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Identification of potential early biomarkers of preeclampsia

Angelika V. Timofeeva [a, *], Vladyslava A. Gusar [a], Nataliya E. Kan [a], Kseniya N. Prozorovskaya [a], Anna O. Karapetyan [a], Oleg R. Bayev [a], Vitaliy V. Chagovets [a], Sergei F. Kliver [b, c], Daria Yu. Iakovishina [c], Vladimir E. Frankevich [a], Gennadiy T. Sukhikh [a]

[a] Federal State Budget Institution "Research Center for Obstetrics, Gynecology and Perinatology", Ministry of Healthcare of the Russian Federation, Oparin Street, 4, Moscow, 117997, Russia
[b] Theodosius Dobzhansky Center for Genome Bioinformatics, Saint Petersburg State University, 41A Sredniy Avenue, St. Petersburg, 199004, Russia
[c] Ksivalue LLC, Russia

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ABSTRACT

Introduction: It is thought that poor placental perfusion caused by inadequate remodelling of the maternal spiral arteries leads to preeclampsia (PE). To identify novel signalling pathways that contribute to PE pathogenesis and to create prerequisites for the non-invasive diagnosis of PE before clinical manifestations of the disease, this study aimed to evaluate miRNA expression levels in the placenta and blood plasma of pregnant women.

Methods: miRNA deep sequencing followed by real-time quantitative RT-PCR was applied to compare miRNA expression profiles in the placenta and blood plasma from women with early- and late-onset PE relative to the control group.

Results: A more than two-fold decrease in miR-532-5p, -423-5p, -127-3p, -539-5p, -519a-3p, and -629-5p and let-7c-5p expression levels was observed in the placenta, while a more than two-fold increase in miR-423-5p, 519a-3p, and -629-5p and let-7c-5p was observed in the blood plasma of pregnant women with PE. The above-listed miRNAs are associated with PE for the first time in this study, except for miR-519a-3p, whose role in PE has already been postulated. Using a logistic regression, plasma samples were classified into the early-onset PE group (probability $p = 0.01$, 80% specificity, 87.5% sensitivity and 87.5% precision) and showed increased miR-423-5p expression levels that were confirmed by the 9.8-fold up-regulation ($p = 0.0002498$) of miR-423-5p expression observed in the blood plasma at 11–13 GW by RT-PCR in a group of pregnant women manifesting severe PE clinical signs at 28–33 GW.

Conclusions: miR-423-5p may be considered a potential candidate for the early diagnosis of PE during the targeted management of high-risk pregnancies.

1. Introduction

Preeclampsia (PE), a multisystem pathological condition that occurs in 3–5% of pregnant women, is one of the leading causes of maternal and perinatal mortality [1]. Typically, the disease manifests in the second half of pregnancy with a classic triad of symptoms including hypertension, proteinuria and peripheral oedema [2]. Although the aetiology and pathogenesis of PE have not been fully elucidated, the development of PE is considered to occur following a two-step process. The first stage involves a placentation defect, probably as a result of a maladjustment of the maternal local immune response against foetal tissues [3] coupled with an abnormal differentiation of cytotrophoblast cells during their invasion of the spiral uterine arteries [4–7]. This symptom results in a decreased placental size and restricted utero-placental blood flow, which does not meet the needs of the growing foetus [8]. As a consequence, ischaemia/hypoxia of the placenta is followed by an increase in syncytiotrophoblast apoptosis and necrosis and by the release of microvesicles and cellular debris by the trophoblasts [9]. There is also a release of damaging factors of placental origin, such as soluble fms-like tyrosine kinase 1 (sFlt1), proinflammatory cytokines, antibodies to the angiotensin 1 receptor, soluble endoglin, tumour necrosis factor alpha, interleukin 1, fibronectin, and blood coagulation factor VIII into the mother's bloodstream [10]. Overall, these changes lead to the onset of the second stage of PE that occurs

* Corresponding author.
E-mail address: avtimofeeva28@gmail.com (A.V. Timofeeva).

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after 20 weeks of gestation (GW) and is characterized by systemic endothelial dysfunction in many organs and systems including the kidneys, cardiovascular system, liver, brain, and others [11,12]. The application of different molecular biology approaches has enabled the identification of the entire gamut of differentially expressed genes in the placenta of pregnant women with PE compared to that of women with physiological pregnancies [13,14]. Nevertheless, the genetic and epigenetic mechanisms regulating these differences in gene expression are still poorly understood. miRNAs form a class of small non-coding RNAs with a length of 21–25 nucleotides that epigenetically control of the expression target genes, mainly at the post-transcriptional level by destabilizing mRNAs and inhibiting protein translation [15]. The role of miRNAs in the regulation of placental development and function has been analyzed in several recent studies. In one of these studies, changes in the levels of 33 miRNA clusters including miR-17-92, C14MC, miR-371-3, the C19MC clusters, the miR-29 cluster, the let-7 family, miR-195 and miR-181c were observed in the placenta depending on 35 gestational age [16]. Large-scale studies of miRNA gene expression in the placenta of pregnant women with physiological pregnancies and pregnant women with PE [17–20] and premature births [21] allowed for the identification of more than a dozen differentially expressed miRNAs in each study. Overall, the lists of identified miRNAs differed between the studies with minimal overlap. The causes for these discrepancies may be associated with the use of different test systems for the analysis of specific sets of miRNAs or to differences in the topology of the studied fragment of placental tissue. The aim of the present study was to perform a miRNA screen on placental tissue and blood plasma for pregnant women with early- and late-onset PE using miRNA deep sequencing. This method allows us to simultaneously analyse all the miRNAs in a sample with high accuracy and specificity, rather than a defined number of molecules, such as the ones present in available microarrays. Additionally, microarrays may not have high specificity due to the possible cross-hybridization of homologous miRNAs. Some of the miRNA that we identified appear usable in early terms of pregnancy and are therefore interesting potential biomarkers. The functional analysis of the identified differentially expressed miRNAs while considering their potential gene-targets will provide additional information on deregulated signalling pathways which have not been previously considered in terms of PE pathogenesis.

