Prenatal diagnosis of 21 trisomy by quantification of methylated fetal DNA in maternal blood: study on 10 pregnancies

Diagnosticul prenatal al trisomiei 21 prin cuantificarea ADN-ului fetal metilat din sângele matern: studiu pe 10 sarcini

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Abstract

Background. The Down syndrome is a severe disease without pathogenic therapy. The only possibility to reduce the consequences of disease is prenatal screening and diagnosis. The gold standard in prenatal diagnosis is the conventional banding cytogenetic analysis of fetal cells obtained by invasive procedures. To reduce the complications, in the last years different methods to detect fetal cells or DNA in maternal blood were developed.

Aim. The aim of study was to verify the reliability of quantification by immunoprecipitation of methylated fetal DNA in maternal blood in the prenatal diagnosis of 21 trisomy. Method. We analyzed probes from 12 pregnant women (7 with confirmed 21 trisomy pregnancy and 5 with normal pregnancy), with two being rejected for technical considerations. For each probe we carried out: extraction of total DNA (maternal and fetal), DNA fragmentation, immunoprecipitation of methylated DNA, washing, isolation of DNA and qPCR for immunoprecipitated DNA. To highlighting specific methylated regions on fetal 21 chromosome we used eight pairs of specific primers for chromosome 21. Finally we analysed the results of qPCR applying the formula D=–6.331+0.959XEP4+0.424XEP6+0.621XEP7+0.028XEP8+0.387XEP10–0.683XEP11+0.897XEP12, where XEPi= fraction value for each marker. Results. In all normal pregnancies the value of D factor was negative concordant with absence of trisomy (100% specificity). In 5 from 6 pregnancies with 21 trisomy the value of D factor was positive, which indicated a high sensibility. However, to a precise estimation of this method is required a larger number of cases that allowing the obtaining of statistically validated results.

Keywords: non-invasive prenatal diagnosis of 21 trisomy, methylated fetal DNA, immunoprecipitation

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Rezumat

Introducere. Syndromul Down este o boală gravă, lipsită de terapie patogenică. Singurele posibilități de reducere a consecințelor bolii le reprezintă screeningul și diagnosticul prenatal. Standardul de aur în diagnosticul prenatal este analiza cromosomică a celulelor fetale obținute prin proceduri invazive. Pentru reducerea complicațiilor, în ultimii ani au fost dezvoltate diferite metode de detectie a celulelor sau ADN-ului fetal în sângele matern. Scopul studiului a fost verificarea aplicabilității metodei de cuantificare prin imunoprecipitare a ADN-ului fetal metilat din sângele matern în diagnosticul prenatal al trisomiei 21. Metodă. Am analizat probe de la 12 gravide (7 cu făt cu trisomie 21 și 5 cu făt euploid confirmate citogenetic), două probe fiind eliminate din considerente tehnice. Pentru fiecare probă am efectuat: extracția ADN-ului total (matern și fetal), fragmentarea ADN-ului, imunoprecipitarea ADN-ului metilat, spălarea, izolarea ADN-ului și qPCR pentru ADN-ul imunoprecipitat. Pentru evidențierea regiunilor metilate specifice pe cromosomii 21 fetali am utilizat opt perechi de amorse specifice cromosomului 21. În final, am analizat rezultatele qPCR aplicând formula: \[ D = -6,331 + 0,959X_{EP4} + 1,188X_{EP5} + 0,424X_{EP6} + 0,621X_{EP7} + 0,028X_{EP8} + 0,387X_{EP10} - 0,683X_{EP11} + 0,897X_{EP12} \] unde \( X_{EPi} \) = valoarea fracției pentru fiecare marker. Rezultate. În toate sarcinile normale, valoarea factorului \( D \) a fost negativă concordant cu absența trisomiei (specificitate 100%). În 5 din 6 sarcini cu trisomie 21 valoarea factorului \( D \) a fost pozitivă, ceea ce indică o sensibilitate crescută. Totuși, pentru estimarea preciză a metodei este necesară analiza mai multor cazuri, ceea ce ar permite obținerea de date valabile statistice.

