In this issue: Non-coding RNAs as potential laboratory biomarkers

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Cell-free nucleic acids in prenatal diagnosis and pregnancy-associated diseases

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ABSTRACT

There is a great effort to find out the biological role of cell-free nucleic acids (cfNAs). They are considered very promising targets in the diagnosis of genetic diseases. Non-invasive sampling (liquid biopsy) has recently become a very popular method, and new molecular biological techniques have been developed for these types of samples. Application of next-generation sequencing (NGS) and massively parallel sequencing (MPS) is spreading fast. These are the part of the arsenal of the modern prenatal genetic diagnostic laboratories by now. Cell-free DNA based non-invasive prenatal testing accounts for more than half of the prenatal genetic tests performed, it is gradually replacing the invasive amniocentesis or chorionic villus sample-based diagnostics. Besides that, new non-coding RNAs are taking more attention: microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) are in the focus of the clinical research to detect the most common pregnancy-associated diseases, like preeclampsia, fetal growth restriction, congenital heart diseases and gestational diabetes. The research is at advanced stage on the

use of microRNAs, while lncRNAs and circRNAs are still promising targets. In this review, comprehensive information is given about the recent developments on this field.

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INTRODUCTION

There is a great effort to determine the biological role and the clinical applicability of the cell-free nucleic acids. These molecules could be DNA, mtDNA, mRNA, miRNA, lncRNA, circRNA and other nucleic acids. They are present in different body fluids and offer the possibility to use them in diagnosis of different diseases. Liquid biopsy became very popular sampling method recently. The first non-invasive method in prenatal diagnosis was introduced by Dennis Lo from the University of Hong Kong, he was able to detect the fetal sex and RhD blood group in 1997 (1). He introduced the real-time PCR technique at that time for that purpose. Researchers tried to detect genetic diseases from maternal plasma applying similar technique (trisomies), but they were not very successful. There was a real breakthrough in 2011, when the first report was published on determination of trisomy 21 using massively parallel sequencing (MPS) (2). Newer genetic diseases were then detected prenatally. The results of a special interesting prenatal case called the attention for the possibility of diagnosis of oncological diseases by this technique. The next-generation sequencing (NGS) reading pattern warned for the mother's hemato-oncological disease, which was not diagnosed yet (3). This observation opened the door for new clinical applications of MPS in the field of oncology, and later for cardiovascular diseases, neurological diseases, infectious diseases, etc. The application of cfDNA is already

Table 1 The type of cell-free nucleic acids molecules with present and possible prenatal diagnostic application

Full name	Abbreviation	Size	Function	Prenatal application
Genomic DNA	gDNA	166 - >10,000 bp	unknown	trisomy, mutation, deletion, microdeletion
Mitochondrial DNA	mtDNA	20-100 bp; <1 – 21 kbp	unknown	preterm prelabour rupture
Messenger RNA	mRNA	varies	coding	not used
MicroRNA	miRNA	18-25 bp	regulation	preeclampsia, congenital heart diseases, gestational diabetes
Circular RNA	circRNA	varies	regulation	congenital heart diseases, gestational diabetes
Long non-coding RNA	lncRNA	over 200 bp	regulation	congenital heart diseases

a success story in prenatal diagnosis of genetic diseases, while there are other non-age-related pregnancy-associated diseases, which cause high maternal and fetal mortality and morbidity. It seems that non-coding RNAs could help to solve these problems. Table 1 shows those cell-free nucleic acids that have prenatal diagnostic potential.

