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NON-INVASIVE PRENATAL DNA SCREENING USING HIGH-THROUGHPUT SEQUENCING IN PREGNANT WOMEN WITH RECURRENT MISCARRIAGE

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Aim. *To investigate the effectiveness of non-invasive prenatal DNA screening (NIPS) for aneuploidy using high-throughput sequencing for the detection of trisomy 21, 18, 13 and fetal sex chromosomes in maternal plasma of women both with and without a history of recurrent pregnancy loss.*

Material and methods. *NIPS using the high-throughput sequencing was carried out and the results obtained from 600 pregnant women with recurrent miscarriage (n=270) and uncomplicated obstetric history (n=330). To confirm chromosomal aneuploidy (CA), karyotyping was used.*

Results. *After 12 weeks of gestation, the frequency of CA in each study group was 3.0% and did not differ between women with recurrent miscarriage and uncomplicated obstetric history. The sensitivity and specificity of NIPS in detecting the most frequent CA was 92% and 99%, respectively. Down syndrome was determined with 100% specificity.*

Conclusion. *The study finding showed that NIPS is accurate in screening for fetal CA from early pregnancy, which is especially important in patients with recurrent miscarriage since it spares them from invasive testing thus preventing iatrogenic complications.*

Keywords*: noninvasive prenatal DNA screening, screening, NIPS, aneuploidy, pregnancy, recurrent miscarriage, threatened miscarriage.*

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Studies of recent years have shown that about 60 % of sporadic and 12.5% of recurrent miscarriages are caused by aneuploidy. Evaluation of the embryo/fetus, including the diagnosis of chromosomal aneuploidy (CA) in women with threatened and recurrent miscarriage, is extremely relevant. Social conditions in the contemporary world allow women to delay childbearing and plan a pregnancy at an advanced maternal age, which increases the risk of fetal CA. Pregnancies with chromosomal abnormalities are often complicated by threatened miscarriage, recurrent bleeding, and pregnancy loss [1]. Accurate diagnosis of fetal CA offers an opportunity to choose a rational management strategy in women with a complicated pregnancy. At the same time, in women with threatened and recurrent miscarriage, invasive methods of prenatal diagnosis are difficult to perform and, without valid medical reasons, they are contraindicated. Although invasive testing is established as a diagnostic standard, it carries a risk of pregnancy loss in 1,0-2,0% of cases. Therefore, the invasive diagnosis should be carried out after careful justification, with detailed consideration of the screening results.

The most frequent aneuploidies associated with congenital malformations and developmental disabilities are autosomal trisomies involving chromosomes 21, 18, and 13. They affect one of every 650-1000, 6000-8000 and 10000-16000 newborns, respectively [2-4]. The risk of these fetal chromosomal abnormalities significantly increases in frequency with advancing maternal age. Partial aneuploidies may also cause Down's, Edwards', and Patau's syndromes in children with clinically healthy parents who carry balanced chromosomal translocations involving chromosomes 21, 18, and 13.

In the past few years, Russia implemented a new for prenatal examination, regulated by Government Decree No. 572 of 12.11.2012. It relies exclusively on biochemical and ultrasound measurements in the first trimester aimed to rule out late-manifesting fetal anomalies in the second trimester. Screening is based on indirect markers and has limited sensitivity and specificity; fluctuations in blood biochemistry depend on both the fetal chromosomal status and the maternal hormonal balance. As a result, combined screening of the first trimester (combination of biochemical testing and ultrasound examination), even with strict observance of timeliness of the examination detects only about 80% of pregnancies with fetal trisomy 21 that need to be assigned to a risk group for subsequent invasive diagnosis [5, 6].

Therefore, there is a need to develop and introduce into clinical practice a more advanced non-invasive screening modality providing greater accuracy in identifying women with high risk for fetal anomalies.

The most promising in this direction seems to be the non-invasive prenatal DNA screening for aneuploidy (NIPS), based on the analysis of cell-free fetal DNA in maternal blood (from now on "DNA screening") [7, 8]. Free fetal DNA circulating in maternal plasma originates mainly from apoptotic trophoblasts in the placenta [9]. The amount of circulating free fetal DNA increases with gestational age, and starting from 10-11 weeks gestation it is sufficient to be detected by molecular testing. This provides an opportunity to use maternal blood for noninvasive prenatal DNA screening for fetal aneuploidy, including in multiparous women [10-14].