Cuvinte cheie: diagnosticul prenatal nonininvaziv al trisomiei 21, ADN fetal metilat, imunoprecipitare

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Introduction

Down syndrome (DS) is a public health problem both by high frequency - 1/700 – 1/800 live births - and by comorbidities and intellectual disability (1, 2). In the absence of pathogenic therapies, the need for performance prenatal prophylaxis of trisomy 21 is evident. Prevention of DS uses secondary prophylaxis requiring prenatal detection of the disease. Methods for screening and prenatal diagnostic (PD) have been developed for this purpose. The screening identifies high-risk pregnancies by noninvasive methods, using biochemical and ultrasound parameters. Combined test applied during the first trimester, assess nuchal translucency, free human chorionic gonadotropin (free \( \beta \)-hCG), pregnancy-associated plasma protein (PAPP-A) and maternal age. The quadruple test, applied in the second trimester of pregnancy, measures alpha-fetoprotein (AFP), unconjugated estradiol (UE3), the unbound fraction of \( \beta \)-hCG and inhibit A, all correlated with maternal age. Integrated test totalizes measurements at various stages of pregnancy linking nuchal translucency and PAPP-A with quadruple test markers in the second quarter (2). Prenatal diagnosis is based on genetic analysis of fetal cells obtained by invasive methods that generate risks for the pregnant woman and the fetus. The methods of obtaining fetal biological material for prenatal diagnosis are chorionic villus sampling (I trimester), amniocentesis and cordocentesis (II trimester of pregnancy). All the invasive methods to obtaining fetal cells could induce bleeding, miscarriage and fetal malformations (3, 4). However, classic prenatal chromosome analysis has the advantage of 100% accuracy, allowing the detection of chromosomal abnormalities in the resolution limit of optical microscopy. The major disadvantage is the long period of 10-14 days required to complete the cell culture (5).

The introduction of molecular cytogenetic tests reduced the time to achieve results within two days, but have the disadvantage of targeted detection of certain chromosomal regions. The FISH technique, based on the hybridization of a labeled fluorescent probe and a specific chro-
mosome, is applicable for the routine prenatal detection of aneuploidies of chromosome 13, 18, 21, X and Y. The method has a high specificity and sensitivity (near to 100%), but is expensive, time-consuming and cannot detect all chromosomal anomalies. QF-PCR and MLPA techniques will produce rapid results at low cost, but are characterized by low sensitivity and specificity (5, 6).

Starting from the prerequisites of necessity for a prenatal diagnosis fast, cheap, safe and less traumatic for the pregnant woman and the fetus, noninvasive prenatal diagnostic methods (NPD) based on isolation of fetal cells or detection of free fetal DNA in maternal circulation (7) were implemented lately.

The passage of fetal cells in maternal circulation during pregnancy is low, so there is about 1 fetal cell/ml of maternal blood. Their frequency slightly increases in the third trimester of pregnancy and in certain disorders associating placental dysfunction (8). The concentration methods of fetal cells - magnetic cell sorting (MACS) and fluorescent activated cell sorting (FACS) – increase the number of fetal cells, but also produce the loss of a large number of cells (7, 9).

Different studies proved that free fetal DNA disappears rapidly after birth, the mean half-life being 16.3 minutes, which creates prerequisites to an improvement in eliminating false positive prenatal diagnosis of fetal cells generated from a previous pregnancy (10). The amount of free plasma DNA in adult women is about 10-100 ng, but during the pregnancy there is a significant increase due to bone turnover and cell apoptosis (11). Free fetal DNA in maternal blood can be detected after the 5th week of amenorrhea, after which there is a steady increase during pregnancy to peak at the end of pregnancy, but not more than 3.4 to 6.2% of the total free circulating DNA (12). Increased concentration of fetal DNA was identified in different obstetrical conditions (preeclampsia, preterm delivery, fetal-maternal hemorrhage, polyhydramnios etc.) but also in trisomy 21 (13-17).

