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Short Communication

Potential role of circulating microRNAs as early markers of preeclampsia

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ABSTRACT

Objective: To identify microRNAs (miRNAs) differentially expressed at early stages of gestation (12–14 weeks) in the serum of pregnant women, who later developed severe preeclampsia (sPE) in the third trimester of pregnancy ($n = 24$) compared to women with normal pregnancy ($n = 24$).
Materials and Methods: Sera from 12–14-week-gestation whole blood were subjected to microarray analysis with TaqMan Low Density Array chips (human microRNA panel V3.0), and to quantitative real-time polymerase chain reaction.
Results: By using the TaqMan Low Density Array chip technology, 19 mature miRNAs appeared differentially expressed in the group of women who later developed sPE as compared to normal women. The expression of four miRNAs (miR-1233, miR-520, miR-210, miR-144) was validated by quantitative real-time polymerase chain reaction analysis. MiR-1233 was the most overexpressed in the serum of women who later developed sPE.
Conclusion: Circulating miRNAs deserve further investigation in order to explore their potential role in the pathogenesis of preeclampsia. In particular, miR-1233 might represent a potential marker of early sPE.

Introduction

Preeclampsia (PE) affects approximately 4–5% of all pregnancies worldwide [1]. It is a common pregnancy-related disease characterized by hypertension and proteinuria, and a major cause of maternal mortality, morbidity, perinatal death, preterm birth, and intrauterine growth restriction [2]. Therefore, the identification of early biomarkers for PE is urgently needed. In this respect, in a previous study by our group, we have investigated the role of the TNF family member TRAIL, a pleiotropic cytokine showing remarkable regulatory activities in the immune and cardiovascular systems [3,4], as a potential marker of PE. Even though TRAIL is expressed by placenta [5] and although its levels are modulated by antiphospholipid antibodies [5], and are high in recurrent miscarriage [6], we did not find any association between levels of circulating TRAIL determined at 12–14 weeks of gestation and subsequent development of PE [7].

Several studies have shown differential expression of given microRNAs (miRNAs), which are a conserved group of ~22-nucleotide regulatory RNAs that play an important role in regulating gene expression, in the human placentas of patients with PE [8–13]. Interestingly, miRNAs specifically expressed in human placentas were also detected in the sera of pregnant women and found to be significantly higher if compared to those of nonpregnant women. Furthermore, their levels were reported to decrease after delivery [13]. However, whether plasmatic miRNAs can represent sensitive and early biomarkers for PE as proposed for other pathologies [14], is currently unknown.

On these bases, we carried out a pilot study to evaluate the potential usefulness of circulating miRNAs as early biomarkers of severe PE (sPE). To this purpose, we have performed a microarray analysis of circulating miRNAs at early gestational times (12–14

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weeks) in order to investigate the variability expression in the sera of pregnant women with normal pregnancies versus women who developed sPE later in gestation.

Materials and methods

We have carried out a retrospective study to identify differentially expressed miRNAs at early stages of gestation (12–14 weeks) in the serum of pregnant women, who later developed sPE in the third trimester of pregnancy. The procedures complied with the Declaration of Helsinki and had been approved by the institutional review board (Institute for Maternal and Child Health, IRCCS Burlo Garofolo of Trieste). All participants signed a written informed consent. Sera from whole blood of 12–14 weeks of gestation were obtained and stored at −80°C. For RNA isolation, pools of sera were created by combining 40 μL of each sample, including 24 women with normal pregnancies and 24 who developed sPE in the third trimester of pregnancy. After the initial denaturizing step, we routinely spiked in synthetic *Caenorhabditis elegans* miR-39 (cel-miR-39) to a final concentration of 10^{-4} pmol/μl for all samples in order to control variations in RNA extraction and/or purification procedures because of the absence of homologous sequences in humans. The pools were lysed with an equal volume of 2× Denaturing Solution (Ambion, Austin, TX, USA). RNA was isolated using the mirVana PARIS kit, modified so that samples were extracted twice with an equal volume of acid-phenol chloroform. RNA was eluted with 100 μL of Ambion solution. We used TaqMan Low Density Array (TLDA) chips (human microRNA panel V3.0; Applied Biosystems Inc, Norwalk, CT, USA) to screen 754 unique specific human miRNAs from the two pooled samples. A 300 ng sample of human serum RNA from each pool was reverse transcribed using the TaqMan miRNA RT Kit, and the TaqMan miRNA Multiplex RT Assays, Human Pool Set. 250 ng of human plasma RNA was added to each of the two multiplex RT reactions (pool A and pool B Megaplex RT-primers). Preamplification reactions were run according to the manufacturer's protocol. A 75 μL aliquot of 0.1× TE was added to the PreAmp product, and 9 μl diluted PreAmp product was used to run the real-time polymerase chain reaction (RT-PCR) reactions by dispensing 100 μl of the PCR reaction mix into each port of the TaqMan MicroRNA Array. The default PCR procedure was carried out, and the analysis was performed using RQ manager software (Applied Biosystems). ΔC_T and $\Delta\Delta C_T$ were calculated using the following mathematical formula: $\Delta C_T = C_{T\ sample} - C_{T\ RNU6B}$, $\Delta\Delta C_T = \Delta C_{T\ case} - \Delta C_{T\ control}$. Finally, the $\Delta\Delta C_T$ was normalized against the cel-miR-39. Quantitative (q)RT-PCR was performed to validate the 19 differentially expressed miRNAs identified in the miRNAs microarray analysis. qRT-PCR was performed using universal Taq-man mix and miRNA-specific primers according to the manufacturer's protocol. Reactions were run on an ABI Step 1 Plus RT-PCR machine (Applied Biosystems) using the default cycling conditions. All reactions were analyzed by using the $2^{-\Delta\Delta C_T}$ calculation procedure. Normalization of experimental data was performed using a spiked-in *C. elegans* control.