2. Materials and methods

2.1. 1st patient cohort

In total, 54 pregnant women aged between 27 and 40 years with Caesarean section indications were enrolled in the study and comprised the following four groups (Table 1): 1) women with full-term physiological pregnancy (n = 16, 37–40 GW); 2) pregnant women with an indication for an emergency Caesarean section due to the lack of prolonging the pregnancy because of cervical insufficiency, placental abruption or premature rupture of the foetal membrane (n = 10, 24–34 weeks) without clinical manifestations of PE; 3) pregnant women with moderate (n = 2/16) and severe (n = 14/16) early-onset PE (n = 16) manifesting the disease at 24–34 weeks; and 4) pregnant women with moderate (n = 11/12) and severe (n = 1/12) late-onset (n = 12) PE manifesting the disease after 34 GW. Exclusion criteria were the application of assisted reproductive techniques to increase fertility, multifoetal pregnancies, severe somatic pathology in pregnant women, foetal aneuploidy, and vaginal delivery. PE was diagnosed by the elevation of blood pressure above 140/90 mm Hg measured at four-hour intervals and proteinuria above 0.3 g/l after 20 GW [22]. Severe PE was diagnosed by the presence of one or more of the following clinical manifestations: 1) systolic blood pressure above 160 mm Hg or diastolic pressure above 110 mm Hg, 2) proteinuria above 5 g/l, 3) very low levels of daily diuresis (less than 500 ml in 24 h), 4) respiratory disorders (pulmonary oedema or cyanosis), 5) liver dysfunction, 6) central nervous system malfunction (severe headache or visual disturbances), 7) pain in the epigastric region or right upper quadrant, 8) thrombocytopenia and 9) the presence of severe intrauterine growth retardation [22]. The diagnosis of “PE” was confirmed by a microscopic examination revealing an increased number of syncytial buds (pronounced Tenney-Parker symptom), branched-type angiogenesis in the terminal villi, the presence of non-functioning areas of chorionic villi with collapsed villi capillaries, focal atherosis of vessels in the stem villi and desquamation of villi syncytiotrophoblasts. The placental and peripheral blood plasma samples were taken from each pregnant woman at the time of delivery. The study was performed according to the verdict of the Local Ethics Committee of Federal State Budget Institution “Research Centre for Obstetrics, Gynaecology and Perinatology” after informed consent was signed by the patients.

2.2. 2nd patient cohort

In total, 16 pregnant women aged between 26 and 46 years underwent a three-stage screening at 11–13, 24–26 and 30–32 GW and comprised the following two groups (Table 2): 1) women with physiological pregnancy (n = 10) and 2) women with severe early-onset PE (n = 6) manifesting the disease at 28–33 GW.

2.3. RNA isolation from the placental tissue

Placental tissue samples were taken for research no later than 10 min after delivery. A 5 mm thick tissue slice was taken by passing through the entire placenta from the foetal surface down through the maternal surface at a location roughly halfway between the umbilical cord attachment site and the placental margin in an area free from any obvious abnormalities as recommended by Burton GJ et al. [23]. Sampled placental tissue free of foetal membranes was washed in 0.9% NaCl and immediately frozen in liquid nitrogen for subsequent storage at −80 °C. Total RNA was extracted from 20 to 40 mg of placental tissue using the miRNeasy Micro Kit (Qiagen, Germany) followed by the RNeasy MinElute Cleanup Kit (Qiagen, Germany). The RNA concentration was measured using a Qubit fluorimeter 3.0 (Invitrogen™, USA). The sample quality of the total RNA was examined on the Agilent Bioanalyzer 2100 using the RNA 6000 Nano Kit (Agilent Technologies, USA). Total RNA samples with a 28S/18S ribosomal RNA ratio equal to 1.5–1.8 were used for further studies.

2.4. RNA isolation from peripheral blood plasma

Venous blood samples from pregnant women were collected into VACUETTE® tubes containing EDTA, centrifuged for 20 min at 300 g (4 °C) followed by plasma collection and re-centrifugation for 10 min at 14500 g. The RNA was extracted from 200 μl blood plasma using the Serum Plasma kit (Qiagen, Germany) with the pre-addition of 5.6×10^8 copies of synthetic cel-miR-39 (Qiagen, Germany) into the mixture containing plasma and the phenol reagent Qiazol.

2.5. miRNA deep sequencing

cDNA libraries were synthesized using 500 ng total RNA from the placenta and 7 of 14 μl total RNA column eluate (Serum Plasma kit, Qiagen, Germany) extracted from 200 μl blood plasma using the