetal DNA and a mix of maternal and fetal DNA, we created artificial mixtures of DNA from *FGFR3* WT lymphocytes with DNA from *FGFR3* mutant lymphocytes at very low concentrations and variable proportions of mutation. We tested our ddPCR multiplex assays with artificial mixtures of 0.5 ng/µL DNA (containing 50%, 25%, 12%, 6%, 3%, 1.5%, 0.8%, 0.4% or 0% of mutant allele). As shown in Figure 2, both ddPCR *FGFR3* mutant A and mutant C allele specific assays are able to discriminate as low as 0.8% and 0.4% of mutant DNA, respectively. Similarly, we tested our minisequencing assays with artificial mixtures of 0.5 ng/µL DNA (containing 50%, 25%, 12%, 6%, 3%, 1.5%, 0.8% or 0% of mutant allele). As shown in Figure 3, both minisequencing *FGFR3* mutant A and

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mutant C allele specific assays are able to discriminate down to 1.5 and 3% of mutant DNA, respectively. Altogether, these results show that these methods are sensitive and specific enough to detect small amounts of *FGFR3* mutation in a background of WT DNA in large excess.

Genotyping the *FGFR3* c.1138G>A mutation by ddPCR and minisequencing in cfDNA from maternal blood

We also studied a healthy pregnant woman with 22 weeks of pregnancy of a fetus with characteristic skeletal features of achondroplasia and whose husband carried the c.1138G>A mutation to determine whether ddPCR and minisequencing assays were able to discriminate the fetal *FGFR3* c.1138G>A mutation from maternal plasma. As shown in figure 4, both assays are able to detect the *FGFR3* mutant A allele in cfDNA from maternal blood, thus confirming the affected status of the fetus (Figure 4). This case is considered as our positive control (Table 2, case 22).

Non-invasive prenatal diagnosis of achondroplasia by ddPCR and minisequencing in cfDNA from maternal blood

We collected 25 additional plasmas from women carrying fetus at risk of achondroplasia, on the basis of abnormal prenatal ultrasound findings in the third trimester of pregnancy. All samples were tested with both ddPCR and minisequencing assays. As shown in table 2, ddPCR and minisequencing analysis classified correctly the healthy and the affected fetuses, as compared to the results of Sanger sequencing from fetal DNA obtained through conventional prenatal screening. In addition to the case based on family history, which is considered as our positive control, four other pregnant women carrying *FGFR3* fetuses were classified as mutant, being clearly differentiated from negative controls. Altogether, the

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sensitivity and specificity of our test are 100% respectively [95% confidence interval, 56.6 to 100%] and 100% [95% confidence interval, 84.5 to 100%].

Discussion

The study of cfDNA is becoming a crucial tool for diagnosis and management in various clinical disorders. Indeed, increased levels of cfDNA have been reported in a number of clinicopathological conditions such as cancer, stroke, trauma, myocardial infarction, autoimmune disorders and pre-eclampsia[8-15]. Additionally, cfDNA from fetal origin can also be detected in the plasma of the mother as early as from the 5th week of gestation[16] opening the field for many applications of non-invasive prenatal testing (NIPT) such as fetal gender determination[16,17] or RHD genotyping[18], chromosomal aneuploidies[19-23], and an increasing number of single gene disorders[24-30].

In this work, we developed a novel and original strategy based on droplet digital PCR combined with minisequencing, allowing the non-invasive prenatal diagnosis of achondroplasia. 26 samples were collected from women carrying fetus at risk of achondroplasia, on the basis of family history or abnormal prenatal ultrasound findings in the third trimester of pregnancy, such as rhizomelic shortening of the long bones below the first percentile associated with macrocephaly. This allowed us to diagnose to date a total of five cases of achondroplasia in maternal blood. Four of these were detected by abnormal prenatal ultrasound findings in the third trimester of pregnancy and were done in parallel to conventional prenatal diagnosis, offering a rapid and definitive diagnosis. The fifth was performed at the beginning of the second trimester of pregnancy because of familial history, the father being achondroplastic. This diagnosis did not change the management of pregnancy; however, it allowed an early diagnosis and an appropriate clinical management of

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the newborn. This diagnosis has an important psychological impact since parents can then prepare themselves before the baby is born.

