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TECHNICAL ADVANCE

Validation of Extensive Next-Generation Sequencing Method for Monogenic Disorder Analysis on Cell-Free Fetal DNA

Noninvasive Prenatal Diagnosis

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During pregnancy, a percentage of the cell-free DNA circulating in the maternal blood is represented by the cell-free fetal DNA (cffDNA), constituting an accessible source for noninvasive prenatal genetic screening. The coexistence of the maternal DNA, the dominant fraction of cell-free DNA, together with the cffDNA component and the scarcity of the cffDNA itself make applying traditional methods of genetics and molecular biology impossible. Next-generation sequencing methods are widely used to study fetal aneuploidies. However, in monogenic disorders, there have been relatively few studies that analyzed single mutations. We present a method for the analysis of an extended group of gene variants associated with recessive and dominant autosomal disorders using next-generation sequencing. The proposed test should allow a complete analysis of common genetic disorders and pathogen-associated variants for diagnostic use. The analysis of cffDNA for single gene disorders may replace invasive prenatal diagnosis methods, associated with the risk of spontaneous abortion and psychological stress for patients. The proposed test should assess reproductive risk for both genetic family disorders and de novo occurrences of the disease. The application of this method to a case of beta-thalassemia is also discussed. *(J Mol Diagn 2019, 21: 572—579; https://doi.org/10.1016/j.jmoldx.2019.02.010)*

The discovery of the presence of cell-free fetal DNA (cffDNA) in blood circulation was of immense significance in the context of prenatal diagnosis.[1] However, the impossibility to separate maternal DNA from cffDNA, the low amount of cffDNA, and the fragmentation of cffDNA (which, on average, is 143 bp) have made the manipulation of the sample, its analysis, and the processing of the results particularly challenging. Applications of massively parallel sequencing methods to cffDNA analysis have allowed the ever-increasing use of noninvasive prenatal tests (NIPTs). Screening primarily detects the main and most widespread chromosomal aneuploidies (chromosomes 21, 18, and 13). Recently, the validation of the sequencing methods and bioinformatics pipelines have allowed the analysis to be extended to the detection of aneuploidy associated with sex chromosomes, whole karyotypes, and subchromosomal abnormalities. The study of chromosomal deletions or duplications allows identification of syndromes associated with copy number variation, introducing a much more detailed level of study compared with the aneuploidy survey alone.[2] However, NIPT remains a nondiagnostic test, in which the highest reliability of results is associated with Down syndrome detection (sensitivity often >99%, specificity nearly as high) and will always be affected by its biological limit, the placental origin of cffDNA.

Current research focuses on verifying the feasibility of cffDNA analysis for the detection of monogenic disorders.

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The aim is to obtain a test capable of identifying the presence of genetic mutations associated with autosomal dominant paternal or de novo transmission disorders and autosomal recessives. In the latter case, verifying the presence of mutations of paternal origins is important for the diagnostic management of the couple in whom both are carriers of a recessive disorder; this allows earlier assessment of the real reproductive risk compared with conventional prenatal diagnosis methods and pregnancy management.

So far, the methods used include droplet-digital PCR, PCR-RED, high-resolution melting, whole-genome genotyping using single-nucleotide polymorphism, real-time quantitative PCR, targeted and dosage haplotyping, and next-generation sequencing. These methods obtain a good degree of sensitivity and specificity but limit the analysis to a single variant or a limited number of variants for a single gene and a single genetic disorder, without using an analytical approach for a large number of genes and mutations associated with recessive and dominant disorders.[3–11]

Extending the number of genes and variants that can be analyzed would make it possible to offer the test for wider screening of pathologies. The future goal should be to have a test that is useful in cases in which it is necessary to verify the presence of a dominant disorder both with paternal and de novo transmission and that is able to analyze the main pathogenetic variants described in the literature in the case of recessive disorders. Given the gestational age at which the test is performed, around the 10th week, such an in-depth analysis might be possible before the clinical signs that can be obtained through ultrasound.