Cleavage of maternal DNA generated fragments longer than 201 bp. The fetal DNA is cleaved into short fragments, usually less than 193 bp (18).

Quantitative changes in DNA associated with fetal aneuploidies can be identified by indirect methods aimed at determining the allelic fraction (placental messenger RNA analysis, the DNA methylation and methylation specific fraction of fetal DNA) and direct methods molecular quantification (digital PCR and massive parallel sequencing) (19). Analysis of free fetal messenger RNA targeting genes expressed exclusively fetal DNA as PLAC4 gene (located on chromosome 21) or maspin gene (located on chromosome 18) (12, 20).

Direct detection methods require expensive equipment and reagents, are laborious, require advanced statistical processing and have not been validated by extensive studies (12, 19). Digital PCR accurately quantify very small amounts of DNA and allows detection of trisomy 21 by counting the number of target sequences on chromosome 21 compared to similar loci on other chromosomes, but the utility of analysis is probed in presence of more than 10,000 DNA sequences (7). Massive parallel sequencing solves the problem of scarceness of fetal DNA in maternal circulation, but has a low sensitivity and requires statistical methods for data processing (21).

A new approach to NPD was conducted by Papageorgiou et al. (2009) and focused on identifying localized regions on chromosome 21 that are differentially methylated (DMR), hypermethylated in the placenta and hypomethylated in peripheral blood. In addition, the methylation status must be maintained the same throughout pregnancy (22). For this, the first step performed was physical separation of methylated and nonmethylated DNA, obtaining a methylated sample. For this sample was quantified the level of hypermethylation for several fetal/maternal differentially methylated regions and the result relates to a reference value representing hypermethylation in pregnancies with normal fetuses (23).
Material and method

The study group consisted of 12 pregnant women investigated between January and November 2012 in the Central Laboratory of Immunology and Genetics of University of Medicine and Pharmacy “Grigore T. Popa” Iași. The prenatal diagnosis was made on grounds of: advanced maternal age, biochemical screening and / or ultrasound positive signs for 21 trisomy. All pregnant women agreed to participate in the study and signed an informed consent (approved by the Bioethics Commission of University of Medicine and Pharmacy “Grigore T. Popa” Iași) after providing genetic counseling, knowing the significance of prenatal diagnosis. In 7 cases we discovered a trisomy 21 confirmed by FISH or conventional chromosomal analysis (Table 1).

The method used for NPD was that established by Papageorgiou et al. (2011) (23).

Working protocol has 6 stages: extraction of total DNA (maternal and fetal), DNA fragmentation, immunoprecipitation of methylated DNA, washing, isolation of DNA and qPCR for DNA immunoprecipitated.

For each pregnant were collected on EDTA 2 ml of peripheral blood and DNA extraction from whole blood has been done up to 6 hours after blood sampling, using a kit QIamp DNA Blood Mini Kit® (Qiagen;West Sussex, UK). Finally, the DNA was quantified, divided into samples, labeled and stored in the collection of DNA in compliance with the quality and quantity of DNA evidence, traceability, anonymity, and the ergonomics.

The genomic DNA was fragmented by sonication at 40 Hz, using a sequence of 15 sonication and 15 s rest alternative for 10 minutes, using a MRC Scientific Instrument Ultrasonic Cleaner.

The DNA sample was diluted in GenDNA TE to reach 0.1 µg/µl, to get a final volume of 300 µl of DNA (30 µg). Verification was done by migration in Agarose gel using a 50 bp ladder. Because there was no complete fragmentation of all samples, the procedure was repeated, and finally we obtained DNA fragments with sizes of 150-500 bp.

