Six consecutive false positive cases from cell-free fetal DNA testing in a single referring centre

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Abstract

Introduction: **recent studies have proposed the introduction of cell-free fetal DNA testing (NIPT - Non Invasive Prenatal Testing) in routine clinical practice emphasizing its high sensibility and specificity. In any case, false positive and false negative findings may result from placental mosaicism, because cell-free fetal DNA originates mainly from placenta.**
Case: **we report six cases of women who underwent chorionic villus sampling (CVS) or amniocentesis to confirm the results from NIPT: two Turner syndromes, two Triple X, one Patau syndrome, one Edward syndrome.**
Results: **using classic cytogenetic analysis and, also, Array - Comparative Genomic Hybridization (Array CGH) the karyotype of all 5 fetuses was found to be normal.**
Conclusion: **results from NIPT must always be confirmed by invasive prenatal diagnosis. It is mandatory to inform the patient that the CVS and amniocentesis still represent the only form of prenatal diagnostic test available.**

Key words: cell-free fetal DNA, NIPT, chorionic villus sampling, amniocentesis, aneuploidy.

Introduction

Trisomy 21 (Down's syndrome) occurs in 1 out of every 800 live births, trisomy 13 (Patau syndrome) in about 1 out of every 10,000 newborns and the incidence of trisomy 18 (Edwards syndrome) is estimated to be 1 in 6,000 live births (1).
Diagnosis of such fetal chromosomal aberrations is an important point in prenatal diagnosis. Conventional invasive prenatal diagnostic methods, such amniocentesis or chorionic villus sampling (CVS), present a risk of miscarriage respectively of about 0.03% (2) and 0.8% (3).
In the past 20 years, many screening tests for fetal aneuploidies have been introduced in routine prenatal care to improve the identification of high-risk pregnancies.
The combination of maternal age, fetal nuchal translucency thickness (NT) and maternal serum free β-human chorionic gonadotropin (free β-hCG) and pregnancy-associated plasma protein-A (PAPP-A) in the first trimester achieved a detection rate (DR) for Down syndrome of about 90% with a false positive rate (FPR) of 5% (4).
For the first time in 1997 (5), cell-free fetal DNA was shown to be present in the plasma of pregnant women, and this finding has opened up new possibilities for non-invasive prenatal diagnosis (6).
Cell-free DNA from the fetus found in the plasma of pregnant women has also been used successfully for the non-invasive determination of the fetal sex and fetal RhD genotype in RhD negative women (5, 7-9).
More recently the same approach of searching for fetal-specific nucleic acids, such as DNA methylation and mRNA markers in maternal plasma, has been proposed the for non-invasive detection of fetal aneuploidies (10-14).
An alternative approach proposed for a non-invasive prenatal diagnosis of fetal trisomy 21 was to show the presence of an elevated amount of chromosome 21 sequences in maternal blood (13).
Some recent studies have proposed to increase the use of free-cell testing in routine clinical practice as a first-line method of screening or contingent on the results of the combined test in the first trimester, emphasizing the high detection rate and the low incidence of FPR, reported to be 99.0% and 0.08%, respectively, for trisomy 21, 96.8% and 0.15% for trisomy 18, 92.1% and 0.20% for trisomy 13, 88.6% and 0.12% for monosomy X, and 93.8% and 0.12% for sex chromosome aneuploidies other than monosomy X (15-19).
It is important to note that as of today, there are no clear percentages of false negatives. Only one arti-

cle, published in February 2014, describes a case report of a false negative for Down's Syndrome (20).
Many national and international associations of fetal-maternal and genetic medicine, such as The Italian College of Fetal Maternal Medicine, the International Society for Prenatal Diagnosis, the American College of Medical Genetics and Genomics, the American College of Obstetricians and Gynecologists Committee on Genetics, the California Technology Assessment Forum and the National Italian Guide Lines, have issued statements in this regard in the last year (21-25).
In particular, these recommendations emphasize that the only accredited screening tests for aneuploidies are the "combined tests", based on the evaluation of nuchal translucency and maternal serum placental proteins, and that the only diagnostic tests for fetal genetic and/or genomic anomalies are the chorionic villus sampling and the amniocentesis. Moreover, the recommendations stressed that the routine utilization of cell-free DNA in maternal blood can no longer be proposed as a first choice test. Furthermore, a very recent study hypothesized that false positive and false-negative findings may result from placental mosaicism, because cell-free fetal DNA is mainly of placental trophoblastic origin (26).
In support of these issues, we present 6 case reports concerning patients who performed amniocentesis or chorionic villus sampling at our center from January 2014 to March 2014 and who had previously undergone at NIPT with a positive finding of aneuploidy.