Herein, we describe a next-generation sequencing method for the analysis of a large group of genes and pathogenetic mutations associated with recessive and dominant disorders. Disorders present in this panel include cystic fibrosis, beta-thalassemia, congenital autosomal recessive deafness, adrenal hyperplasia, hemochromatosis, achondroplasia, hypochondroplasia, thanatophoric dysplasia, Apert syndrome, Crouzon syndrome, Pfeiffer syndrome, Noonan syndrome, Leopard syndrome, phenylketonuria, Rett syndrome, and polycystic kidney recessive disease.

The method's application to a diagnostic case for beta-thalassemia, in which both parents were healthy carriers, has also been described. Beta-thalassemia is one of the most widespread autosomal recessive genetic disorders. At present, only genetic analysis is possible using invasive procedures (amniocentesis and villocentesis) in pregnancies with reproductive risk associated with beta-thalassemia; however, these procedures are associated with a risk of abortion, ranging from 0.1% to 0.2%, and with psychological stress for the couple. So far, studies that have focused on the analysis of beta-thalassemia using cffDNA have used methods able to consider only one or a few genetic variants of many widespread pathogenetic ones.[12–16] This method includes all of the genetic variants considered pathogenetic and associated with beta-thalassemia, as is the case for the other diseases represented in the panel.

Materials and Methods

Sample Collection

Approximately 10 mL of maternal peripheral blood samples was collected into Streck tubes (Streck, La Vista, NE) at the 10th week of gestation.

DNA Extraction

Cell-free DNA was extracted from 10 mL of maternal plasma using the QIAamp Circulating Nucleic Acid Kit, according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). After DNA isolation, DNA was quantified by the Agilent Technologies 4200 TapStation System (Agilent Technologies, Santa Clara, CA), using the High Sensitivity D1000 ScreenTape System (Agilent Technologies). The minimum DNA concentration to subject the sample to analysis was found to be between 80 and 120 pg/μL.

Design of Next-Generation Sequencing Gene Panel

A panel of genes, detailed in Table 1, was designed and synthesized by using the custom IonAmpliseq Designer system version 6.0 from Thermo Fisher Scientific (Waltham, MA), and in particular using the hot spot system with an amplicon size of between 125 and 175 bp. The result of the design was a panel consisting of 98 amplicons with a size of approximately 12 Kb in total. The panel includes *CFTR* (cystic fibrosis), *HBB* (thalassemia, beta), *GJB2* (deafness, autosomal recessive), *CYP21A2* (adrenal hyperplasia), *HFE* (hemochromatosis), *FGFR3* (achondroplasia, hypochondroplasia, and thanatophoric dysplasia), *FGFR2* (Apert, Crouzon, and Pfeiffer syndromes), *PTPN11/RAF1/SOS1* (Noonan and Leopard syndromes), *PAH* (phenylketonuria), *MECP2* (Rett syndrome), and *PKHD1* (polycystic kidney disease). The total number of variants analyzed was 337, including all those classified as pathogenic for each gene in the Online Mendelian Inheritance in Man database.

The selection of genetic disorders was based on the incidence in the population and on the role that the diagnosis has at the prenatal age in combination with ultrasound. For the choice of the mutations inserted, the Online Mendelian Inheritance in Man, Human Genetic Mutation Database (HGMD) professional version 2018.3, and ClinVar National Center for Biotechnology Information databases were referred to.