CELL-FREE FETAL DNA (cffDNA)

The presence of cell-free DNA (cfDNA) was observed by Mandel and Metais in the sera of cancer patients in 1948 (4). Tan *et al.* reported a higher concentration of cfDNA in samples of cancer patients in 1966 (5). Later the first clinical application was introduced by Leon *et al.*, but did not get larger attention because of technical reasons (6). They measured the concentrations of cfDNA in blood samples from oncological patients. The real clinical application started when Dennis Lo detected the fetal gender and RhD group in the maternal plasma in 1997 (1). The maternal blood contains cfDNA molecules which originate in 90-95% from the mother and in 5-10% from the fetus. Introduction of the massively parallel sequencing made wider prenatal clinical application possible from 2011 (2). The number of the performed non-invasive prenatal tests is growing steadily, at least 50% of the genetic tests are made by this method nowadays. They have a very high sensitivity and specificity. There are several reports showing high number of non-invasive tests performed in different countries (7,8). It is possible to detect trisomies, mutations, deletions, etc. with NGS.

However, there are a few drawbacks in non-invasive prenatal testing (NIPT). Low fetal DNA content in those patients who are having high body mass index (BMI), or in the case of early pregnancy, these could cause false negative results. Placental mosaicism, vanishing twin could cause false negative or false positive results. These factors should be considered during the evaluation of the NIPT results.

CELL-FREE FETAL MITOCHONDRIAL DNA (cffmtDNA)

Higher level of nuclear DNA (nDNA) was observed in several pregnancy-related complications like in preeclampsia, fetal growth restriction and preterm delivery. Little is known about cfmtDNA in these syndromes. Recently, Kacerovsky *et al*. studied the nDNA and mtDNA levels in amniotic fluid samples obtained from preterm prelabor rupture of membrane cases (9). They observed higher levels of these DNA molecules in these cases. They suppose these are connected to the intra-amniotic inflammatory response. There are only a few studies related to the mtDNA and prenatal diagnosis of diseases.

CELL-FREE RNA (cfRNA)

mRNA

According to the latest results, there are about 23,000 genes in the human genome, which encode several types of RNAs, including mRNAs. Different tissues have their own mRNA profile. They are present in the serum and in other biological fluids and could be measured. During the pregnancy, placental markers are detectable in the maternal circulation. A paper was published on the application of single nucleotide polymorphism (SNP) for the detection of trisomies using allelic ratio of specific heterozygous SNP on cffmRNA. There are altered ratios in the case of trisomies, 1:1 shows diallelic, while 1:2 or 2:1 shows trisomic sample (10). Somehow mRNAs are not used in prenatal diagnosis of genetic diseases as yet.

miRNAs

miRNAs are short ribonucleic acid molecules with the size of 18-25 bp. They belong to the non- coding RNAs, produced from longer precursors. They

play a pivotal role in gene regulation. miRNAs are present in different biological fluids (plasma, liquor, saliva, seminal fluid, etc.) and they are considered as ideal molecules from the laboratory point of view: they are stable following freezing and thawing cycles, and it does not have an effect on their quality and concentration. Their encapsulation into extracellular vesicles during apoptosis and necrosis allows these stabilized miRNAs to reach any part of the body. The other possible way for their transport, formation of macromolecule complexes with Argonaute2 (Ago2), LDL and HDL (11).

Genomic studies identified several hundreds of miRNAs in the placenta (12), some of them expressed only in that tissue, while some in other tissues. Their role and function are not well known, probably they take part in the regulation of placentation (13).

Most key molecules from the biogenesis of miRNAs are detectable in the placenta (14, 15, 16). Placenta specific miRNAs appeared in the latest time of the evolution and they are present only in mammals (17). They are expressed differently in the certain parts of the placenta and secreted from the trophoblast layer in different concentrations during the periods of pregnancy (18, 19). This concentration depends on the signal transduction cascades and environmental factors (hypoxia, oxidative stress, etc.) (13).