Digital PCR has already been proposed as an alternative technique to other quantitative techniques for NIPT of aneuploidies[31-33] as well as monogenic disorders[26-29]. Several groups have reported on the diagnosis of achondroplastic fetuses using different analytical techniques such as MALDI-TOF, restriction analysis, QF-PCR, quantitative real time PCR and more recently NGS[30,34-39]. The early PCR-based methods appear to be suitable in diseases for which there is a single hotspot mutation. But because of the inherent properties of cffDNA which is characterized by very low absolute and relative concentrations, such methods may not be sufficiently sensitive for the delivery of accurate results in all cases. Droplet digital PCR, which is based on the partitioning of biological samples, allows to optimize the sensitivity, specificity and accuracy for the detection and analysis of nucleic acids of low abundance, such as cffDNA. When compared with the traditional technique of real time quantitative PCR, the partitioning step of ddPCR reduces the effects of competition due to the presence in plasma of a large excess of maternal DNA, and thus increases the specificity and sensitivity of the detection of cfDNA of fetal origin. Likewise, minisequencing assays have demonstrated a poorer sensitivity as compared to digital PCR, probably because of the effects of competition above mentioned. In order to avoid false negative results with samples containing a low cfDNA fraction, a second sample will be requested two weeks later in case of discordant results between ddPCR and minisequencing assays. Because of the low prevalence of this disorder and the subsequent rare recruitment of patients at our national level, and given that a single nucleotide accounts for approximately 99% of cases, the implementation of achondroplasia diagnosis in our routine clinical testing by NGS approach appears to be costly and time-consuming.Our combined approach by ddPCR and minisequencing is found to be cost-effective and judicious.

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One limit to the digital PCR approach is that a cfDNA molecule can be counted only if it contains the binding sites for both of the digital PCR primers. Owing to the random fragmentation of cfDNA, only very short cfDNA molecules, are analyzed by a given digital PCR assay. Because of this constraint, the choice of primers and probes is crucial: these must target small sequences. Hence, the amplicons used in the present study did not exceed 90 bp in length. Furthermore, a non-negligible risk of false-negative sample could be a fetal fraction being too low to be detected, even by ddPCR and minisequencing assays. In order to prevent this, we used the amplification of a differentially methylated sequence within the *RASSF1A* gene's promoter, a universal marker useful for the detection of false-negative results caused by low fetal DNA concentrations in maternal plasma[8]. In order to prevent false negative results due to low fetal fraction, a second sample 2 weeks later is recommended in case of fetal fraction <4%. Finally, as national French guidelines promote the use of two independent techniques in the context of prenatal diagnosis (www.anpgm.fr), we chose to develop minisequencing assays specific for the target mutations, and these assays appeared to be as effective as ddPCR.

In conclusion, in this work we have developed a novel, original and cost-effective strategy based on droplet digital PCR combined with minisequencing, allowing the non-invasive prenatal diagnosis of achondroplasia from pregnant woman plasma. Although this cohort is of moderate size, it is important to note that no false positive or false negative were observed (sensitivity 100% [95% confidence interval, 56.6 to 100%] and specificity 100% [95% confidence interval, 84.5 to 100%]). Promising properties of ddPCR and minisequencing, in terms of accuracy, sensitivity and specificity observed for achondroplasia detection allow us to consider extending the applications of these technologies in non-invasive prenatal diagnosis of many other monogenic diseases.