Cell-free fetal DNA analysis of maternal plasma can be performed using high-throughput sequencing techniques [15, 16]. At present, this method is becoming more widely accepted in laboratory practice for solving clinical problems. High-throughput sequencing of the total cell-free maternal and fetal DNA with subsequent analysis using powerful bioinformatics tools allows for the possibility to tally the total number of fragments per chromosome and diagnose fetal chromosomal aneuploidy. This fundamentally new approach in prenatal screening can be an effective tool for identifying fetal CA in pregnant women. Numerous studies [13, 17-21] reported a high diagnostic performance of the method, which, however, may differ depending on the studied chromosomes. From now on, the diagnostic characteristics are given with a 95% confidence interval according to the "Rules for assessing the clinical performance of laboratory tests. GOST R 53022.3-2008".

Given the high accuracy of the NIPS, it is important to assess its clinical value in women with recurrent miscarriage.

This study aimed to investigate the effectiveness of NIPS using high-throughput sequencing to detect trisomy 21, 18, 13, and fetal sex chromosomes in maternal blood of women with recurrent miscarriage and complicated course of the current pregnancy.

Material and methods

The study comprised 600 pregnant women at 11-16 weeks and six days gestation with (n=270) and without (n=330) a history of recurrent miscarriage. Diagnostic evaluation included detailed medical history, physical examination, and the first-trimester screening (maternal serum marker tests and ultrasound evaluation of the fetus). When selecting patients, the limitations of the method were taken into account: oncological diseases in pregnancy; multiple-gestation pregnancy, including cases of the spontaneous demise of one of the fetuses; the body mass index greater than 30 kg/m2.

The study was carried out at the Department of Clinical and Molecular Genetics of the V.I. Kulakov NMRC for OGP. Samples of maternal peripheral blood (10 ml EDTA) of 600 singleton pregnant women were analyzed. Information was provided for each blood sample, the patient's code was defined automatically, taking into account the age of the woman, the gestational age, the date of blood sampling. All women participating in the study gave written informed consent for the use of their blood samples.

The analysis of circulating cell-free DNA was performed using high-throughput semiconductor sequencing. Maternal blood samples were tested for the presence or absence of fetal chromosomal aneuploidy and fetal sex as follows. At the first stage, plasma was separated from the maternal blood. In the second stage, cell-free DNA containing the maternal and fetal fractions was isolated. After that, DNA libraries for high-throughput sequencing, emulsion polymerase chain reaction (PCR), and sequencing on the Ion Proton (Life Technologies, USA) were sequentially prepared. Bioinformatics analysis of circulating cell-free DNA sequencing data was performed to evaluate the results. The risk of aneuploidy was assessed using the software developed by the authors.

Results

All women participating in the study were able to complete their pregnancies, and we analyzed the pregnancies' course and outcomes.

Clinical data and health history of pregnant women included in the study based on the above criteria were analyzed. The analysis included careful examination of patients' demographics such as age, socioeconomic status, occupational hazards, heredity; extragenital and gynecologic comorbidity, menstrual function, and a reproductive anamnesis.

The mean age of women in the whole cohort, study groups with and without recurrent miscarriage was 34.9 ± 5.5, 33.9 ± 5.5, and 35.1 ± 5.5 years, respectively. The distribution of women by age is presented in Table 1.

Table 1. Distribution of the study participants by age

Age	With recurrent miscarriage, *n*=270		Without recurrent miscarriage, *n*=330		*p*
(years)	*n*	%	*n*	%	
18–24	13	4,8	9	2,7	0,19
25–29	41	15,2	56	17,0	0,55
30–34	100	37,0	91	27,6	0,01
35–39	78	28,9	113	34,2	0,16
40–44	37	13,7	51	15,5	0,54
45	1	0,4	10	3,0	0,01

In the group of women with recurrent miscarriage, there were more participants in the 30-34 years maternal age group, while among women without recurrent miscarriage there were more participants aged 45 years.