Case presentation

Case 1: a 30-year-old nulliparous woman was referred to the Artemisia Fetal-Maternal Medical Centre at the 12th week of gestation. She underwent chorionic villus sampling in order to confirm the diagnosis of Turner syndrome (45, X0) obtained through the research of Cell-free DNA. Her past medical history was negative. Both parents were healthy and non-consanguineous. The pregnancy was uncomplicated and the patient was taking regular supplements of folic acid and magnesium from the beginning of pregnancy. As routine practice, previously, our medical equipe performed an ultrasound examination. Ultrasound indicated a normal pregnancy regarding the uterus, placenta and fetal morphology and biometric data compatible with the period of amenorrhea. Then, chorionic villus sampling was performed. Using Quantitative Fluorescent Polymerase Chain Reaction (QFPCR) a preliminary result was obtained which showed a normal XX karyotype. This result was, then, confirmed through classic cytogenetics using three different cell cultures. This analysis also confirmed a normal 46,XX karyotype.

Case 2: a 33-year-old nulliparous woman was referred to the Artemisia Fetal-Maternal Medical Centre at the 16th week of gestation. She underwent an amniocentesis in order to confirm the diagnosis of triple X syndrome (47, XXX) obtained through the research of Cell-free DNA. Her past medical history was negative. Both parents were healthy and non-consanguineous. The pregnancy was uncomplicated and the patient was taking regular supplements of folic acid from the beginning of pregnancy and also performed antibiotic prophylaxis with azithromycin (three days before the procedure). As routine practice, previously our medical equipe had performed an ultrasound examination. Ultrasound indicated a normal pregnancy with regular fetal growth, no apparent structural malformation and female genitalia were visible. Then, an amniocentesis was performed. Using Quantitative Flourescent Polymerase Chain Reaction (QFPCR) a preliminary result was obtained that showed a normal XX karyotype. This result was, then, confirmed through classic cytogenetics using three different cell cultures. This analysis also confirmed a normal 46,XX karyotype. Moreover, Array - Comparative Genomic Hybridization (Array CGH) was performed and the results indicated that the fetus was a heterozygous carrier of the connexin 26 gene (GJB2) 35delG mutation associated with congenital deafness.

Case 3: a 31-year-old nulliparous woman was referred to the Artemisia Fetal-Maternal Medical Centre at the 16th week of gestation. She underwent an amniocentesis in order to confirm the diagnosis of triple X syndrome (47, XXX) obtained by analyzing the Cell-free DNA. She was carrier of spinal muscular atrophy (SMA) and affected by thyroid diseases which were being treated with 37,5 mcg of levothyroxine daily. Her husband was a carrier of cystic fibrosis. Both parents were healthy and non-consanguineous. The pregnancy was uncomplicated and the patient performed antibiotic prophylaxis with azithromycin (three days before the procedure). As routine practice, previously our medical equipe had performed an ultrasound examination. The ultrasound revealed a normal pregnancy with regular fetal growth, no apparent structural malformation and female genitalia were visible. Then, an amniocentesis was performed. Using Array CGH, a result was obtained which highlighted a normal XX karyotype. This result was, then, confirmed through classic cytogenetics using three different cell cultures. This analysis also confirmed a normal 46, XX karyotype.

Case 4: a 40-year-old nulliparous woman was referred to the Artemisia Fetal-Maternal Medical Centre at the 12th week of gestation. She underwent chorionic villus sampling in order to confirm the diagnosis of Patau syndrome (47, +13) obtained by research of the Cell-free DNA. Her past medical history was negative. Both parents were healthy and non-consanguineous. The pregnancy was uncomplicated and the patient was taking regular supplements of folic acid from the beginning of pregnancy. As routine practice, previously our medical equipe had performed an ultrasound examination. Ultrasound indicated a normal pregnancy regarding the uterus, placenta and fetal morphology and biometric data was compatible with 13 weeks of gestation. Then, chorionic villus sam-

pling was performed. Using Array CGH, a result was obtained that indicated a normal XX karyotype. This result was, then, confirmed through classic cytogenetics using three different cell cultures. This analysis also confirmed a normal 46,XX karyotype.

Case 5: a 41-year-old multiparous woman was referred to Artemisia Fetal-Maternal Medical Centre at the 13th week of gestation. She underwent chorionic villus sampling in order to confirm the diagnosis of Turner syndrome (45, X0) obtained through the analysis of the Cell-free DNA. Her past medical history was negative. Both parents were healthy and non-consanguineous. The pregnancy was uncomplicated and the patient was taking regular supplements of folic acid from the beginning of pregnancy. As routine practice, previously, our medical equipe performed an ultrasound examination. Ultrasound indicated a normal pregnancy regarding the uterus, placenta and fetal morphology and biometric data compatible with the period of amenorrhea. Then, chorionic villus sampling was performed. Using Quantitative Fluorescent Polymerase Chain Reaction (QFPCR), a preliminary result was obtained which highlighted a normal XX karyotype. This result was, then, confirmed through classic cytogenetics using three different cell cultures. This analysis also confirmed a normal 46,XX karyotype.