Next-Generation Sequencing

Gene Panel Validation

For the initial validation of the panel, DNA chimeras were used, prepared by mixing a DNA wild type with decreasing percentages of mutated DNA. This simulated the conditions present in the analysis of cffDNA in which, on average, the

Table 1 Genetic Variants Used for Panel Design

***CFTR* (cystic fibrosis)**					
rs113993960	*rs11971167*	*rs121909015*	*rs77010898*	*rs78802634*	*rs121908769*
rs121908745	*rs1800098*	*rs121909016*	*rs213950*	*rs75789129*	*rs121909045*
rs77101217	*rs75039782*	*rs121909017*	*rs1800091*	*rs121909031*	*rs113857788*
rs113993958	*rs77902683*	*rs121909018*	*rs74571530*	*rs121908753*	*rs121909046*
rs78655421	*rs121908748*	*rs121909019*	*rs267606722*	*rs76554633*	*rs121909013*
rs77932196	*rs75053309*	*rs121909020*	*rs121909008*	*rs121909033*	*rs75961395*
rs74551128	*rs121909021*	*rs143570767*	*rs121909009*	*rs75115087*	*rs79850223*
rs76713772	*rs121909023*	*rs78194216*	*rs121909010*	*rs79633941*	*rs121908804*
rs113993959	*rs141158996*	*rs121908744*	*rs80034486*	*rs121909034*	*rs121908777*
rs121908755	*rs121908766*	*rs267606723*	*rs74767530*	*rs121908773*	*rs121908788*
rs121909005	*rs121909025*	*rs121909047*	*rs121909011*	*rs121909035*	*rs77646904*
rs75527207	*rs121909026*	*rs36210737*	*rs121908776*	*rs121909036*	*rs121908754*
rs74597325	*rs121908750*	*rs121909042*	*rs121909012*	*rs121909037*	*rs121908764*
rs75549581	*rs121909028*	*rs121908775*	*rs121909040*	*rs79635528*	*rs1800123*
rs80055610	*rs121908787*	*rs121909043*	*rs74503330*	*rs121908761*	*rs75389940*
rs121909006	*rs121908751*	*rs75096551*	*rs76649725*	*rs77409459*	*rs121908784*
rs121908758	*rs79282516*	*rs121909044*	*rs121908811*	*rs121908752*	*rs1800111*
rs121909041					
***CYP21A2* (21-hydroxilase deficiency)**					
rs6475	*rs6445*	*rs9378252*	*rs7769409*	*rs267606756*	*rs6467*
rs6471	*rs151344503*	*rs267606757*	*rs9378251*		
***FGFR2* (Apert syndrome)**					
rs77543610	*rs79184941*	*rs121918498*			
***FGFR2* (Crouzon syndrome)**					
rs121918489	*rs121918501*	*rs121918497*	*rs121918493*	*rs121918488*	*rs121918496*
rs121918490	*rs121918487*	*rs121918507*	*rs121918494*	*rs121918491*	*rs121918500*
***FGFR2* (Pfeiffer syndrome)**					
rs121918495	*rs121918510*	*rs121918505*	*rs121918499*	*rs281865420*	*rs121918502*
rs121918503	*rs121918506*				
***FGFR3* (achondroplasia)**					
rs75790268	*rs267606809*	*rs28931614*	*rs121913114*		
***FGFR3* (hypochondroplasia)**					
rs28933068	*rs121913115*	*rs78311289*	*rs77722678*	*rs121913116*	*rs28928868*
rs80053154					
***FGFR3* (thanatophoric dysplasia)**					
rs121913484	*rs267606808*	*rs121913483*	*rs121913101*	*rs121913482*	*rs121913479*
rs121913103	*rs121913485*				
***GJB2* (deafness)**					
rs35887622	*rs80338939*	*rs80338945*			
***HBB* (beta-thalassemia)**					
rs34502690	*rs35497102*	*rs33943001*	*rs33944208*	*rs63750099*	*rs33986703*
rs33915217	*rs35662066*	*rs34451549*	*rs33941377*	*rs34533941*	*rs11549407*
rs35724775	*rs80356820*	*rs33913712*	*rs33994806*	*rs36107977*	*rs63750783*
rs33913413	*rs63749819*	*rs63750475*	*rs33981098*	*rs63751201*	*rs33974936*
rs34527846	*rs33969853*	*rs33952266*	*rs33980857*	*rs63750532*	*rs33922842*
rs35004220	*rs35225141*	*rs33930702*	*rs34598529*	*rs35532010*	*rs33995148*
rs35328027	*rs281864901*	*rs63751218*	*rs33931746*	*rs33941849*	*rs33982568*
rs34690599	*rs34889882*	*rs35477349*	*rs63751128*	*rs33971440*	*rs193922563*
rs33951465	*rs34856846*	*rs41443947*	*rs34500389*	*rs33945777*	*rs33914668*
rs63751208	*rs267607297*	*rs35894115*	*rs35949130*	*rs33956879*	*rs33931779*
rs34883338	*rs35383398*	*rs267607298*	*rs34999973*		
***HFE* (hereditary hemochromatosis)**					
rs1799945	*rs28934595*	*rs28934597*	*rs1800758*	*rs28934596*	*rs111033557*
rs1800730	*rs111033558*	*rs111033563*	*rs28934889*		