All women lived in similar climatic conditions, mostly in Moscow and the Moscow region, had secondary and higher education. None of them had a history of professional hazards.

The results of cell-free embryonic DNA (cfDNA) analysis of the maternal blood were obtained for 600 women. In 12 women (2%), the result was not initially obtained due to an insufficient amount of cfDNA (less than 4%) in the sample. Repeat samples of the venous

blood were collected from 10 pregnant women and showed an increased cfDNA fraction; therefore the results were finally obtained. In 2 women (0.3%) the results were not obtained; in these cases, an invasive testing (amniocentesis) was performed, normal fetal karyotype was detected. The mean level of cfDNA in the 12 women mentioned above was 2.3%, in contrast to the results of other study participants (11.1%).

After the combined screening, women who were found to have high risk of fetal CA were strongly recommended to undergo invasive testing followed by karyotyping. They refused it and signed refusal forms in their medical cards.

For research purposes, taking into account all the necessary conditions for the test, the study participants were offered NIPS with a mandatory explanation of the benefits and limitations of the method. After that, NIPS was conducted with the informed consent of participating women. The interpretation of the results was carried out by a geneticist. In all cases, when a change in the pregnancy management strategy was required, the patients were referred to an invasive testing (amniocentesis) followed by karyotyping.

Eighteen women (3%) with high risk of Ca identified by NIPS were recommended to undergo invasive prenatal testing (Table 2). Only in 5 out of 53 women (9.4%), who were at high risk according to the results of the combined screening, high risk was confirmed by NIPS; in all cases CA was confirmed by karyotyping. While among 547 low-risk women, according to combined screening, NIPS identified 13 patients with high risk of aneuploidy. In all cases, trisomies 21 and 13 were confirmed by karyotyping. In cases of monosomy X, the data were inconsistent due to mosaicism.

In a comparative analysis of the results of combined screening and NIPS in different subgroups of patients, it was found that in the group with recurrent miscarriage, among 24 women with high risk according to combined screening, only 2 had high risk identified by NIPS. In the group without recurrent miscarriage, 3 out of 29 women had positive NIPS results. In the group of low individual risk, six women with high risk of aneuploidy were identified by NIPS (Table 3) based on the results of combined screening in the group of recurrent miscarriage. Out of 301 women without recurrent miscarriage at low risk according to combined screening data, seven women had high risk established by NIPS.

Patients with recurrent miscarriage and low risk of CA, as calculated by the ASTRAIA software, significantly more often had abnormal levels of β-hCG, which is probably due to placental implantation abnormalities in pregnant women with recurrent pregnancy loss.

Therefore, NIPS identified 18 patients at high risk of CA, including 9 cases of trisomy 21, 3 cases of trisomy 13 - 3, and 6 cases of sex chromosomes.

The course and outcomes of pregnancies in women in the study groups were analyzed. Compared with women without recurrent miscarriage, pregnant women with

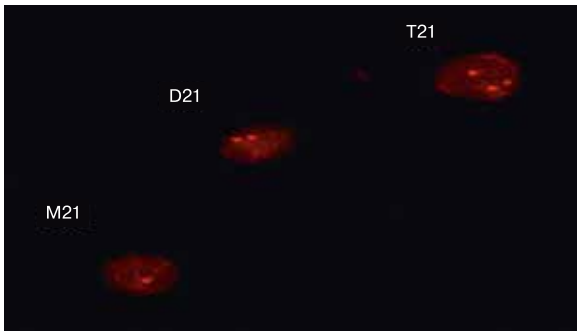
Table 2. Comparative analysis of the results of combined screening and NIPS

	With recurrent miscarriage, n=270		Without recurrent miscarriage, n=330		p
	n (%)	Positive NIPS test	n (%)	Positive NIPS test	
High individual risk (ASTRAIA software)	24 (8,8)	2 (0,7) 47,XX+21 47,XX+21	29 (8,8)	3 (0,9) 47,XX+13 47,XY+13 45,X	0,96/ 0,81
Low individual risk (ASTRAIA software)	246 (91,1)	6 (2,2) 47,XX+21 45,X 47,XX+21 45,X 47,XY+21 45,X	301 (91,2)	7 (2,1) 47,XY+21 47,XX+21 47,XY+13 45,X 47,XY+21 45,X 47,XX+21	0,96/ 0,93

