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SHORT COMMUNICATION

The End of Amniocentesis? From TriTest to PrenaTest™

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ABSTRACT

Purpose: The rapid development in molecular biological technologies makes it possible to screen and to diagnosis thousands of genetic conditions, mutations and also predispositions to chronic diseases or traits, either prenatally or after birth. Clinical application of noninvasive prenatal diagnosis (NIPD) using fetal deoxyribonucleic acid (DNA) in maternal plasma has become a reality. We review the latest developments in screening and diagnosis of chromosomal diseases and a new noninvasive method of prenatal diagnosis.

Materials and methods: PrenaTest™ (LifeCodexx AG, Konstanz, Germany, patented and licensed by Sequenom Inc., San Diego, California, USA) is a molecular genetic test that can detect fetal trisomy 21 with a high precision level from maternal blood using new generation multiplex genome sequencing techniques. The test is based on the analysis of extracellular fetal DNA measured in the blood of pregnant women: Cell-free fetal DNA (cffDNA).

Results: In the case of trisomy 21, there were found 105 specific fragments of chromosome 21, 15 of fetal origin and 90 of maternal origin. The validity of PrenaTest™ has been demonstrated by many tests (427 cases), and the sensitivity was 95%, with a false negative rate of 5% and a specificity of 99.5%. Anyway, an additional ultrasound can always be performed to assess fetal morphology.

Conclusion: The arrival of the molecular genetic era also leads to many new ethical, social and medicolegal problems and dilemmas that obstetricians will have to face in the near future. There is an urgent need for the development of a new model for provision of genetic screening and diagnosis.

Keywords: Amniocentesis. Cell-free fetal DNA, First trimester screening, Pregnancy, Prenatal diagnosis.

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INTRODUCTION

Pregnancy screening for fetal aneuploidy started in the mid 1960s, using maternal age as the screening test. Down syndrome (DS) screening has been an integral part of routine prenatal screening for the last three decades. Recent efforts have been directed at developing additional noninvasive prenatal screening techniques that could not only improve sensitivity of prenatal screening, but also be employed in the first trimester to offer earlier diagnostic and interventional opportunities. Nuchal translucency (NT) has proven to be an effective and cost-effective screening test that, when combined with serum markers β-human chorionic gonadotropin (hCG) and pregnancy-associated plasma protein (PAPP-A) in the first and/or second trimester, broadens the diagnostic possibilities and improves the diagnostic capabilities of current prenatal DS screening methods. Despite the potential benefits, significant operational issues regarding access to and availability of such testing may limit its widespread application and necessitates the maintenance of both nonsonographic and second trimester screening methods. The implementation of first trimester DS testing requires the development and maintenance of nationally standardized quality control systems to ensure the reliability of serum and ultrasound measurements and the accurate assessment of risk.[1] Major recent advances include the completion of the Human Genome Project, the use of microarray and related technologies for mass screening and diagnosis of thousands of genetic abnormalities and noninvasive prenatal diagnosis (NIPD) using fetal DNA in maternal plasma.[2]

Since the decree of June 23rd, 2009, the obstetrician/gynecologist or the general practitioner offers a combined screening in the first trimester of pregnancy (from 11^{+0} to 13^{+6} weeks of gestation): Ultrasound measurement of NT and crown-rump length (CRL) are performed prior to analysis of biochemical serum markers (two or three hormones of placental origin: α-fetoprotein (AFP), estriol (E3), hCG). The final result allows to estimate the individual risk of carrying a child with trisomy 21, or more specifically belonging to a statistically high-risk group. This calculation of risk is performed by a software.