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	Oligonucleotidic Sequences of Primers and Probes		Cycling Conditions
***FGFR3* amplification before Sanger Sequencing**	**Forward Primer**	5' GCG TGC TGA GGT TCT GAG 3'	1 cycle of 95°C for 7 min/ 40 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec/ 1 cycle of 72°C for 7 min
	Reverse Primer	5' GAA TGT TTC GTG CCC CAA AG 3'	
Sanger Sequencing Assay FGFR3	**Forward Primer**	5' GCG TGC TGA GGT TCT GAG 3'	1 cycle of 96°C for 30 sec/ 30 cycles of 96°C for 20 sec, 50°C for 5 sec, and 60°C for 4 min
	Reverse Primer	5' GAA TGT TTC GTG CCC CAA AG 3'	
ddPCR Assay *FGFR3*_mutant A allele	**Forward Primer**	5' CAG TGT GTA TGC AGG C 3'	1 cycle of 95°C for 10 min/ 40 cycles of 94°C for 30 sec and 57°C for 1 min/ 1 cycle of 98°C for 10 min
	Reverse Primer	5' ACC ACC AGG ATG AAC AG 3'	
	Mutant A Probe	5' AGC TAC aGG GTG GGC (Fam) (IowaBlack) 3'	
	Wild-type G Probe	5' AGC TAC GGG GTG GGC (Hex) (IowaBlack) 3'	
ddPCR Assay *FGFR3*_mutant C allele	**Forward Primer**	5' CAG TGT GTA TGC AGG C 3'	1 cycle of 95°C for 10 min/ 40 cycles of 94°C for 30 sec and 57°C for 1 min/ 1 cycle of 98°C for 10 min
	Reverse Primer	5' ACC ACC AGG ATG AAC AG 3'	
	Mutant C Probe	5' CTA CcG GGT GGG CTT (Fam) (IowaBlack) 3'	
	Wild-type G Probe	5' CTA CGG GGT GGG CTT (Hex) (IowaBlack) 3'	
ddPCR Assay *RASSF1A* *	**Forward Primer**	5' AGC CTG AGC TCA TTG AGC TG 3'	1 cycle of 95°C for 10 min/ 40 cycles of 94°C for 30 sec and 57°C for 1 min/ 1 cycle of 98°C for 10 min
	Reverse Primer	5' ACC AGC TGC CGT GTG G 3'	
	Probe	5' (Fam) CCA ACG CGC TGC GCA T (MGB) 3'	
ddPCR Assay *ACTIN B**	**Forward Primer**	5' GCG CCG TTC CGA AAG TT 3'	1 cycle of 95°C for 10 min/ 40 cycles of 94°C for 30 sec and 57°C for 1 min/ 1 cycle of 98°C for 10 min
	Reverse Primer	5' CGG CGG ATC GGC AAA 3'	
	Probe	5' (Vic) ACC GCC GAG ACC GCG TC (MGB) 3'	
***FGFR3* amplification before Minisequencing Assay**	**Forward Primer**	5' CAG GCC TCA ACG CCC ATG TC 3'	1 cycle of 95°C for 10 min/ 55 cycles of 95°C for 10 sec, 68°C for 10 sec and 72°C for 15 sec/ 1 cycle of 72°C for 7 min
	Reverse Primer	5' GGG GAG CCC AGG CCT TTC TT 3'	
Minisequencing Assay *FGFR3*	**Forward Primer**	5' aaaaaaaaaaaaaaaaaaaaaaaaaaaaa ATG CAG GCA TCC TCA GCT AC 3'	1 cycle of 96°C for 10 sec/ 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 30 sec
	Reverse Primer	5' ttttttttttttttttttttttttttttt GAA CAG GAA GAA GCC CAC CC 3'	

Table 1. Oligonucleotidic sequences of primers and probes, and cycling conditions used in the different assays.