Table 3. Analysis of the results of NIPS in different subgroups of patients at low risk according to ASTRIA

Combined screening markers	With recurrent miscarriage, *n*=270		Without recurrent miscarriage, *n*=330		*p*
	n (%)	Positive NIPS test	n (%)	Positive NIPS test	
Nuchal fold thickness (⩾3 mm)	7 (7,6)	1 (0,4) 47,XX+21	11 (3,3)	2 (0,6) 47,XY+13 45,X	0,83 14,3%/18,2%
Deviation of β-hCG and PAPP-A levels from normal values	41 (15,2)	1 (0,4) 45,X	63 (19,1)	2 (0,6) 45,X 47,XX+21	0,82 2,4%/3,2%
Isolated change in β-hCG values	75 (27,8) P=0,001	1 (0,4) 47,XY+21	45 (13,6)	1 (0,3) 45,X	0,73 1,3%/2,2%
Isolated change in the PAPP-A values	48 (17,8)	1 (0,4) 45,X	59 (17,9)	1 (0,3) 47,XX+21	0,88 2,1%/1,7%

Figure. Three clones in peripheral blood cells of a newborn (FISH)



recurrent miscarriage had a significantly higher incidence of gestational complications: threatened miscarriage (31.9% vs 13.6%, p⩽0.0001), early premature birth (31.9 vs 13.6%, p⩽0.0001), retroplacental and extraovular hematomas, second trimester bleeding (22.2 vs 7.6%, p⩽0.0001), isthmic-cervical insufficiency (42.6 vs 17.9%, p⩽0.0001), and premature birth (8.1 vs 2.4%, p = 0.0023). In this regard, invasive diagnostic testing in pregnant women with recurrent miscarriage should be limited to the approved indications.

Among 15 women, who underwent invasive diagnostic testing, aneuploidies were confirmed in 12 patients. Of them, ten women underwent abortion due to medical indications, while two patients refused to terminate their pregnancies and gave birth to live children with Down syndrome and Shereshevsky-Turner syndrome (monosomy X).

In 3 cases the patients refused to undergo invasive testing; all these cases were associated with monosomy X. These patients were monitored until completion of pregnancy, and outcomes were analyzed. In 2 cases, the karyotype was confirmed, and in 2 cases there was a false positive result for monosomy X, which was caused by placental mosaicism [12, 13, 15-25]. Trisomies 13 were diagnosed; however, due to single observations on these chromosomes, the sensitivity and specificity of the method in the described case cannot be defined. Medical indications for pregnancy termination were aneuploidy, fetal malformations, and maternal conditions. All cases of multiple fetal malformations were observed in the group without recurrent miscarriage.

The frequency of aneuploidy after 12 weeks of gestation did not differ significantly between the group of women with (3.0%) and without (3.0%) recurrent miscarriage (Table 4).

One false negative result of NIPS related to mosaic form of trisomy 21 was due to fetal mosaicism. FISH test of peripheral blood cells of the newborn allowed the detection of 3 different cell clones. In addition to normal and trisomal cells, a small number of monosomal cells were detected (Fig. A repeat testing of the child at 2 months of age showed that the proportion of trisomic cells increased, and the proportion of monosomal and normal cells decreased (Table 5).

After birth, all newborns were evaluated phenotypically, and 547 (91.2%) of them had a normal phenotype.

Based on the study findings, the sensitivity, specificity, positive and negative prognostic values of the NIPS for different CAs were determined. The results are shown in Table 6.

The findings of our study support the recommendation to use NIPS in women with recurrent miscarriage, taking into account existing limitations.