(*table continues*)

Table 1 (*continued*)

***MECP2* (Rett syndrome)**					
rs28934905	*rs61749721*	*rs179363901*	*rs28934907*	*rs28935168*	*rs28934906*
rs61750240	*rs104894864*	*rs61749743*	*rs267608434*	*rs61748421*	*rs61751362*
rs28935468					
***PAH* (phenylketonuria)**					
rs5030861	*rs5030850*	*rs118203925*	*rs5030851*	*rs62516097*	*rs62642930*
rs5030858	*rs199475566*	*rs62516151*	*rs62514927*	*rs118203923*	*rs118203921*
rs62642936	*rs5030859*	*rs74486803*	*rs62508588*	*rs62514893*	*rs78655458*
rs62508698	*rs62642933*	*rs76212747*	*rs62514959*	*rs62507344*	*rs62508727*
rs76296470	*rs62514955*	*rs62508689*	*rs79931499*	*rs62516060*	*rs62642926*
rs5030849	*rs62508646*	*rs62642941*	*rs5030860*	*rs5030856*	*rs5030855*
rs5030847	*rs62514958*	*rs62644473*	*rs62516095*	*rs5030854*	*rs5030841*
rs62514891	*rs62644499*	*rs75193786*	*rs62514952*	*rs62517167*	*rs62514934*
rs5030843	*rs62642934*	*rs62507347*	*rs62516096*	*rs62642937*	*rs74603784*
rs5030846	*rs62516101*	*rs5030852*	*rs62514953*		
***PKHD1* (polycystic kidney disease)**					
rs28937907	*rs137852948*	*rs137852947*	*rs137852945*	*rs137852949*	*rs137852950*
rs137852946					
***PTPN11* (Leopard syndrome)**					
rs121918456	*rs121918469*	*rs121918468*	*rs121918457*	*rs121918470*	
***PTPN11* (Noonan syndrome)**					
rs121918466	*rs121918458*	*rs121918461*	*rs267606990*	*rs121918460*	*rs121918463*
rs121918467	*rs121918459*	*rs121918462*	*rs121918453*	*rs121918455*	*rs28933386*
rs121918454					
***RAF1* (Noonan syndrome)**					
rs80338796	*rs80338799*	*rs80338797*	*rs121434594*		
***SOS1* (Noonan syndrome)**					
rs137852812	*rs267607079*	*rs137852814*	*rs137852813*	*rs267607080*	

dbSNP, gene name, and associated genetic disorder are reported for each variant.

fetal fraction in the maternal DNA is included in a range of between 2% and 20%. These chimeras were prepared for c.1521_1523 del CTT mutations in the *CFTR* (cystic fibrosis) gene (p.Phe508del; NM_000492.3), c.118C>T p.Gln40Ter in the *HBB* (beta-thalassemia) gene (p.Gln40Ter; NM_000518.4), c.1138 G>A in the *FGFR3* (achondroplasia) gene (p.Gly380Arg; NM_000142.4), c.417 G>C in the *PTPN11* (Noonan syndrome) gene (p.Glu139Asp; NM_002834.4), c.35delG (NM_004004.5) in the *GJB2* (congenital deafness) gene, and c.755C>G in the *FGFR2* (Apert syndrome) gene (p.Ser252Trp; NM_000141.4). Using these chimeras showed detection of low frequency variants, up to 1%.

