MICRO-RNAs IN OBSTETRICS DIAGNOSTIC: ARE WE THERE YET?

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Abstract

MicroRNAs are small non-coding RNAs, involved in the post-transcriptional regulation of gene expression, intensely studied for their usefulness as biomarkers. Their high stability in various biological fluids is one of the main rationale behind the attempts to introduce them in clinical practice in general and, given the increased requirement of early, non-invasive diagnostics of pregnancy associated pathologies, in obstetrics in particular. Among these pathologies, pre-eclampsia was one of the most studied, for its major impact on fetal and maternal morbidity. In the present article, we review the data on circulating placenta-specific microRNAs, as well as their involvement in the pathogenesis of pre-eclampsia, with the aim of evaluating their possible role as non-invasive biomarkers.

Rezumat: Micro ARN-ul în diagnosticul obstetrical

MicroARN sunt molecule mici, non-codante de ARN, implicate în reglarea expresiei genice la nivel post-transcripţional, intens studiaţi pentru utilitatea lor ca biomarkeri. Stabilitatea lor crescută în lichide biologice argumentează pentru introducerea lor ca biomarkeri în practica clinică în general şi, în contextul necesităţii stabilirii unui diagnostic precoce şi neinvaziv al patologiilor asociate sarcinii, în obstetrică în special. În acest sens, una dintre cele mai studiate patologii asociate sarcinii este pre-eclampsia, din cauza morbidităţii materne şi fetale asociate. În acest articol, analizăm datele cunoscute cu privire la microARN specific-placentari, cât şi rolul lor în patogenia pre-eclampsiei, cu scopul de a evalua utilitatea acestora ca biomarkeri neinvazivi.

Cuvinte cheie: microARN, placenta, obstetrică, pre-eclampsie

Introduction

MicroRNAs are endogenous, small non-coding RNAs involved in the regulation of a myriad of biological processes. The miRBase release of 21st of June 2014 contains 28645 microRNA precursors, 35828 mature microRNAs from 223 species, 1881 of which are human microRNAs.

The small non-coding 22 nucleotides long RNA is the mature form of a larger precursor that gets through multiple steps of processing which could follow different pathways. Most microRNAs follow the canonical way of maturation, in which the primary transcript is cleaved by RNase III endonuclease Drosha and the double stranded RNA binding domain protein Pasha/DGCR8 (DiGeorge Critical Region 8), and then exported in the cytoplasm by exportin 5 (XPO5), where is further processed by the RNase III enzyme Dicer. The mature product is loaded onto the RNA-induced silencing complex (RISC), a multiprotein complex, where it exerts its function. The main microRNA function is posttranscriptional

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regulation of gene expression, being able to inhibit translation or to degrade target mRNAs, depending on the degree of complementarity with the target mRNA (1-3).

Except for their functional role as transcriptional regulator, microRNAs were intensively investigated as possible biomarkers for specific diseases. Their usefulness as non-invasive biomarkers, resides in their stability in the blood, a feature first questioned, and later confirmed by multiple reports. Incubation at room temperature and freeze-thawing cycles did not alter microRNA presence in plasma and serum. Rapid degradation of the synthetic microRNA used in the experiment, but not of the endogenous microRNAs, suggests that the later are present in the blood in a form protected from RNase activity (4, 5). Several studies proved that the microRNA's increased stability is given by the complexes that it forms with proteins or the packing upon secretion into exosomes (or other microvesicles), otherwise, in the naked form, microRNAs would be rapidly degraded (6-9).

MicroRNA utility as biomarkers in the clinical practice attracted attention in obstetrics, were the need for an early, non-invasive diagnostic is in great demand.

From all obstetrics pathologies, our attention was directed towards one of the most important, pre-eclampsia, which has a strong impact on feto-maternal morbidity and mortality. There is an urgent need for specific non-invasive biomarkers to diagnose and/or predict pre-eclampsia and microRNAs are very good candidates. In the last years, microRNAs were intensely studied for this purpose. Here, we analyze the functional, diagnostic and prognostic significance of placenta specific microRNAs in the context of pre-eclampsia.