There are placenta-specific miRNAs that are not expressed in other tissues, these are located on chromosome 14 and 19 in clusters (C14MC, C19MC and miR-371-3). The C14MC includes 34 mature miRNAs and these are evolutionarily conserved in mammals having placenta (20). The C19MC has 46 different spin-like structure miRNAs and from these 59 mature miRNAs are formed, this is the biggest known cluster in placental mammals (21). Both clusters are imprinted, while they show altered expression during the pregnancy. The C14MC miRNAs expressed from the maternal allele and their level is the highest in the first trimester and it decreases later (21). The C19MC is the opposite, the paternal allele is active (22) and the expression is increasing during the pregnancy (21). It is detectable even in the maternal circulation (23, 24). Less is known about the miR-371-3 cluster, which is located also on chromosome 19 (25).

They are expressed in the placenta and in embryonic stem cells (26). There is a great effort to find out the biological function of miRNAs and their diagnostic applicability in clinical practice. They could be classified as placenta specific, placenta-associated and placenta-derived miRNAs (27).

There are pregnancy-related complication and an intensive research performed to find out the utility of miRNAs in the diagnosis of preeclampsia, congenital heart diseases, gestational diabetes and fetal growth restriction.

PREECLAMPSIA

Preeclampsia is a serious pregnancy-associated disease and occurs in about 3-5% of the pregnancies. This is the main cause of maternal, neonatal morbidity and mortality. There is no reliable biomarker for the prediction of the development of this disease. There are numerous publications on the determination of miRNA expression in preeclampsia, even from the Central-Eastern European region several groups performed active research in this field (28). There is an agreement that placental dysfunction is the main cause of the development of this disease, while the pathogenesis is not clearly understood yet. Genetic predisposition, immune factors, and inflammation related causes are well studied. A number of research groups reported abnormal expression of miRNAs in the pathophysiological process of the disease (29, 30). These are involved in metabolic changes, immune function, cell adhesion, cardiovascular development, etc.

miR-210 is widely studied in preeclampsia as it is proved to be induced by hypoxia. It is upregulated in various tumors and cardiovascular diseases, and similarly in pregnancies with preeclampsia. miR-155 is upregulated along with transcription factor 1 and NF-κB protein, it may also inhibit trophoblast proliferation and invasion (30).

Skallis *et al.* published a review recently on miRNAs in preeclampsia. They divided their effect according to play a role in impaired trophoblast migration and invasion (miR-195, miR-276C, miR-278a-5p, miR-210), impaired angiogenesis (miR-210, miR-21, miR-22) and dysregulation of maternal immune system (miR-223, miR-148a, miR-152) (31).

CONGENITAL HEART DISEASES (CHD)

This is the most common congenital malformation with an incidence of 4-5% in the general population (32). The exact etiology of this disease group is not known yet. CHD causes a serious health issue accounting for 30–50% of mortality among newborns and infants (33). Unfortunately, the misdiagnosis is very high besides the use of fetal ultrasound echocardiography, the diagnostic efficiency is about 6-35% (34). There are other not very specific biomarkers in the clinical practice, like acylated ghrelin, beta human chorionic gonadotropin and pregnancy-associated plasma protein A (PAPP-A). Early prenatal diagnosis of CHDs may reduce postnatal morbidity and mortality (35-39). Zhu et al. performed a SOLiD sequencing for comparison of miRNA profile from women having a fetus with CHD and healthy pregnant women. These were ventricular septal defect, atrial septal defect, or teratology of Fallot cases. They found miR-19b, miR-22, miR-29c, and miR-375 significantly upregulated in the patient group (40). Our research group found elevated miR-99a level as a possible biomarker for the detection of CHD by analyzing maternal plasma samples (41). The miR-99a/let7c miRNA cluster is located in the chromosome region 21q21.1 and has been shown to control cardiomyogenesis in embryonic stem cells (42). We analyzed also let-7c expression in the maternal circulation and found that similarly to miR-99a, it is also overexpressed in cases of fetal cardiac malformations (43). CHDs are the most common cause of birth defects; however, present prenatal screening methods are not able to detect high-risk cases effectively. MiRNA studies in the maternal circulation could improve the efficacy of diagnosis and give new opportunities for CHD research and diagnosis.