Furthermore, the average base coverage depth of the panel for each mutation of interest was monitored using the Integrative Genomics Viewer software version 2.3.20 (Broad Institute, Cambridge, UK; *http://software.broadinstitute.org/software/igv*, last accessed October 9, 2018), resulting in a value of approximately 10,000.

At the end of this first validation phase, 125 cffDNA samples, obtained from patients who underwent villocentesis, were processed. In these samples the absence of mutations was confirmed in 95 samples, the presence of a mutation associated with cystic fibrosis of paternal origin was confirmed in 8 samples, the presence of a mutation associated with beta-thalassemia of maternal origin was confirmed in 9 samples, the presence of a mutation associated with beta-thalassemia of paternal origin was confirmed in 3 samples, and the presence of a mutation associated with hereditary hemochromatosis was confirmed in 10 samples. Results matched those of chorionic villus sampling.

Library Preparation and Sequencing Conditions

The Thermo Fisher Scientific Ion Ampliseq kit was used to prepare libraries from extracted samples, integrated with our custom panel for the analysis of monogenic disorders.

For the optimization of the amplification of the targets, the following PCR conditions were used: 4 μL of 5× IonAmpliseq HiFi mix, 4 μL of 5× Custom IonAmpliseq Primer Pool (Thermo Fisher Scientific) (containing our regions of interest), and 12 μL of cffDNA. The following PCR conditions were used: enzyme activation for 2 minutes at 99°C, denaturation for 15 seconds at 99°C, and annealing and extension for 4 minutes at 60°C for 24 cycles. After the amplification, to the same reaction, 2 μL of FuPa reagent (Thermo Fisher Scientific) was added and the incubations were completed in a thermal cycler: 50°C for 10 minutes, 55°C for 10 minutes, and 60°C for 20 minutes. After incubation, to ligate adapters to amplicons, 4 μL of Switch Solution, 2 μL of Ion Xpress Barcode adapter mix, and 2 μL of DNA ligase (all from Thermo Fisher Scientific) were