Pregnancy specific microRNAs

Placenta specific microRNAs

Placenta, an organ of both fetal and maternal origin, develops after blastocyst implantation in the uterin wall. Chorion, the fetal part of the placenta, derives from the external cell layer of the 16-cells embryo, and differentiates into villous and extravillous cytotrophoblast. Cytotrophoblast cells from trophoblast trabeculae further proliferate and differentiate into finger-like protrusions (primary villi) that are invaded by extra-embryonic mesenchyme (secondary villi) and then by capillaries (tertiary villi), and form the villous trophoblast. Next, the cytotrophoblastic progenitor cells go through differentiation and fuse with the syncytium resulting the syncytiotrophoblat (10). A study that determined the mitotic index in the trophoblastic cells showed that DNA replication take place only in the cytotrophoblast (mitotic index is 1,5-2,9% in cytotrophoblast and 0 in syncytium), explaining why the main part of fetal genetic material found in maternal blood is mitotically incompetent (11). The extravillous trophoblast, on the other hand, distinguishes by invasion of the decidua and the myometrium and has an important impact on the vascular remodelling processes. It creates plugs that obstruct the spiral arteries, decrease the blood flow and the levels of O_2 to protect the embryo from the harmful effects of the oxidative stress. After the 10th week of gestation, the plugs disintegrate and the blood circulation is established, and the O_2 partial pressure significantly increase (12).

MicroRNAs expression in placenta, intensively studied in recent years, seems to have a decisive influence on the placental growth and physiological processes. Proliferation, migration, apoptosis and invasion of the trophoblast cells, angiogenesis and cell metabolism are just some of the biological processes controlled by microRNAs in the placenta (13, 14).

Most of the genes encoding placenta specific microRNAs are located on chromosomes 14 and 19, grouped into 3 clusters: the chromosome 14 microRNA cluster (C14MC), the chromosome 19 microRNA cluster (C19MC) and microRNA-371-3 cluster (15).

C14MC is expressed predominantly in the placenta and epithelial tissues. It is exclusively expressed from the maternal inherited chromosome and is regulated by methylation. C19MC is exclusively expressed from the paternal inherited chromosome and is also regulated by methylation. It is mainly

expressed in the reproductive system and placenta (16).

MiR371-3 cluster comprises mainly three microRNA coding genes: hsa-miR-371, hsa-miR-372, hsa-miR-373. It is located on chromosome 19, in the vicinity of C19MC and is predominantly expressed in the placenta, although some of the members of this cluster are also expressed in the embryonic stem cells (16).

Both clusters, C14MC and C19MC, are involved in cellular differentiation and immunomodulation. C14MC expression seems to be increased in the first trimester of pregnancy and decreases progressively to term, while C19MC and cluster miR-371-3 expression is increased mainly in the second part of the pregnancy (15). These results are inconsistent with another study that showed that C19MC expression together with C14MC, miR-17-92 and miR-371 clusters are initially increased and decrease during pregnancy, while let-7, miR-195, miR-29, miR-34 and miR-181c clusters are increased in the third trimester (17). However, one should mention that the work methodologies in the two studies are different as well as the biological samples used (fresh tissue and cultured cells, respectively); the contradictory results were attributed to the influence of the cell culture environment on microRNA expression.

In another study, the let-7c expression was significantly decreased in choriodecidua compared to placenta and amnion, but there was no difference in expression between labour onset and delivery. Its overexpression in the mature oocyte and zygote decreases at the 2-cell stage (18).

A microarray analysis of microRNA expression in chorioamniotic membranes shows significantly downregulation in term versus preterm labor pregnancies. miR-338, miR-136, miR-449 and miR-199a* expressin levels were confirmed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (19).