The threshold that has been set by the French Social Security (SSF) to support an amniocentesis, which then allows a definitive diagnosis, is 1/250. When the risk is less than 1/250, it is still possible to undergo amniocentesis, but costs (approximately € 500) are not reimbursed. If the screening is done in good conditions, it can detect 70% of trisomic children, but it also produces, with the cutoff chosen in France (1/250), a significant number of unnecessary amniocentesis. In fact, the test can be normal while the baby is carrying an anomaly, and abnormal while the baby is fine. It is therefore a probability and not a 100% diagnosis.[3-6]

MATERIALS AND METHODS

PrenaTest™ (LifeCodexx AG, Konstanz, Germany, patented and licensed by Sequenom Inc., San Diego,

California, USA) is a molecular genetic test that can detect fetal trisomy 21 with a high precision level from maternal blood using new generation multiplex genome sequencing techniques. The test is based on the analysis of extracellular fetal deoxyribonucleic acid (DNA) measured in the blood of pregnant women: cell-free fetal DNA (cffDNA).

Small fragments of fetal DNA circulate freely in maternal blood (on average they represent 10% of total circulating DNA). These fetal fragments come from of dead cells from the placenta which are continuously discharged into the maternal circulation. The life cycle of these fragments is of less than 2 hours and in no cases they can be found some hours after birth.

This test works in the following way:

- Withdrawal of 20 ml of maternal blood
- Isolation of plasma in the laboratory
- Purification of free DNA (cffDNA)
- Creation of a 'genomic library' which is then amplified
- The DNA fragments are then decoded by the technique of 'massive parallel sequencing' (MPS), using the Illumina HiSeq2000 technology
- Analysis of data by PrenaTest™ DAP.21 software, that will calculate the statistic z-score (Fig. 1).

The result is considered positive if the z-score calculated is ≥ 3, with a probability of more than 99.87% (Figs 2 to 6).

RESULTS

Assuming that a plasma sample from a pregnant woman contains 100 fragments specific to a chromosome, about

$$z(\text{Chr21})_{\text{sample}} = \frac{\%(\text{Chr21})_{\text{sample}} - \text{median}(\text{Chr21})_{\text{reference}}}{\text{MAD}(\text{Chr21})_{\text{reference}}}$$

Fig. 1: z-score calculation formula (MAD = mean absolute deviation)