Discussion

Currently, the common combined prenatal screening for fetal CA estimates only indirect markers, and therefore it has low sensitivity and specificity [5-7]. Invasive testing has high accuracy and is considered the gold standard for prenatal genetic testing. However, it carries a risk of post-procedural miscarriage, estimated to be about 0.5 – 2.0 % of the general female population and higher among women with a history of recurrent mis-

Table 4. Assessment of the frequency of CA in women in the study groups after 12 weeks gestation

Aneuploidy	With recurrent miscarriage, *n*=270	Without recurrent miscarriage, n=330
Trisomy 21	5 (1,9%)	4 (1,2%)
Trisomy 13	-	3 (0,9%)
Monosomy X	3 (1,1%)	3 (0,9%)
Total	8 (3,0%)	10 (3,0%)

Table 5. Mosaic false negative specimen (child blood FISH test)

Clones of cells	Immediately after birth	At the age of 2 months
T 21	77 cells (76%)	256 cells (85%)
M 21	6 cells (6%)	5 cells (2%)
D 21	18 cells (18%)	56 cells (13%)

Table 6. Accuracy of the NIPS for various CAs

Aneuploidy	Sensitivity	Specificity	PPV (positive predictive value)	NPV (negative predictive value))
Trisomy 21	89%	100%	100%	99%
Trisomy 13	100%	100%	100%	100%
45, X0	100%	99%	37%	100%
Total	92%	99%	76%	99%

carriage [9, 26]. In women with recurrent miscarriage, whose pregnancy proceeds with bleeding and signs of possible miscarriage, the decision to offer invasive testing must be strictly scrutinized.

In our study, NIPS was compared with combined screening in groups of women with and without recurrent miscarriage. The choice of women with recurrent miscarriage was because they have a higher incidence of obstetric complications and supposedly higher incidence of CA. After 12 weeks of gestation, the frequency of CA in each study group was 3.0% and did not differ between women with recurrent miscarriage and uncomplicated obstetric history. In this regard, it becomes clear that after 12 weeks of gestation, the frequency of CA is similar among women with and without recurrent miscarriage. Therefore, high incidence of gestational complications among women with recurrent miscarriage is due to placenta-related problems. The study findings suggest the need for careful evaluation of indications for invasive testing in women with recurrent miscarriage.

The analysis of the rate of false-positive results combined the screening of the first trimester showed that out of 53 patients with high risk of CA according to the ASTRAIA program, only 5 had CA. Therefore, 48 women with a normal fetal karyotype could undergo invasive testing. The rates of false positive results of combined screening were similar among women with (8.8%) and without (8.8%) recurrent miscarriage.

Particularly noteworthy is that among 547 women, who, according to conventional screening, had low risk, NIPS identified 13 patients with high risk of aneuploidy. With the conventional prenatal examination, the above-mentioned pregnant women would not have been offered invasive testing and could not have had the correct diagnosis.

Low-risk women with recurrent miscarriage had significantly higher levels of β-hCG ($P = 0.001$), which is indicative of abnormalities of placental processes, but not fetal CA.

Though having high specificity, NIPS has some limitations. While the results of NIPS are highly accurate, false-positive or false-negative results may occur due to placental, maternal, or fetal mosaicism. In our study, the false negative result of NIPS occurred due to the presence of 3 pools of fetal cells – disomic, trisomic, and monosomal, which together indicated a low risk of CA. Such limitations should be taken into account, and patients should be notified of them during genetic counseling. At the same time, the leaders of international research teams agree that prenatal genetic counselors should provide pregnant women information about the opportunities, limitations, and benefits of NIPS allowing them to choose a screening program.

Based on the results of this study, the use of high-throughput sequencing for non-invasive screening on aneuploidy 21, 18, 13 and sex chromosomes can be recommended, followed by invasive testing in women identified as having a high risk.

The method can be used to screen and determine the risk in all pregnant women before the final diagnosis is made, as an independent method, and in addition to conventional methods.

Researchers believe that NIPS can eventually replace or improve existing screening tests [12, 19, 27-30]. Similar conclusions were drawn based on the results of several prospective studies, in all cases confirmed by classical karyotyping [27, 28]. The findings of our study suggest that in women with recurrent miscarriage, NIPS can reduce the level of anxiety in parents, improve strategies for the management of complicated pregnancy without prolonging it if CA is detected, and most importantly, to spare women with recurrent miscarriage from unnecessary invasive testing that carries a high risk of iatrogenic complications.

Conclusion

This study showed that NIPS is a promising method for screening for fetal CA in women with recurrent miscarriage that can be recommended as a highly effective prenatal test.

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