Although the current dogma stipulates the main placental microRNA source is the trophoblast, especially syncytiotrophoblast, microRNA isolation from mesenchymal stem cells suggests that microRNAs are expressed also in other placental cells (20, 21). Also villous and extravillous trophoblast have the same embryonic origin, they express different functions, so in a report it was evaluated if there is a difference in microRNA expression between the villous and extravillous regions. Using laser microdissection, a significant increase of some C19MC microRNAs (miR-517–3p, miR-518b, miR519d, miR-520g, miR515–5p, and miR1323) expression was found in the villous regions of the paraffin-embedded placental slides, when compared with extravillous trophoblast, with no difference between first and third trimester. The same study demonstrated that C19MC attenuates trophoblast migration and that they could play a role in the differentiation between villous and extravillous trophoblast (22).

Placenta-specific microRNAs in maternal blood

Trophoblastic and villous stromal cells release fetal microRNAs containing exosomes into the maternal circulation, which arguments in favor of their use as biomarkers for pregnancy-associated pathologies (23). Based on TSG101 exosome marker expression, it was shown that cultured primary human (PHT) and BeWo trophoblastic cells secrete microvezicles enriched in placenta-specific microRNAs containing exosomes (24) (20).

Maternal blood analysis of microRNA levels shows a progressive increase, proportional with gestational age, peaking towards the end of the third trimester. However, only some of these microRNAs (miR-526a, miR-527 and miR-520d-5p) could differentiate pregnant from non-pregnant status (5).

Plasma clearance after placenta delivery revealed that 24, out of the 82 microRNAs expressed both in maternal plasma and placenta, are associated to pregnancy, 21 of which are mapped to chromosomes 14 and 19. Interestingly 5 of these microRNAs (miR-515-3p, miR-517a, miR-517c, miR-518b, miR-526b) show a significant increase with gestational age. On the other hand, the concentration of the majority of the 82 microRNAs didn't decreased markedly postpartum, suggesting a possible expression in other tissues (25). The correlation between gestational age and hsa-miR-517a, hsa-miR-518b

levels and their clearance from maternal blood after delivery was confirmed by other study (20). A qRT-PCR comparison of microRNAs levels in plasma from pregnant and non-pregnant women identified 7 pregnancy associated microRNAs (miR-516- 5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525, and miR-526a) (26).

Among the 157 microRNAs profiled by microarray in 5 full-term placentas, only 17 were undetectable in maternal plasma at 24 hours after delivery. Out of the 4 microRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) with the highest concentration in the placenta and present in the plasma, only one miR-141, shows a progressive increase during pregnancy and a marked decrease after delivery (fold change ł 18) (27).

Next generation sequencing followed by qRT-PCR validation shows significant difference in the concentrations of microRNAs in serum versus plasma. All the 36 microRNAs abundant in maternal plasma were also found in the serum, 19 of them significantly overexpressed and 6 under-expressed. Of note, 77 microRNAs were detected only in the serum and not in maternal plasma. Interestingly, hsa-let-7 family was the most abundant in the serum, , with hsa-let-7b and hsa-let-7c upregulated and hsa-let-7e downregulated when compared to maternal plasma (28).

Abnormal microRNAs expression in preeclampsia

Pre-eclampsia is a pregnancy-specific disease and is characterized by hypertension and proteinuria after the 20th week of gestation. The pathogenic mechanisms that cause the disease are not completely elucidated, although an abnormal development of the placenta with inadequate trophoblast invasion has been repeatedly reported. Features like hypoxia due to deficient perfusion, endothelial dysfunction, immune system deregulation and abnormal inflammatory response, were associated with multiple factors, and further complicates the picture and preventing the finding of an efficient therapy (29). Hypoxia, one of the factors with a significant impact on the feto-placental unit, is associated with deregulation of at least 90 microRNAs, including one of the most studied, hsa-miR-210, specifically over-expressed in hypoxic placentas, in particular in pre-eclampsia (30).