Methylated fetal DNA extraction was done by using magnetic beads and 5-methylcytosine antibody extraction according to the protocol - MagMeDIP (Magnetic Methylated DNA Immunoprecipitation kit - Diagenode®). For this purpose, the DNA from each fetus was divided into three samples: two used for immunoprecipitation (IP) and one considered as evi-

<table>
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<th>Motivation of performing prenatal diagnosis</th>
<th>Fetal status</th>
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<td>16 weeks</td>
<td>DTP</td>
<td>T21*</td>
</tr>
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<td>18 weeks</td>
<td>DTP</td>
<td>T21*</td>
</tr>
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<td>PN 3</td>
<td>16 weeks</td>
<td>DTP</td>
<td>T21*</td>
</tr>
<tr>
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<td>11 weeks</td>
<td>nuchal fold &gt; 3 mm</td>
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</tr>
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<td>16 weeks</td>
<td>AMA, pathological fetal ultrasound</td>
<td>T21*</td>
</tr>
<tr>
<td>PN 6</td>
<td>16 weeks</td>
<td>AMA, DTP, pathological fetal ultrasound</td>
<td>T21**</td>
</tr>
<tr>
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DTP – pathological double test, AMA – advanced maternal age, TTP – pathological triple test; T21 – trisomy 21, N-euploid fetus; * confirmed by FISH; ** confirmed by fetal chromosome analysis
Each IP contained 1 µg of DNA. For immunoprecipitation was carried out a mixture of 75 µl DNA, 5 µl mixture of 20 µl of diluted antibody of magnets (100 µl per reaction) which was stirred magnetically at 4°C for 12 hours.

Washing was carried out at cold, in magnetic stirrer in four successive stages (the first three using MagWash-1 buffer and the last using MagWash-2 buffer). At the end of washing, centrifugation was performed by keeping the sediment.

In the next step we isolated both immunoprecipitated DNA, as well as sample input.

To IP samples added 100 µl of buffer DIB with proteinase K (1 µl proteinase K to 100µl buffer DIB). To input sample added 92.5 µl buffer DIB to 7.5 µl of DNA. The samples IP and input were incubated 15 minutes at -55°C, then another 15 minutes at 100°C, followed by rapid stirring 5 minutes, centrifugation at 4°C with 14,000 rpm and the supernatant was finally transferred to labeled tubes, stored at -20°C, representing prepared DNA for qPCR.

To achieve qPCR analysis of immunoprecipitated DNA we used a kit containing four pairs of specific primers validated for four types of DNA: methylated control DNA (primer pair 1) unmethylated control DNA (primer pair 2), human DNA methylated (TSH2B) and human DNA unmethylated (GAPDH promoter). DNA from IP and input samples was diluted in 25 µl solution containing each: 1µl of primer pair (Forward and Reverse) 12.5 µl SYBR Green PCR master mix, 5 µl DNA sample and 6.5 µl water.

Polymerase chain reaction (PCR) was carried out in the following sequence of steps: amplification at 95°C for 7 minutes (one cycle), amplification at 95°C for 15 s (40 cycles), amplification for 60 seconds at 60°C (40 cycles), and amplification at 95°C for 1 min (one cycle). After amplification was done 60 successive cycles of melting of 1 minute each, with gradual increase of temperature by 0.5°C, the first cycle at 65°C. The analysis of efficiency for immunoprecipitation was done by calculating the ΔCT between IP and input for all four types of DNA: methylated DNA, unmethylated DNA, TSH2B and GAPDH promoter (Figure 1, Supplementary Table 1). Analysis of imunoprecipitation efficiency for a given locus was calculated using the following formula:

\[ \% \text{ (meDNA-IP/Total input)} = 2^{\left( \Delta \text{CT}_{10\%\text{input}} - 3.32 \right)} \times 100\% \]

where 2 is the AE (amplification efficiency), CT (meDNA-IP) and CT (10% input) are threshold values obtained from exponential phase of qPCR for the methyl DNA sample and input sample respectively; the compensatory factor (3.32) is used to take into account the dilution 1:10 of the input.