Case 6: a 44-year-old nulliparous woman was referred to the Artemisia Fetal-Maternal Medical Centre at the 12th week of gestation. She underwent chorionic villus sampling in order to confirm the diagnosis of Edwards syndrome (47, +18) obtained by analyzing the Cell-free DNA. She had had 3 previous pregnancies, one ending in a termination of pregnancy (TOP) for Down's Syndrome (47, +21) and the other two in miscarriages at the first trimester. The material of the second miscarriage was used to perform karyotyping with the detection of a trisomy of chromosome 15 (47, +15). Her husband was a heterozygous carrier of the human hemochromatosis gene (HFE) H63D and C282Y mutation associated with hemochromatosis. Both parents were healthy and non-consanguineous. The pregnancy was uncomplicated and the patient was taking regular supplements of folic acid from the beginning of pregnancy. As routine practice, previously our medical equipe had performed an ultrasound examination.
Ultrasound evidenced a normal pregnancy regarding the uterus, placenta and fetal morphology and the biometric data was compatible with the period of amenorrhea. Then, chorionic villus sampling was performed. Using Array CGH and classic cytogenetics discordant results were obtained; both tests showed a mosaicism (46, XX/47, XX +18), but in different proportions. For this reason, we decided the patient should undergo an amniocentesis at the 16th weeks of gestation. Using Array CGH, a result was obtained that highlighted a normal 46,XX karyotype. This result was, then, confirmed through classic cytogenetics using three different cell cultures. So the result of the chorionic villus sampling was classified as placental mosaicism of trisomy 18.

Discussion

In our case report, we discuss 6 false-positive cases at NIPT that came to our center in the last two months.
It's well known that with NIPT, there is a risk of false positive cases due to the fact that the analyzed fetal DNA has a placental origin and another important factor is that placental mosaicism can give discordant, and therefore, invalid results (26-30). This thesis is confirmed by the results of our six cases, in which patients underwent chorionic villus sampling or an amniocentesis to confirm two cases of Turner syndrome, two cases of triple X syndrome and one case of Patau syndrome.
In all six cases, the fetal karyotype was normal at invasive prenatal diagnosis.
It is important to note that as of today, there are no percentages of false negatives. Only one article, published in February of 2014, describes a case report of a false negative for Down's Syndrome (20). Moreover, in view of the early gestational age in which the test is performed, the quantity of circulating fetal DNA is very low, significantly increasing the false negatives. In fact, many published studies on fetal DNA have been based on samples taken in the second and third trimester in order to obtain a larger share of DNA (31).
We wanted to publish our cases given that the clinical use of NIPT has become increasingly common but it is important to understand the benefits, risks and limitations in order to guide parents in making an informed decision.
The Italian Society of Human Genetics has suggested changing the acronym NIPT to NIPS (Non Invasive Prenatal Screening) in order to avoid confusion. In addition to the current state of the art, this method appears to have a reliability comparable to already existing screening tests, but is limited exclusively to the screening of Down syndrome and Trisomy 18. Doubts remain for Trisomy 13 and there are difficulties due to the large number of false positives in sex chromosome abnormalities (28-30). In particular, despite the FPR of 0.12% for sex chromosome aneuploidies reported in a meta-analysis published in 2014 (19), a prospective NIPT study, also published in 2014, reports that 16 (8.6%) of 181 positive sex chromosome aneuploidies were due to an abnormal maternal chromosome X karyotype that masked the true contribution of the fetal chromosome X DNA fraction (29).
Also, considering the high cost of the test, NIPT couldn't be offered as a form of screening, because, by definition, screening must be inexpensive so it can be applied to a large number of people. Therefore, it should be reserved for cases in which combined or integrated screening gives anomalous results; and it is necessary to emphasize that the test does not give results in 2 to 11% of cases (27).
Moreover, in recent years, with the introduction of genomic technologies that can be used in prenatal diagnosis in the form of chorionic villus sampling and amniocentesis, it is now possible to diagnose thousands of diseases. Thus, prenatal diagnosis no longer simply excludes Down's syndrome only. Furthermore,

thanks to introduction of array CGH, micro deletions and micro duplications can be diagnosed.
Therefore, at the moment, there is no test that can guarantee a diagnosis of hundreds of genetic or chromosomal disorders if we exclude amniocentesis and chorionic villus sampling (2, 15, 19, 26).

Conclusion

It is important to bear in mind that NIPT does not provide a specific diagnosis and merely screens a limited number of compared diseases. Placental mosaicism can give discordant and invalid results. Pretest counseling must be provided, and a positive NIPS result should be confirmed with invasive diagnostic testing.

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