The implication of miR-210 in the pre-eclamptic pathological processes was confirmed in a study that demonstrated not only increased levels of miR-210 in pre-eclamptic placentas and plasmas, but also its upregulation in cytotrophoblastic cultured cells and JAR cells exposed to hypoxia. In the same experiment, the migration and invasion of first trimester cytotrophoblast cultured cells was inhibited by both miR-210 overexpression and hypoxia. In hypoxic cultured cells inhibition of miR-210 restored the cytotrophoblastic migration and invasive ability, which suggest that miR-210 is responsible for the placental injuries under hypoxic conditions. Expression of miR-210 is regulated by hypoxia inducible factor (HIF) 1a and HIF2a and requires NF-ęB p50 direct binding to its promoter (31). HIF1 controls oxygen homeostasis and under hypoxic conditions the two subunits HIF1a and HIF1b form a complex that acts as a transcriptional activator for genes involved in correcting hypoxia. Conversely, under hypoxic conditions, miR-210 indirectly increases HIF-1a levels through Glycosylphosphatidylinositol Specific Phospholipase D1 (GPD1L) inhibition and therefore promotes the hypoxic response (ectopic GPD1L can repress hypoxic effects by causing HIF-1a degradation) (32). MiR-210 overexpression in pre-eclamptic placentas was confirmed by yet another study, this time in association with downregulation of miR-455-5p and miR-455-3p, the expression during *in vitro* syncytialization of which suggests a possible implication in placental development. Further experiments demonstrate that mucin 1 (MUC1) inhibition by miR-455-3p induces the decrease of HIF1A levels, suggesting that miR-455-3p could also represses the hypoxic response to promote trophoblast differentiation (33).

The strong expression in term placentas and in maternal plasma (starting with the 28th week of gestation, but not at 16 weeks) of miR-206 suggests that miR-206 overexpression may be rather a response to pre-eclampsia than a trigger. Two of the miR-206 predicted target genes are involved in the

pathogenesis of pre-eclampsia, vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1), were deregulated (overexpressed, and under-expressed, respectively) in the same placenta samples. Whether miR-206 could influence these expression of these genes deregulation remains to be clarified (34).

qRT-PCR analysis of full-term placentas harvested from women with severe pre-eclampsia show a strong downregulation of 6 microRNAs (miR-195, miR-223, miR-218, miR-18a, miR-379, and miR-411) and over-expression of 7 microRNAs (miR-210, miR-30a-3p, miR-518b, miR-524, miR-17-3p, miR-151, and miR-193b). Further qRT-PCR analysis of hsa-miR-17-92 cluster in both chorionic and basal plates of the severe pre-eclamptic placentas confirmed the down-regulation of miR-17, miR-18a, miR-19b1 and miR-92a1 only in chorionic samples. The analysis of the same cluster in second trimester and term maternal plasma show similar results for miR-18a, miR-19b1 and miR-92a1, and for miR-210. In an attempt to clarify these microRNAs involvement in placental developmental processes, Peng Xu YZ et al. demonstrated that Smad2 inhibition by miR-18a could be associated with trophoblastic invasion (35). miR-210 over-expression was also noted in a microarray study performed on pre-eclamptic placentas, along with the down-regulation of other microRNAs (miR-328, miR-584, miR-139-5p, miR-500, miR-1247, miR-34c-5p and miR-1) (36).

In contradiction, another microarray/qRT-PCR study describes the down-regulation of miR-210, miR-15b, miR-181 and miR-483–5p, in placentas from women with pre-eclampsia and preterm labor (37). However, when PHT cells were exposed to hypoxic conditions, with the notable exception of miR-520c-3p, there was no difference in expression of the entire C19MC cluster (24). Yet another microarray study (followed by Northern Blot validation) on hypoxic PHT cells found no change in miR-210 levels, with the concomitant overexpression of miR-93, miR-205, miR-224, miR-335, miR-451, miR-491 and downregulation of miR-424. Mouillet et al. evaluated if changes in miR-205 levels (miR-205 is an epithelial cell specific marker, and trophoblast cells are epithelial) have an impact on placental development. A gain and loss of function analysis demonstrated that the modulation of DEM1 gene expression by miR-205, influences trophoblast differentiation (38). Of note, miR-205 was also found (by sequencing) expressed in placenta samples, in a later study (39).