Results

The clinical characteristics of each individual patient and control are summarized in Table 1. miRNAs microarray was performed on 48 serum samples obtained at 12–14 weeks of gestation, including 24 from women who developed sPE in the third trimester of gestation and 24 from women with normal pregnancies. We decided to pool the serum in order to bypass the high variability present in individual samples. A total of 754 unique specific human miRNAs were assayed. Bonferroni test correction to p value was applied and only significant miRNAs with $p < 0.00007$ were selected. Nineteen differentially expressed miRNAs were identified out of these, including 12 upregulated (miR-1233; miR-650; miR-520a; miR-215; miR-210; miR-25; miR-518b; miR-193a-3p; miR-32; miR-204; miR-296-5p; miR-152) and 7 downregulated (miR-126; miR-335; miR-144; miR-204; miR-668; miR-376a; miR-15b) in sPE, using TLDA chips (human microRNA panel V3.0). Four miRNAs (miR-1233, miR-520a, miR-210, miR-144) were validated in sPE serum sample by qRT-PCR. In particular, results summarized in Table 2 show that circulating miR-1233 displayed the greater

Table 1
Main characteristics of enrolled women: 24 preeclamptic and 24 controls, unless specified.

Characteristics	Preeclamptic women	Controls	p
Maternal age at delivery, y	34.4 (33.0–36.8)	33.7 (30.3–36.1)	0.216
Nulliparous	18 (75.0%)	15 (62.5%)	0.534
Prepregnancy BMI, kg/m^2	22.4 (18.9–26.4)	23.0 (21.4–27.3)	0.288
BMI ≥ 30 kg/m^2	2 (8.3%)	3 (12.5%)	1.000
Nonspontaneous conception	0 (0.0%)	2 (8.3%)	0.489
Not Caucasian	2 (8.3%)	1 (4.2%)	1.000
Smoking at 12th wk of gestation	2 (8.3%)	0 (0.0%)	1.000
Pregestational diabetes	0 (0.0%)	1 (4.2%)	1.000
Gestational diabetes	1 (4.2%)	0 (0.0%)	1.000
Birthweight below 10th centile for gestational age	5 (20.8%)	0 (0.0%)	0.050
Percentile of birthweight for gestational age	82.9 (41.5–94.1)	27.2 (11.1–37.3)	0.000
Gestational age at delivery	40.6 (39.3–41.1)	36.7 (35.1–39.0)	0.000
Induction of labor	9 (37.5%)	4 (16.7%)	0.193
Mode of delivery			
Spontaneous vaginal	14 (58.3)	7 (29.2%)	0.080
Elective CD	2 (8.3%)	7 (29.2%)	0.137
Urgent CD	5 (20.8%)	10 (41.7%)	0.212
Operative vaginal	3 (12.5%)	0 (0.0%)	0.234
Live births	24 (100.0%)	24 (100.0%)	1.000
Umbilical pH < 7.1	0/18 (0.0%)	2/23 (8.7%)	0.495
Umbilical BE < −12	0/18 (0.0%)	3/22 (13.6%)	0.238
Apgar 1st minute < 5	0 (0.0%)	6 (25.0%)	0.022
Apgar 5th minute < 7	0 (0.0%)	2 (8.3%)	0.489
Fetal distress	7 (29.2%)	9 (37.5%)	0.760

Data in the table are frequencies and percentages or medians and interquartile ranges. For dichotomous variables, p is from Fisher's exact two-tailed test; for continuous variables, p is from Mann–Whitney's rank-sum test.
BMI = body mass index; CD = cesarean delivery.