GESTATIONAL DIABETES (GDM)

Another serious pregnancy-related complication is gestational diabetes (GDM). Early and effective diagnosis of the disease is an urgent need. About 7% of pregnancies effected by GDM and the number of cases growing year by year (44). Even some calculations predict 25% prevalence in the USA (45, 46). The screening of GDM is performed between the 24th-28th gestational weeks all over the world. Naturally it means late diagnoses, so the treatments usually do not start before the end of the first trimester. Screening strategy involving earlier detection could help in the proper diagnosis and treatment of the GDM.

Irregular expression of circulating miRNAs has been associated with GDM. They could serve as potential early biomarkers. The miR-518d was the first recognized miRNA showing altered expression in GDM, it belongs to the C19MC cluster (47). It seems that this miRNA regulates peroxisome proliferator-activated receptor α (PPARα) gene. Microarray analysis performed on GDM and non-GDM placentas showed dysregulation of miR-508-3p, miR-27a, miR-9, miR-137, miR-92a, miR-33a, miR-30d, miR-362-5p and miR-502-5p. Interestingly these miRNAs

target genes involved in the epidermal growth factor receptor (EGFR) signaling, which could cause e.g. macrosomia (48). Wander et al. reported recently an altered expression of 10 microRNAs, including miR-155-5p and miR21-3p, which showed higher plasma levels in GDM (49).

More extensive research is needed on microRNAs to introduce them as biomarkers in the early GDM diagnosis.

Circular RNAs (circRNAs)

Circular RNAs are special non-coding RNAs with evolutionary conservation, structural stability, and tissue specificity. They act like miRNA sponges and regulate the expression of different genes (50). CircRNAs are present in the placenta and could be involved in pregnancy related pathological processes (51). They have role in the development of tumors and other diseases (52, 53).

A recently published study measured the level of three lncRNAs in GDM and combined these with the expression of 99 miRNAs, however, circ_5824, circ_3636 and circ_0395 levels were significantly lower in GDM (54). CircRNAs could be other interesting molecules for functional studies in GDM.

Long non-coding RNAs (lncRNAs)

LncRNAs are a kind of non-translating RNA having the length of over 200 nucleotides. They are stable in plasma and other biological fluids; they show disease and tissue specificity. There are more than 1,000 lncRNAs which are involved in different biological processes (55). Recent studies call attention for their potential role as biomarkers or prognostic markers.

LncRNAs have a role in the development of the heart and CHD related lncRNAs could be detected in placental tissues and even in the maternal circulation (31). Gu *et al.* performed a study on 62 CHD patients and 62 healthy controls by using microarray and determined 3694 up-regulated and 3919 down-regulated genes. They validated the CHD-associated lncRNAs and found *ENST00000436681*, *ENST00000422826*, *AA584040*, *AA706223* and *BX478947* suitable to use as biomarker (31). There is intensive research to find out the role and clinical applicability of lncRNAs.

CONCLUSION AND FUTURE PERSPECTIVES

Cell-free nucleic acids have a special role in the normal physiological processes and in the development of diseases. CfDNA was the first clinically applicable non-invasively obtained sample type which is already widely used in the prenatal detection of genetic diseases using NGS. The application of different cfRNAs are in experimental phase now, with research groups performing studies to find out their role and clinical utility, like miRNAs, lncRNAs and circRNAs. Cell free miRNAs have a potential diagnostic, prognostic and therapeutic applicability.

They are expressed in all cell types and changes in their expression patterns could call the attention for pathological conditions. We know less about lncRNAs and circRNAs they have a potential for clinical use in the next couple of years. Several pregnancy-associated diseases are in the focus of the research, like preeclampsia, gestational diabetes and congenital heart diseases. Epigenetic changes causing fetal-maternal complications is not well known, additional studies are necessary to provide insight into the molecular pathological mechanisms. There is a critical issue related to the lack of standardized protocols on sample processing, expression profiling, and data analysis.

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