qRT-PCR analysis of microRNA expression in placentas associated with preeclampsia, gestational hypertension and *intrauterine growth restriction* (IUGR) revealed deregulated microRNAs known to be associated with cardiovascular and cerebrovascular diseases. Two upregulated (miR-499a-5p, miR-1-3p) and 3 downregulated (miR-26a-5p, miR-103a-3p, miR-145-5p) microRNAs could significantly distinguish between pre-eclampsia and normal placentas. Both, miR-499-5p overexpression (common to all three pathologies) and miR-1-3p progressive overexpression, suggest rather a compensatory mechanism, than a pathogenic one. Both of them are associated with heart failure and myocardial infarction, and miR-1-3p also with dyslipidaemia, therefore only miR-26a-5p, miR-103a-3p and miR-145-5p seem to be associated specifically with pre-eclampsia (40).

qRT-PCR experiments on full-term placentas confirmed miR-17, miR-20a and miR-20b overexpression in severe pre-eclampsia; gene target analysis suggests a strong impact on angiogenesis and placenta development, as both Ephrin-B2 and EPHB4, are direct targets of miR-20b (41).

High-throughput analysis of microRNA expression in pre-eclamptic placentas identified 25 microRNAs with altered expression, 10 of which were further confirmed by qRT-PCR: 8 overexpressed (miR-148a-3p, miR-210, miR-193b-3p, miR-31-5p, miR-365a-3p, miR-516b-5p, miR-520a-5p and miR-27a-5p) and 2 under-expressed (miR-135b-5p and miR-136-3p). Further analysis of miR-193-3p, a known tumor suppressor alters placental development, with possible suppressive effects on migration and invasion. Overexpression of miR-193-3p in HTR-8/SVneo cells attenuate migration and invasion, while its inhibition has an opposite effect (42).

Another qRT-PCR study found 11 C19MC microRNAs downregulated in pre-eclamptic

placentas (miR-515-5p, miR-517-5p, miR-518b, miR-518f-5p, miR-519a, miR-519d, miR-520a-5p, miR-520h, miR-524-5p, miR-525, and miR-526a). In the same study, decreased expression of *miR-517-5p, miR-519d, miR-520a-5p* and *miR-525 was associated not only with pre-eclampsia, but also with both gestational hypertension (GH) and fetal growth restriction (FGR).* Interestingly, miR-517-5p, miR-520a-5p, miR-524-5p and miR-525 are associated with *both the severe and the mild forms of pre-eclampsia*. However, the gradual decrease of the microRNAs expression correlates with the chronicity of the disease, suggesting that at least for some microRNAs, the down-regulation represents a compensatory mechanism rather than a cause (43).

MicroRNA sequencing of maternal plasma and placenta samples from 4 patients with pre-eclampsia (and, strangely, only one healthy control), describes up-regulation of 17 microRNAs (miR-126, miR-126*, miR-130a, miR-135b, miR-142-3p, miR-149, miR-188-5p, miR-18a, miR-18b, miR-203, miR-205, miR-224, miR-27a, miR-29a, miR-301a, miR-517c, miR-518-3p, miR-518e, miR-519d and miR-93) in both plasma and placenta, with a higher expression in placenta than in maternal plasma. Complementary pathway analysis shows an impact of these microRNAs in focal adhesion, Wnt, MAPK or ErbB signalling pathways as well as other pathways possibly involved in the pathogenesis of pre-eclampsia (39).

Microarray analysis (followed by qRT-PCR validation) of severe pre-eclampsia plasma samples identified 7 microRNAs (miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p) to be upregulated, potentially impacting TGF-b and MAPK signalling, cytokine–cytokine receptor interaction, cancer metastasis or endocytosis (44).