* described by Chan et al, 2006

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Case	Indication of *FGFR3* prenatal screening	Pregnancy Term (weeks of gestation)	Fetal FGFR3 Genotyping by ddPCR				Fetal DNA %	Fetal FGFR3 Genotyping by Minisequencing		Fetal *FGFR3* Status from non invasive plasmatic sample	Fetal *FGFR3* Status from invasive sample
			FGFR3 Mutant A allele (droplets Fam+)	*FGFR3* WT G allele (droplets Hex+)	Total droplets	Observed % Mutant A allele	*RASSF1A / B-ACTIN*	*FGFR3* mutant A allele	*FGFR3* WT G allele		
1	Abnormal ultrasound findings	24+3	0	603	25396	0%	9%	-	+	Not affected	Not affected
2	Abnormal ultrasound findings	33+6	102	1049	24024	9%	8%	+	+	Affected	Affected
3	Abnormal ultrasound findings	33	0	1412	24072	0%	11%	-	+	Not affected	Not affected
4	Abnormal ultrasound findings	34	41	1144	26696	3%	4%	+	+	Affected	Affected
5	Abnormal ultrasound findings	30	0	983	26227	0%	10%	-	+	Not affected	Not affected
6	Abnormal ultrasound findings	18	1	1006	26037	0%	8%	-	+	Not affected	Not affected
7	Abnormal ultrasound findings	23	0	763	29374	0%	9%	-	+	Not affected	Not affected
8	Abnormal ultrasound findings	29	0	1090	29816	0%	12%	-	+	Not affected	Not affected
9	Abnormal ultrasound findings	30	78	1034	26201	7%	7%	+	+	Affected	Affected
10	Abnormal ultrasound findings	30	0	659	24762	0%	11%	-	+	Not affected	Not affected
11	Abnormal ultrasound findings	33	0	1038	25599	0%	11%	-	+	Not affected	Not affected
12	Abnormal ultrasound findings	26	0	557	25088	0%	6%	-	+	Not affected	Not affected
13	Abnormal ultrasound findings	34+3	0	1320	25808	0%	12%	-	+	Not affected	Not affected
14	Abnormal ultrasound findings	34+2	2	1644	25872	0%	11%	-	+	Not affected	Not affected
15	Abnormal ultrasound findings	32+4	0	1024	26193	0%	9%	-	+	Not affected	Not affected
16	Abnormal ultrasound findings	33+5	0	1437	25180	0%	15%	-	+	Not affected	Not affected
17	Abnormal ultrasound findings	32	0	902	26754	0%	10%	-	+	Not affected	Not affected
18	Abnormal ultrasound findings	32+6	0	694	28354	0%	8%	-	+	Not affected	Not affected
19	Abnormal ultrasound findings	30	0	533	22842	0%	8%	-	+	Not affected	Not affected
20	Abnormal ultrasound findings	27	0	518	28889	0%	10%	-	+	Not affected	Not affected
21	Abnormal ultrasound findings	32+3	0	1069	28748	0%	8%	-	+	Not affected	Not affected
22	Family history (Positive Control)	22	366	5107	27598	7%	6%	+	+	Affected	Affected
23	Abnormal ultrasound findings	34+4	65	737	28325	8%	6%	+	+	Affected	Affected
24	Abnormal ultrasound findings	12+4	0	839	24872	0%	6%	-	+	Not affected	Not affected
25	Abnormal ultrasound findings	32	0	1018	26708	0%	12%	-	+	Not affected	Not affected
26	Abnormal ultrasound findings	30+2	0	2002	25862	0%	9%	-	+	Not affected	Not affected

Table 2. FGFR3 fetal genotype classification. Indication for prenatal screening, pregnancy term, results of noninvasive testing from plasmatic samples, ddPCR and minisequencing assays, and results testing from invasive sample are presented. WT = Wild-type.