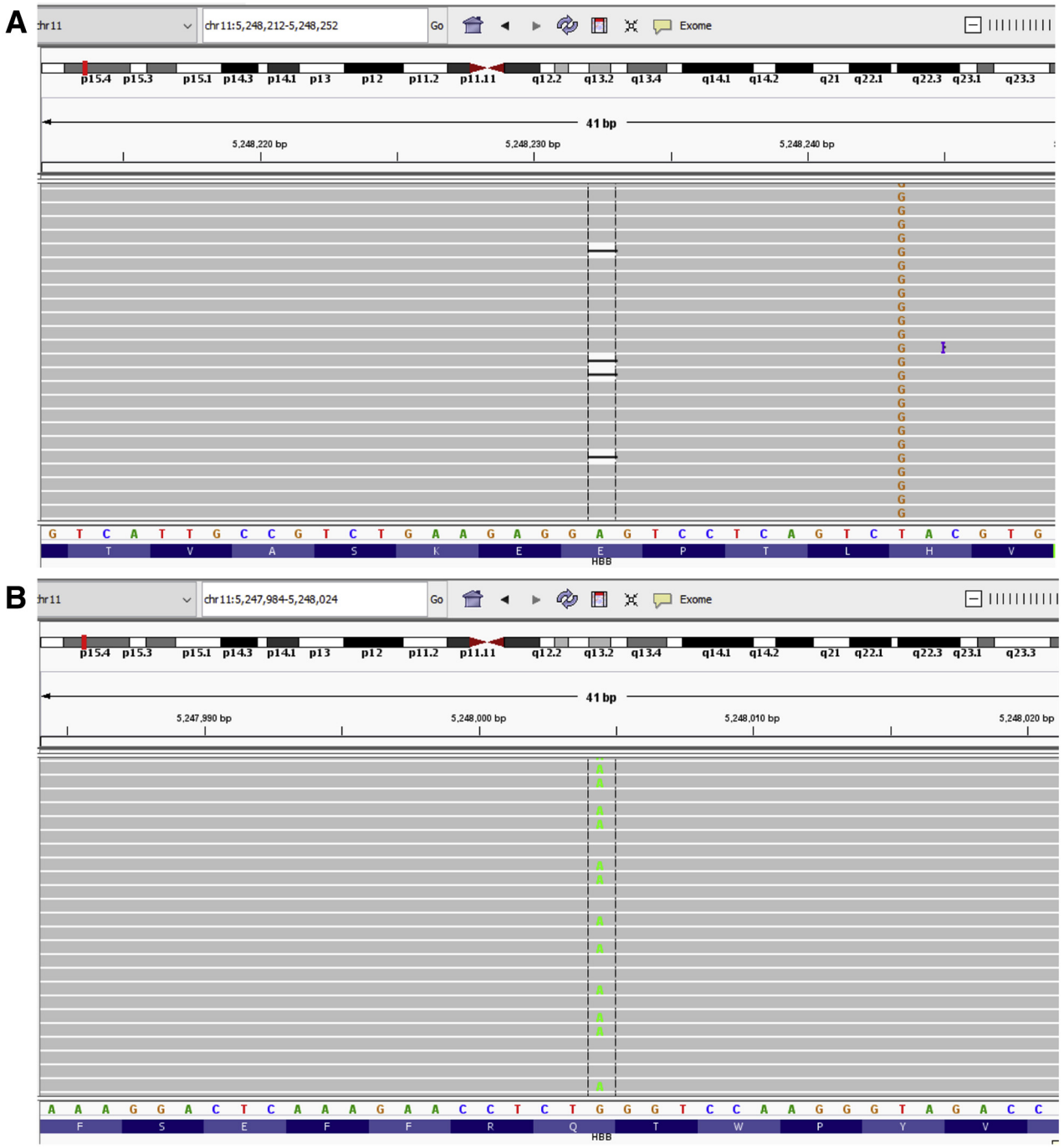

Figure 1 Coverage analysis by Integrative Genomics Viewer software version 2.5x. **A:** c.20 del A mutation (p.Glu7fs; NM_000518.4) in the *HBB* gene. Wild-type allele coverage, 9769; mutant allele coverage, 186; mutated allele detected at 1.86% frequency. **B:** c.118C>T variant (p.Gln40Ter; NM_000518.4) in the *HBB* gene. Wild-type allele coverage, 5811; mutant allele coverage, 4090; mutated allele detected at 41.3% frequency. Chr, chromosome.

added using the following conditions: 22°C for 1 hour, 68°C for 5 minutes, and 72°C for 5 minutes.

The whole volume of library (30 μL) was purified using 1.5× (45 μL) of Agencourt Ampure XP reagent (Beckman Coulter, Brea, CA) and then amplified using 50 μL Platinum PCR SuperMix HiFi (Thermo Fisher Scientific) and 2 μL of Library Amplification Primer mix (Thermo Fisher Scientific), using the following conditions: 98°C for 2 minutes, amplification at 98°C for 15 seconds, and 64°C for 1 minute for five cycles.