To achieve qPCR for highlighting regions on chromosome 21 in the fetal methylated DNA were used eight pairs of specific primers chromosome 21, primers for hypermethylated region on chromosome 13 and hypomethylated region on chromosome 22, according to the proto-
The reaction for PCR was carried out using a device PalmCycler™ (Corbett, Lifesciences / Qiagen, Germantown, MD, USA) and the parameters of amplification were: pre-incubation (95°C/7 min), followed by 40 cycles of amplification (95°C/15 sec with ramp rate of 4.4°/sec and 60°/1 min with ramp rate 2.2°/sec; fluorescent acquisition was done at

Table 2. The values of ΔCT for markers of chromosome 21

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Table 3. The values of normalized ΔCT and for median of normalized ΔCT for markers of chromosome 21

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Median for normalized ΔCT

| 0.042714 | 0.06464 | 0.00286 | 0.02818 | 0.01912 | 0.04373 | 0.00498 | 0.01144 |

T21 – trisomy 21; N – normal

col described by Papageorgiou et al in 2011 (23). Primers were reconstituted according to the standard protocol. For EP4 and EP6 primers was used a concentration of 900nM, for EP5 primer was used a concentration of 450nM, for EP7 primer was used at a concentration of 750nM, while the other primer’s concentrations were 300nM.
465-510), melting (95°C/15 sec with ramp rate 4.4°C/sec, 65°C/1 min with ramp rate 1°C/sec, fluorescence acquisition was made at 465-510), cool (40°C/30 sec, ramp rate of 2.2°C/sec).

The amplification program used a temperature of 60°C for hybridization for all mixtures. In the mix of EP 4 at this temperature were obtained primer dimer thereby making it difficult to interpret. For mix EP4, application of a hybridization temperature of 64ºC eliminated the nonspecific products, so for all mixes we got a single melting peak, which allowed the comparison samples. Samples 1 and 7 were not considered because was not obtained amplification for Input nor controls or for sets of primers used for the analysis of differentially methylated regions on chromosome 21.

Results

For assessment of the results the following parameters were calculated: ΔCT for normal and trisomic pregnancies, Normalized ΔCT for normal and trisomic pregnancies, the ratio value per sample and ratio value per DMR, and the amount of discrimination D. For this we used the formulas:

\[ \Delta CT_{PB}^{Normal} = CT_{PB}^{Normal} \text{IMP} - CT_{PB}^{Normal} \text{IP} \]

\[ \Delta CT_{PB}^{T21} = CT_{PB}^{T21} \text{IMP} - CT_{PB}^{T21} \text{IP}. \]

(where PB=Peripheral Blood, T21= Trisomy 21, IMP – Input; IP= Immunoprecipitated)

\[ \text{Norm ΔCT value}^{PB} = E \Delta CT_{PB}^{Normal} \]

(where Norm= Normalized)

\[ \text{Norm ΔCT value}^{PB} \text{ T21} = E \Delta CT_{PB}^{T21}, \]

(where E= 10 (1/slope) = efficiency of the primer)

\[ \text{Ratio Value Sample} = \text{DMR} = \text{Norm ΔCT}_{PB}^{Sample} / \text{Median (Norm ΔCT)}_{PB}^{Normal} \]

\[ D = -6.331 + 0.959 X_{EP4} + 1.188 X_{EP5} + 0.424 X_{EP6} + 0.621 X_{EP7} + 0.028 X_{EP8} + 0.387 X_{EP9} - 0.683 X_{EP11} + 0.897 X_{EP12}, \]

(ωhere X_{EPi}= fraction value for the 8 markers used: 4, 5, 6, 7, 8, 10, 11 și 12 – Supplementary Table 1).

Applying the above formulas we obtained the results shown in Supplementary Tables 2, 3, and in Tables 2, 3, 4.

For better highlighting the results we achieved the Figure 2, which reflects the graphical
representation of value D for the 10 cases analyzed.

All pregnancies without trisomy 21 were correctly identified by obtaining a value of negative discrimination, the value of D ranging between -1.74 and -6.32.

In five of the six samples with trisomy 21 considered in the final analysis, we discovered a correlation between the presence of trisomy and positive value of factor D: sample 2 – D = 1.56; sample 3 – D = 1.41; sample 4 – D = 12.40; sample 5 – D = 0.34, sample 11 – D = 1.02.

The only case in which we found negative values of factor D was sample 6 - D = -2.96, which was inconsistent with the presence of trisomy.