The immune system alterations as part of the pathogenic mechanism involved in pre-eclampsia bring into question the possible involvement of microRNAs. MiR-30a overexpression in mesenchymal stem cells (MSCs) derived from cord blood harvested from preeclampsia patients was associated with the inhibition of the immunosuppressive function of MSCs. MiR-30a overexpression represses the production of IL-8, IL-6 and COX2, and activation of NF-ęB and JNK in IL1b-elicited MSCs. By targeting cyclin E 2 gene (CCNE2), a cell cycle regulator, miR-30a overexpression also inhibits MSC proliferation (45). This experiment demonstrates miR-30a implication in preeclampsia pathogenesis, by altering the immune processes at the fetal-maternal interface.

Interestingly, miR-144 was found to be not only down-regulated in both mild and severe pre-eclampsia, as well as in the sera of pregnant women at 12-14 weeks of gestation that developed severe pre-eclampsia in the third trimester(46). In the same study, miR-1233, miR-520a and miR-210 were confirmed overexpressed (47). Altogether, miR-144 seems to be involved in the pathological mechanisms of preeclampsia through hypoxia and ischemia regulation.

Sandrim et al found over-expression of miR-885-5p in pre-eclamptic plasma samples, majority of which found in exosomes, but not in microvesicels and/or apoptotic bodies. Interestingly, this upregulation was correlated with AST levels, suggesting not only its origin in liver cells, but also its possible use as predictor for pre-eclampsia aggravation (47).

Maternal leukocytes of pre-eclamptic women after 30 weeks of gestation have lower levels of miR-15a-3p, miR-31-3p underexpression but higher levels of miRNA-451a, miRNA-122-5p. All these microRNAs could entail endothelial injuries by altering the expression of genes involved in endothelial biology. MiR-15a downregulation increases *B-cell lymphoma 2* (BCL2) levels and consecutively the oxidative stress, while miR-31 downregulation strengthens the immune response, both with negative effects on endothelial cells. Overexpression of miR-451a (known to induce apoptosis) and of miR-122-5p (targeting G-protein-coupled receptor kinase-interacting protein 1 -GIT1), a regulator of nitric-oxide synthesis in endothelial cells) could induce endothelial damage (48).

Several studies failed to find any microRNAs associated with pre-eclampsia. Luque et al analyzed by microarray 754 microRNAs but found no significant differences between serum samples from early pre-eclamptic women and healthy controls, questioning microRNA's utility as biomarker for early diagnosis of pre-eclampsia (49). Hromadnikova et al

used qRT-PCR to assess the levels of 7 circulating placenta-specific microRNAs (miR-520a*, miR-520h, miR-525, miR-526a, miR-516-5p, miR-517*, miR-518b) in insufficient, pre-eclampsia and/or IUGR but also found no significant changes (23).

With the exception of one single study (37), miR-210 was the only microRNA that was found upregulated by multiple methods (high-throughput sequencing, microarray and qRT-PCR) in pre-eclamptic plasma, sera and placenta samples in the majority of the studies.

Data for miR-518b and miR-524 expression in term placentas, as well as for miR-17 and miR-18a (in plasma and placentas) were contradictory (35, 39, 41, 43).

MiR-520a was found either upregulated in placenta and sera samples or downregulated in placenta samples, (42, 43, 46). Placenta samples in which was overexpressed were delivered only by caesarean by Han Chinese women (42), while in the second case the delivery was both by caesarean (87,5%) and vaginal by Caucasian women (43) and in both studies the delivery took place before and after 34 weeks of gestation. By contrast, serum samples were collected between 12-14 weeks of gestation, from patients that developed preeclampsia in the third trimester, majority of the women by Caucasian origin (46). These contradictory results question if the different origins of the patients or even the delivery method could affect significantly the microRNAs expression in different diseases.

Conclusions

A large number of microRNAs have been analyzed and associated with preeclampsia, but so far none of them were confirmed as a specific biomarker for this condition. Even so, the results are showing promising results, with some microRNAs were confirmed to be deregulated in multiple studies and demonstrated to be involved in pre-eclampsia pathogenesis.

More studies are needed to clarify microRNAs role in pregnancy and the possibility to be used as biomarkers or therapy in various diseases associated with pregnancy.

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