Table 2
Comparison of microarray and validation of some microRNAs (miRNAs) by quantitative real time polymerase chain reaction. Quantification values are reported in parenthesis.

miRNAs	Fold change >2	Chromosomal location
miR-1233	5.6 (5.4)	15
miR-650	3.7	22q11.22
miR-520a	3.5 (3.2)	19q13.42
miR-215	3.5	17q11.2
miR-210	3.3 (3.1)	17p13.1
miR-25	3.1	7q22.1
miR-518b	3.1	7q22.1
miR-193a-3p	2.6	17q11.2
miR-32	2.6	9q31.3
miR-204	2.5	9q21.12
miR-296-5p	2.4	Xp11.22
miR-152	2.4	17q21.32
miRNAs	**Fold change <0.5**	**Chromosomal location**
miR-126	0.48	9q34.3
miR-335	0.42	7q32.2
miR-144	0.4 (0.39)	7q21-q22
miR-204	0.39	9q21.22
miR-668	0.33	14q32.31
miR-376a	0.32	14q32.31
miR-15b	0.24	3q25.33

differential expression between the group of 24 women who developed sPE in the third trimester of gestation and the group of 24 women with normal pregnancies.

Discussion

Circulating miRNAs have emerged as potential novel diagnostic biomarkers for different human pathologies such as cancer, tissue injury, cardiovascular diseases, and also pathological pregnancy. In this respect, although the mechanisms involved in the development of PE remain poorly understood, our data have displayed novel dysregulated miRNAs at early stages of gestation (12–14 weeks) in the serum of pregnant woman who developed sPE in the third trimester of pregnancy. In particular, we found 12 upregulated and 7 downregulated miRNAs. Among the upregulated miRNAs, we validated miR-210 [13] and miR-520a in sPE. With respect to our current data, it is noteworthy that a previous study reported the elevation of several miRNAs in the sera of women during early gestation (i.e., within the 12^{th} to 16^{th} week of pregnancy) with later onset of PE [14]. In our current study, we have identified miR-1233 through the TLDA chip technology and validated it by qRT-PCR, thus highlighting it as the most over-expressed miRNA in sPE. Although this is the first report suggesting a potential role of miR-1233 in predicting sPE, this miRNA has been already implicated in renal carcinoma [15]. Interestingly, further miRNAs that were upregulated in the serum of pregnant women who later developed sPE, were related to cancer in previous studies: miR-650 to hepatocellular carcinoma [16]; miR-32 and miR-193a-3p to colon cancer [17]; miR-152 to endometrial cancer [18]; miR-215 and miR-204 in metastatic renal cell carcinoma [19]. In addition, in line with our current findings, miR-296-5p and miR-25 have been reported to be elevated in placentas with sPE [20]. By contrast, several downregulated miRNAs in the serum of pregnant women who develop sPE in late onset, have also been previously reported to be modulated in cancer patients. In particular, miR-144 is correlated with several types of cancers: its levels are significantly decreased in bladder cancer tissues [21] and in colorectal cancer [22], while miR-126 [23] miR-335 [24], miR-668 [25], miR-15b [26], miR-204 [27] are considered oncosuppressors. Taken together, our data suggest a protumoral like signature of circulating miRNAs in women who develop sPE at a later stage. Nevertheless, since the majority of these miRNAs was not validated by qRT-PCR but it was only analyzed by TLDA technology, confirmatory studies with data validation by qRT-PCR are needed to better explore the role of miRNAs in PE pathogenesis.

Conclusion

We have shown a potential link between early dysregulated miRNAs expression and subsequent development of PE. We also identified novel candidate miRNAs that may be relevant in the pathogenesis of PE or as early disease's biomarkers. In particular, miR-1233 showed the greatest differences between women who developed sPE in the third trimester of pregnancy and women with normal pregnancy, thus becoming a potential biomarker candidate.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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