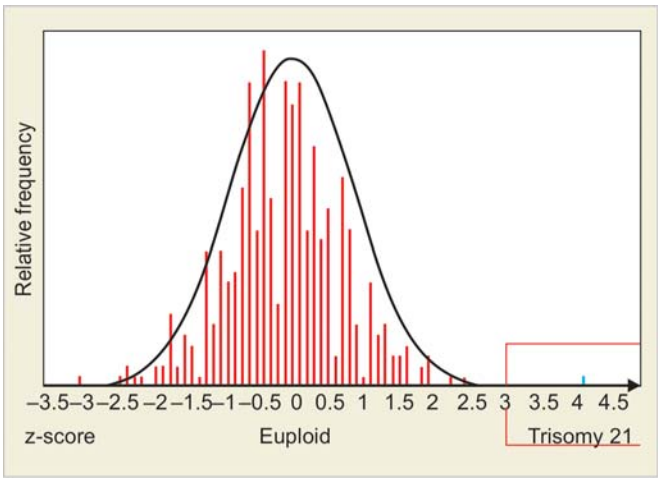

Fig. 2: Example of frequency distribution of z-score

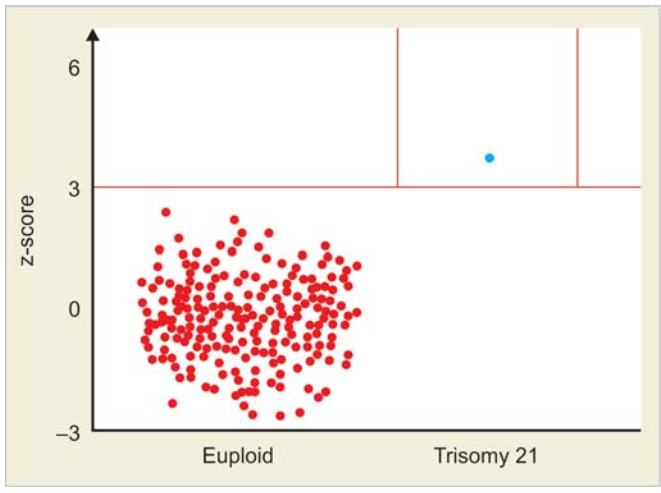

Fig. 3: z-score for a reference euploid group and a trisomy 21 case

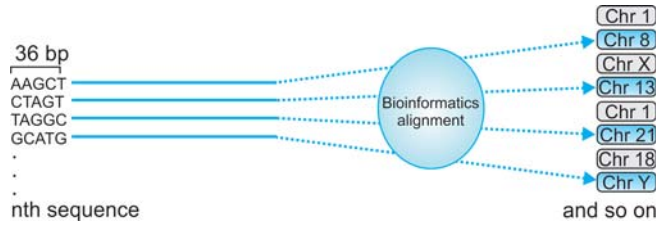

Fig. 4: Sequencing and alignment bioinformatics

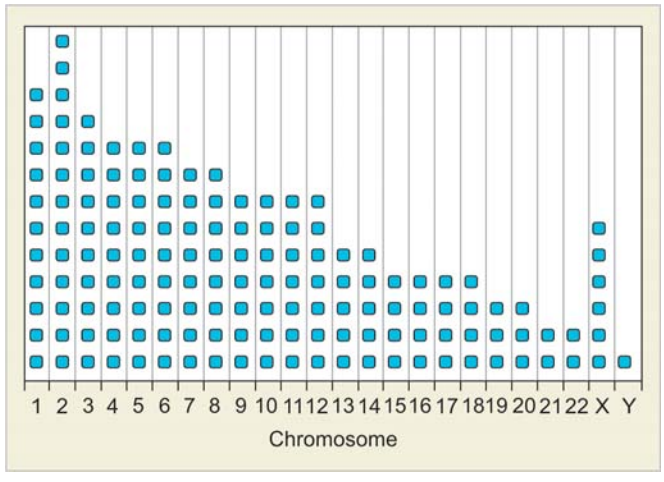

Fig. 5: Counting sequences

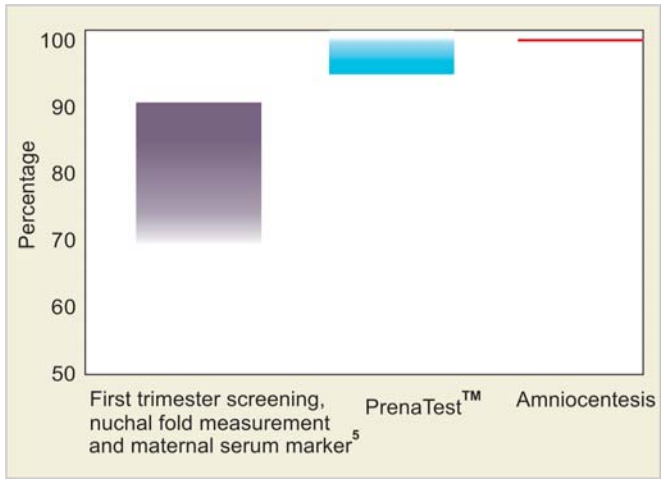

Fig. 6: Detection rate of trisomy 21: comparison of noninvasive and invasive methods

Note: The figures and data are part of the advertising of LifeCodexx AG, Konstanz, Germany

10 fragments will be of fetal origin and 90 will be of maternal origin, if euploid. In the case of trisomy 21, there were found 105 specific fragments of chromosome 21, 15 of fetal origin and up to 90 of maternal origin. The validity of PrenaTest™ has been demonstrated by many tests (427 cases), and the sensitivity was 95%, with a false negative rate of 5% and a specificity of 99.5%. Anyway, an additional ultrasound can always be performed to assess fetal morphology.