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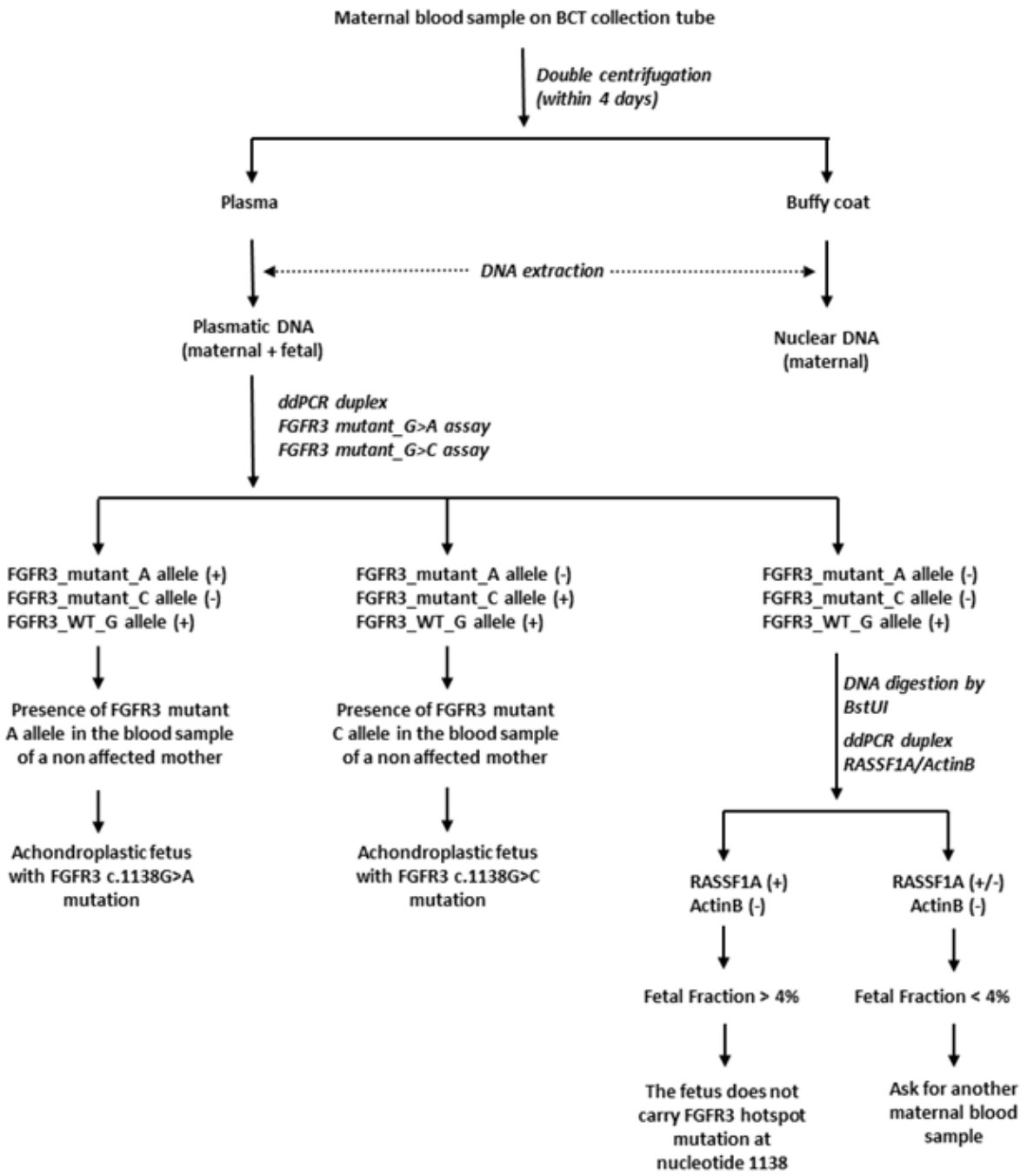

Figure 1. Decision algorithm for ddPCR assay.