At the end, size selection was performed by Agencourt Ampure XP reagent (Beckman Coulter), using, for the first step, 0.5× sample volume (25 μL) and, for the second, 1.2× sample volume (60 μL). After size selection, the library was eluted in 50 μL of Low TE buffer and quantification was performed by Qubit Fluorometer (Thermo Fisher Scientific).

For sequencing, the 520 Chip Kit (Thermo Fisher Scientific) was used, generating 3 to 5 million reads for chip, on the S5 sequencing platform (Thermo Fisher Scientific). In these sequencing conditions, multiplexing eight samples per run, a mean depth of approximately 10,000 was obtained (Supplemental Figure S1).

Data Analysis

The objective of a sequencing with such a high depth is to obtain a high sensitivity in the detection of variants, similar to that required for the search for somatic variants. Variant calling was performed using Thermo Fisher Scientific's Ion Reporter system version 5.10. To increase selectivity and eliminate the risk of incidental findings, a hot spot filter was introduced into the analysis (Supplemental Table S1) to identify only the variants of interest and not all those present in the amplicons. This allows analysis of only the high-coverage regions, and not those variants present in regions with low coverage in the amplicon that could represent false positives.

Results

In the case described, the maternal blood sample was obtained from a 10-week pregnancy in which the mother was the carrier for the c.118C>T mutation (p.Gln40Ter; NM_000518.4) and the father was the carrier of the c.20 del A mutation (p.Glu7fs; NM_000518.4) in the *HBB* gene. Only cffDNA was analyzed and not paternal and maternal genomic DNA; the goal of the test was to identify variants without trio analysis and validate this approach for de novo variant assessment.

After cffDNA extraction, the sample underwent NIPT for the exclusion of chromosomal aneuploidies; the sample was found to be male, devoid of chromosomal aneuploidy, with a fetal fraction of 12%. For fetal fraction determination, two different methods were used; the first was based on a regression model that considers the number of reads aligned on chromosome Y, and the second was based on a regression model based on a 50-Kb bin read count.

The cffDNA was then analyzed for the presence of monogenic disorders. It was compound heterozygous for both mutations. In particular for the c.118C>T mutation, the coverage of the wild-type allele was found to be 5811, whereas that of the mutated allele was 4090; for the c.20 del A mutation, the coverage of the wild-type allele was found to be 9769, whereas that of the mutated allele was 186; sequence coverage by Integrative Genomics Viewer is shown in Figure 1.

The result obtained was confirmed by the analysis of chorionic villi, a sample of which was taken at 12 weeks of pregnancy.

The system was, therefore, sufficiently sensitive to detect the mutation of paternal origin, detecting the mutated allele at 1.86% frequency. The high frequency of the mutated allele of maternal origin equal to 41.3% is due to the presence in the cffDNA sample of both maternal and fetal components, in which the maternal component is clearly predominant.

Discussion

Currently, great efforts are underway so that the NIPT is not only able to determine the presence of trisomy 21 but also to achieve the same degree of reliability for chromosome 18 and 13 trisomies, for sexual aneuploidy, and for whole karyotypes; many tests are already offered to the patients for the detection of subchromosomal abnormalities. The goal is the development of reliable screening tests able to support invasive prenatal diagnosis, albeit without an absolute diagnostic value for biological limits deriving from the placental origin of cffDNA.

Just as NIPT is replacing conventional invasive diagnostic tests (amniocentesis and villocentesis), it is necessary to introduce methods also capable of investigating the presence of monogenic disorders in the fetus. Such investigations are relevant in the case of couples with familial reproductive risk, where both are carriers of a genetic disorder (autosomal recessive disorders) or one of the two is affected (autosomal dominant disorder), or in the case of genetic disorders with de novo onset.