DISCUSSION

Prenatal diagnosis for chromosome abnormalities has been available for over 30 years. The most common referral indication is a raised risk of DS, and diagnosis has, until recently, been carried out by culture of cells from invasive prenatal sampling, followed by full karyotype analysis, with a waiting time of around 2 weeks for results. More recent developments in fluorescence *in situ* hybridization (FISH) and quantitative fluorescence-polymerase chain reaction (PCR) techniques have led to rapid 1-2 d reporting for DS, opening the way to the possibility of targeted testing based on referral indication, thus reducing the incidence of difficult counseling issues and potentially unnecessary pregnancy terminations following the unexpected discovery of anomalies such as balanced chromosome rearrangements.[7] The provision of prenatal diagnosis requires the highest standards in laboratory practice to ensure an accurate result. In preimplantation genetic diagnosis, protocols additionally have to address the need to achieve an accurate result from 1 to 2 cells within a limited time. Emerging protocols of 'noninvasive' prenatal diagnosis, which are based on analysis of free fetal DNA in the circulation of the pregnant mother, also have to achieve a result from a limited quantity of fetal DNA against a high background of maternal free DNA. Real-time PCR uses fluorescent probes or dyes and dedicated instruments to monitor the accumulation of amplicons produced throughout the progress of a PCR reaction. Real-time PCR can be used for quantitative or qualitative evaluation of PCR products and is ideally suited for analysis of nucleotide sequence variations (point mutations) and gene dosage changes (locus deletions or insertions/duplications) that cause human monogenic diseases. Real-time PCR offers a means for more rapid and potentially higher throughput assays, without compromising accuracy and has several advantages over end-point PCR analysis, including the elimination of post-PCR processing steps and a wide dynamic range of detection with a high degree of sensitivity.[8]

The isolation of fetal cells or free fetal DNA in maternal circulation has been for many years an important research in the field of prenatal diagnosis. Researchers from Hong Kong pioneered it 10 years ago: they indeed were the first to successfully detect floating fragments of fetal DNA in maternal blood.[9,10] But to allow this discovery to bear fruit, it still had to be developed a high-throughput sequencer capable of reconstituting fetal genome from the DNA fragments. This advanced technology has been available from some years ago, and the tests used have then multiplied. Thus, the ability to detect the sex of the fetus or Rhesus (Rh) factor are already used in practice due to the 'simple' analysis of these fragments of fetal DNA.[10]

Chitty et al in the American Journal of Obstetrics and Gynecology have recently published in April 2012 an editorial in which they practically gave the green light to the use of this test in clinical practice.[11] Indeed, several groups have sought to confirm that the detection of trisomy 21 could be done in this way: one of the major publications in this field was once again that of Hong Kong researchers in 2011, in the British Medical Journal. The team of RWK Chiu has evaluated the effectiveness of the test in 753 patients.[12] The multiplex sequencing method allowed the detection of fetuses with trisomy 21 with a sensitivity of 100% and a specificity of 97.9%, giving a positive predictive value (PPV) of 96.6% and a negative predictive value (NPV) of 100%. Clinical trials are published in a study called 'noninvasive chromosomal evaluation (NICE) Study: results of a multicenter prospective cohort study for detection of fetal trisomy 21 and trisomy 18,' which came out at the beginning of August 2012.[13] Finally, after years of research, fetal DNA will provide a safe and NIPD.

In summary, the presence of fetal DNA in maternal blood allows to detect an abnormal increase of chromosome 21 in case of fetal trisomy, since there are three copies of chromosome 21 instead of two. Fetal DNA molecules in maternal blood do not count for more than 10 to 20% of total DNA molecules in maternal plasma and a variation in quantity was, until recently, difficult to evidence. The development of genome sequencing has permitted to solve the problem, identifying and quantifying millions of DNA fragments in a few days. It was this sequencing that was used in the study published by the British Medical Journal. It enrolled 753 patients, all at high-risk for trisomy 21. By the multiplex sequencing method, fetuses carrying trisomy 21 were detected with a sensitivity of 100% and a specificity of 97.9%, giving the test a PPV of 96.6% and a NPV 100%.[14-47]