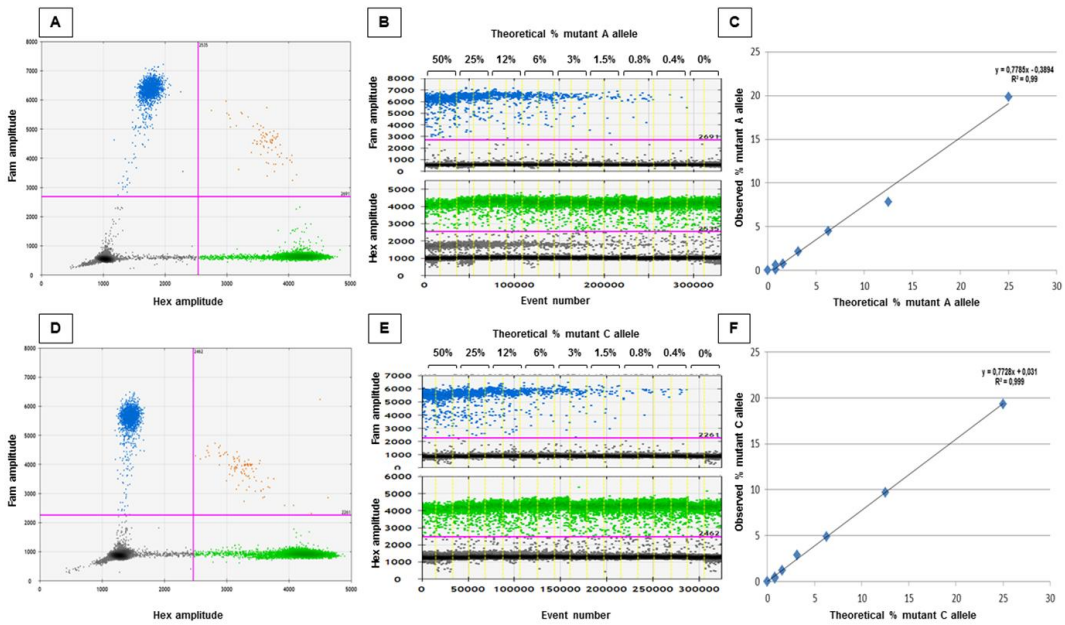

Figure 2. Validation of the ddPCR assays – serial dilutions for each set of probes with artificial mixtures of 0.5 ng/µL DNA from peripheral blood leucocytes with a mutant content of 50%, 25%, 12%, 6%, 3%, 1.5%, 0.8%, 0.4% or 0%. **A and D**. Two-dimensional representation of the fluorescence intensity obtained by ddPCR using *FGFR3* mutant A **(A)** and *FGFR3* mutant C **(D)** ddPCR assays, respectively. The y and x axes correspond to the FAM and HEX intensities, for mutant and WT alleles respectively. Negative droplets (grey dots) and positive ones (blue dots for FAM+ only, green dots for HEX+ only and brown for FAM+ and HEX+) are assigned as a function of the FAM and HEX florescence amplitudes. **B and E**. One dimensional representation of the fluorescence intensity obtained by ddPCR using *FGFR3* mutant A **(B)** and *FGFR3* mutant C **(E)** ddPCR assays, respectively. Each column corresponds to one point of the serial dilution mixture of 0.5 ng/µL DNA with a mutant content of 50%, 25%, 12%, 6%, 3%, 1.5%, 0.8%, 0.4% or 0%. **C and F**. Correlation between observed vs. theoretical ratios using *FGFR3* mutant A **(C)** and *FGFR3* mutant C **(F)** ddPCR assays, respectively. Each point corresponds to one point of the serial dilution mixture of 0.5 ng/µL DNA with a mutant content of 50%, 25%, 12%, 6%, 3%, 1.5%, 0.8%, 0.4% or 0%.

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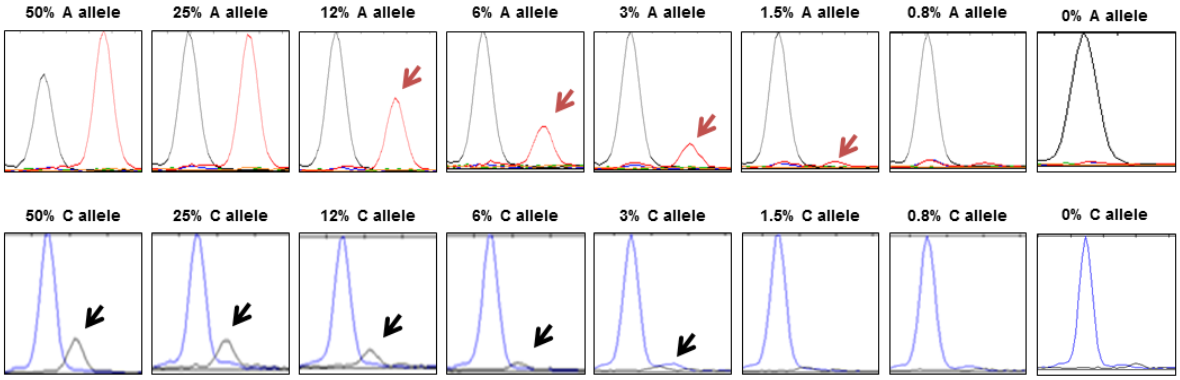

Figure 3. Validation of the minisequencing assays – serial dilutions for each set of probes with artificial mixtures of 0.5 ng/µL DNA from peripheral blood leucocytes with mutant-A allele (up) or mutant-C allele (down) content of 50%, 25%, 12%, 6%, 3%, 1.5%, 0.8% or 0%.