Noninvasive prenatal diagnosis, performed at early gestation stage (10th week), could precede the appearance of pathologic ultrasound signs and, in general, would avoid the risk of abortion associated with invasive prenatal diagnosis. Analysis for monogenic disorders could be indicated in the case of ultrasound anomalies, like shortened long bones or an increase in nuchal translucency, for the exclusion of the presence of de novo variants even in cases in which ultrasound findings are not a reliable indicator, in the case of pregnancy complications, in the case of premature rupture of membranes that preclude prenatal invasive diagnosis, and in the case of familial pathogenic conditions for early assessment of reproductive risk or proper management of pregnancy.

Until now, the diagnostic techniques applied to noninvasive prenatal diagnosis described in the literature attempt to analyze the effect of monogenic disorders on cffDNA, and have been based on diagnostic methods that allow the detection of only one or a small number of mutations. However, this approach does not allow the analysis of a broad spectrum of genes and mutations associated with the most common disorders in the population.

Herein, we introduce the application of next-generation sequencing methods for the diagnosis of single-gene disorders on cffDNA. This approach allows us to perform broad genetic screening, including some of the most widespread and severe syndromes. The evaluation of such a large number of variants allows the analysis of both recessive and dominant disorders and the real evaluation of the reproductive risk of the couple.

The application of the method to a case of beta-thalassemia, in which both parents were carriers, has been

described. The test was able to identify the presence of maternal and paternal mutations, the latter with a frequency of the allele mutated close to 1%, thus demonstrating remarkable sensitivity and specificity.

At present, this method allows the identification of de novo onset disorders (eg, Noonan syndrome; incidence, 1:1000 to 1:2500), autosomal dominant disorders of paternal origin, and paternal mutations associated with recessive disorders. In the case of autosomal recessive genetic disorders in couples in whom both are carriers of the disease, just the analysis of the presence of the mutation of paternal origin makes it possible to establish the extent of the real reproductive risk for the couple and immediately direct it to investigations or toward a safe pregnancy. For example, in the clinical case of beta-thalassemia described, to which the method of analysis was applied, even the detection of the mutation of paternal origin would have given the couple a real reproductive risk of 50% and not the theoretical risk of 25% for recessive disorders; in case of exclusion of mutation of paternal origin, the risk of a pathologic pregnancy would have been eliminated.

In the case of maternal origin variants in autosomal recessive disorders, the frequency of the mutated allele is approximately 40% to 50%, because the maternal component is clearly predominant compared with the amount of cffDNA, whereas the contribution of the fetal alleles is between 4% and 10%, directly proportional to the percentage of cffDNA present in maternal blood. Therefore, the detection of the maternal origin mutation could have resulted in a heterozygous or wild-type fetus.

The evaluation of the frequency rate of the mutated allele will make it possible to distinguish the presence/absence of the mutation in the fetus, both for the mutation of maternal origin and in cases in which the paternal and maternal mutation are equal. To achieve this goal, our center is engaged in the execution of an extended number of samples derived from parents carrying recessive diseases and in the elaboration of suitable statistical methods that will allow tracing the distribution of the values associated with various genetic fetal conditions. All samples will derive from patients subjected to villocentesis. For a statistical evaluation of the frequency rate of fetal maternal-derived mutated allele, only cffDNA samples included in a fetal fraction range >4% will be subjected to sequencing. The limit read depth will be maintained at 10,000, starting from concentration of cffDNA after isolation of at least 80 pg/μL. The bioinformatics pipeline will be perfected by relating the minor allele frequency value and the percentage of fetal fraction in the cffDNA sample. The deadline for this study is scheduled for the second half of 2019.

The described method demonstrates sufficient specificity and selectivity for the detection of pathogenetic variants associated with recessive and dominant disorders, ensuring diagnosis in cases of paternal and de novo onset. This method is the first to be developed for the analysis of a large number of genetic pathologies using cffDNA. It can precede and replace common invasive prenatal diagnosis methods. In the case of maternal origin variants, for the characterization of the fetal genetic condition, the bioinformatics algorithm is being developed to distinguish the maternal and fetal component more accurately.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2019.02.010*.

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