NIPD could significantly change the framework for testing and screening in pregnancy. The ethical implications of this technology include current issues in prenatal diagnosis, implications for informed consent, possible nonmedical uses and options for regulation. The prospect

of NIPD normalizing screening and termination in pregnancy is raised as a concern. NIPD will also require monitoring to ensure women are making well-informed decisions, given that a risk to the pregnancy is absent. The question of whether NIPD will reduce anxiety needs to be established and the prospect that it will increase terminations on the grounds of disability should be recognized. The offer of NIPD external to any clinical oversight might give rise to wider social sex selection, paternity testing or testing 'for information'. The value assumptions of these uses of NIPD need to be addressed.[48-50]

CONCLUSION

The translation of novel genomic technologies from bench to bedside enjoins the comprehensive consideration of the perspectives of all stakeholders who stand to influence, or be influenced by, the translational course. Noninvasive prenatal aneuploidy testing that utilizes cffDNA circulating in maternal blood is one example of an innovative technology that promises significant benefits for its intended end users. However, it is currently uncertain whether it will achieve widespread clinical implementation.[51] cffDNA can be detected in the maternal circulation during pregnancy, potentially offering an excellent method for early NIPD of the genetic status of a fetus. Using molecular techniques, fetal DNA and ribonucleic acid (RNA) can be detected from 5 weeks gestation and are rapidly cleared from the circulation following birth. cffDNA comprises only 3 to 6% of the total circulating cell-free DNA, therefore diagnoses are primarily limited to those caused by paternally inherited sequences as well as conditions that can be inferred by the unique gene expression patterns in the fetus and placenta. Broadly, the potential applications of this technology fall into two categories: first, high genetic risk families with inheritable monogenic diseases, including sex determination in cases at risk of X-linked diseases and detection of specific paternally inherited single gene disorders, and second, routine antenatal care offered to all pregnant women, including prenatal screening/diagnosis for aneuploidy, particularly DS and diagnosis of Rh factor status in RhD negative women. Already, sex determination and Rh factor diagnosis are nearing translation into clinical practice for high-risk individuals. The analysis of cffNA may allow NIPD for a variety of genetic conditions and may in future form part of national antenatal screening programs for DS and other common genetic disorders.[52]

Analysis of cffDNA in maternal plasma provides the opportunity for reliable, timely, safe, and cost-effective diagnosis of single gene disorders. The detection of certain fetal loci using cffDNA and conventional molecular analytic approaches is possible from 4 weeks gestation. To date, noninvasive first-trimester analysis for single gene disorders has been limited by assay sensitivity and specificity, due to the background maternal DNA. The anticipated ability to enrich the fetal component of cell free DNA will increase the robustness of tests and permit semiquantitative analysis, broadening the scope of testing to include recessive disorders such as cystic fibrosis. Testing for large-scale mutations might remain limited by the fragmented nature of cffDNA and, when testing very early in gestation, careful ultrasound examination will be needed to determine the number of gestational sacs, because of the risk of discordant twin pregnancies.[53] NIPD using massive parallel sequencing of cffDNA to test for trisomies 21, 18 and 13 should be an option available to women at increased risk in lieu of amniocentesis. Pretest counseling of these women should include a discussion of the limitations of noninvasive prenatal testing. No irrevocable obstetrical decision should be made in pregnancies with a positive NIPD result without confirmatory invasive diagnostic testing. Although testing of cffDNA in maternal plasma appears very promising as a screening test for DS and other trisomies, studies in average-risk pregnancies and a significant reduction in the cost of the technology are needed before this can replace the current maternal screening approach using biochemical serum markers with or without fetal NT ultrasound.[54] The authors believe that this test would reduce to 0.1% the number of women who should still require amniocentesis or chorionic villus sampling (CVS). In fact, the test will achieve the same results as amniocentesis without putting the fetus at risk and prevent fetal deaths by accidents due to sample of amniotic fluid.

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