Discussions

The results of our experiments confirmed the high specificity of this new method for testing the presence of 21 trisomy by a non-invasive diagnosis. Thus, we obtained a good correlation between the results of analysis of methylation of free fetal DNA from maternal blood and the results of prenatal diagnosis (by FISH method or by conventional karyotype). In all pregnancies with normal fetus, we obtained a negative value for discrimination factor D, concordant with presence of 2 chromosome 21. On the other hand, the analysis of methylation of fetal DNA showed positive values for D factor in 5 from 6 probes from pregnancies with 21 trisomy. These results are concordant with an excess of hypermethylated fetal DNA, characteristic to aneuploidy.

Our study indicated that analysis of methylation status of fetal DNA from maternal blood could have a practical value, allowing a good discrimination between a normal pregnancy and a pregnancy with 21 trisomy’s foetus. These data are concordant with studies of Papageorgiou et al. (23, 24). Although our study considered only ten probes – six from pregnancies with 21 trisomy and four from normal pregnancies – we found two positive aspects. First of all, in normal pregnancies the values of D factor were negatives (concordant with absence of an excess of hypermethylated fetal DNA) and this indicates the absence of false positive results with a 100% specificity. The second aspect was the good detection of 21 trisomy – five from six cases of pregnancies with 21 trisomy were confirmed by analysis of methylation status of fetal DNA. Thus, although the group is not statistically significant, we can say that the sensitivity of the method is quite high, correlated with the presence of a single false negative result. The presence of a sensibility less than 100% was observed also by Tong et al, that indicated a negative value of D factor in two of three cases of 21 trisomy analysed by same method (25).

The main aim of our study was to verify the reliability of quantification by immunoprecipitation of methylated fetal DNA in maternal blood in prenatal diagnosis of 21 trisomy and we had some limitations correlated with type of sampling collection. Thus, we perform the non-invasive prenatal test on blood samples providing from women with knowing fetal chromosomal formula: 7 with trisomy 21 and 5 with nor-
mal fetus. The cohort is very small and the obtaining values for sensibility and sensitivity of method may differ for real values. Thus, it is impossible to estimate a positive or negative predictive value of the test, but such a problem was identified in any type of method for detection of fetal DNA in maternal blood (26-28).

In light of the above findings, it is clear that the validation of the method required several conditions. The first condition was to apply the method on a larger number of unselected cases, allowing the obtaining of statistically validated results. In addition, by finding a correction factor could smoothing the positive and the negative results, which enables a better discrimination between the two type of probes, with increasing the sensitivity and specificity of the method near 100%.

Conclusions

Our study confirmed the reliability of noninvasive prenatal diagnostic method based on the comparison of the methylation status of maternal versus fetal DNA, which could be a future alternative to current invasive prenatal diagnostic methods marked by various incidents, the most serious being miscarriage.

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Abbreviations

AE = amplification efficiency
AFP = Alpha-fetoprotein
AMA = advanced maternal age
bp = base pairs
CT = cycle threshold
DMR = Differentially methylated regions
DS = Down syndrome
DTP = patological double test
FACS = Fluorescent activated cell sorting
FISH = Fluorescence in situ hybridization
free β-hCG = Free human chorionic gonadotropin
GAPDH-glyceraldehide3-phosphate dehydrogenase
IMP = Input;
IP = Immunoprecipitated
MACS = Magnetic cell sorting
MLPA = Multiplex ligation-dependent probe amplification
NPD = Noninvasive prenatal diagnostic
PAPP-A = Pregnancy-associated plasma protein
PB = Peripheral blood,
PCR = Polymerase chain reaction
PD = Prenatal diagnostic
PLAC4-placenta specific 4 gene
QF-PCR = Quantitative-fluorescent polymerase chain reaction
q-PCR = Quantitative real time polymerase chain reaction
T21= Trisomy 21,
TSH2B=testis-specific H2B histone gene
TTP =patological triple test
UE3 = Unconjugated estradiol
$X_{Epi}$ = fraction value for each marker
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