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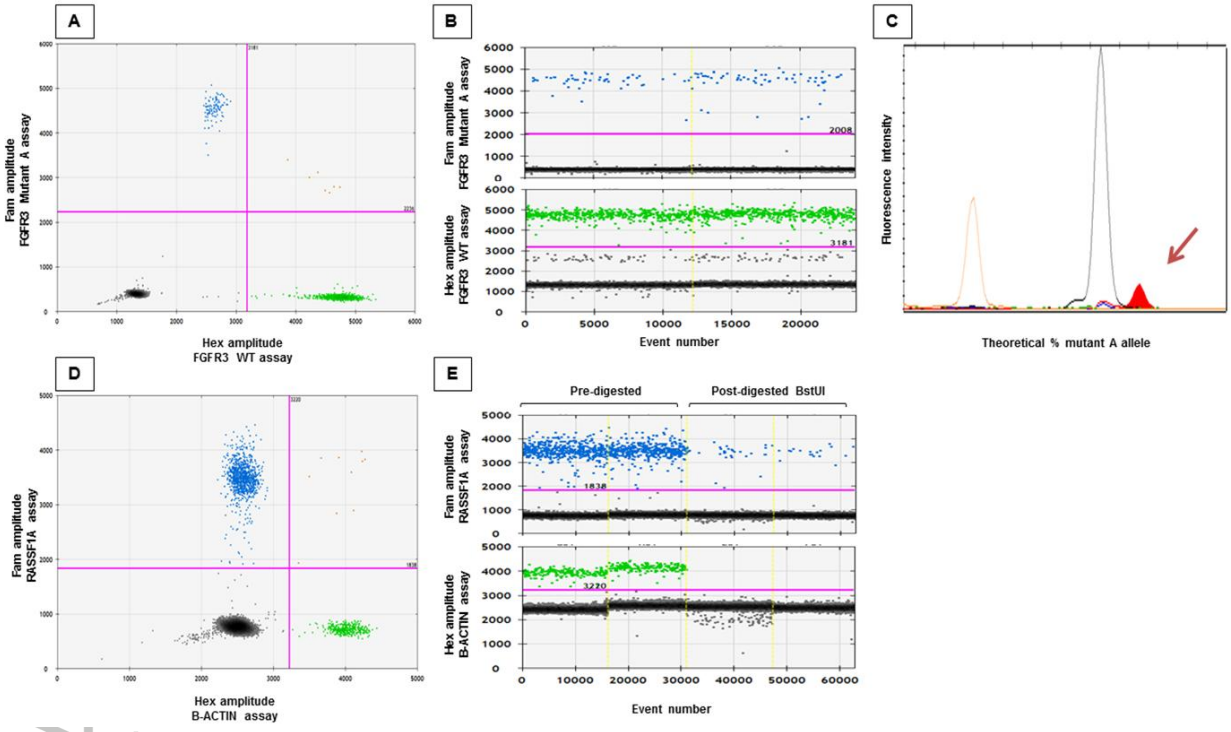

Figure 4. Genotyping the *FGFR3* c.1138G>A mutation by ddPCR and minisequencing assays in plasmatic DNA from maternal blood at 22 weeks of pregnancy. **A and D**. Two-dimensional representation of the fluorescence intensity obtained by ddPCR using *FGFR3* mutant A-allele ddPCR assay **(A)** and RASSF1A/ B-ACTIN assay **(D)**, respectively. **B and E**. One dimensional representation of the fluorescence intensity obtained by ddPCR using *FGFR3* mutant A-allele ddPCR assay (**B**) and RASSF1A/ B-ACTIN assay before and after BstUI digestion **(E)**, respectively. **C**. Minisequencing electrophoregram. The brown pic corresponds to the size marker, the black pic corresponds to the WT G-allele, and the red pic corresponds to the mutant A-allele.

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