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Quantification of circulating miR-517c-3p and miR-210-3p levels in preeclampsia

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ABSTRACT

MicroRNAs (miRNAs, miRs) are small regulatory non-coding RNAs that regulate gene expression by incomplete complementary attachment to the 3′UTR, 5′UTR, ORF and promoter regions of target mRNAs. We compared plasma levels of miR-210-3p and miR-517c-3p as cell-free microRNAs (cfmiRNAs) in preeclamptic (n = 20) and healthy women (n = 20). These miRs are responsible for cell growth and proliferation, placental hypoxia, immune response and apoptosis. We found higher expression levels of miR-210 and miR-517c in preeclamptic cases (+3.34 and +2.27 fold change, respectively). This is the first study that evaluates the plasma levels of miR-517c in preeclamptic cases by real time PCR (RT-PCR) technique. This study can lead to new opportunities for research about the roles of miRNAs in preeclampsia etiology or new biomarkers.

1. Introduction

MicroRNAs are small regulatory non-coding RNAs (19–25 nucleotides) that act as a "hub" in different regulatory networks of genes. These regulatory RNAs can often suppress the expression of the gene at the translational level with incomplete complementary attachment to the 3′UTR regions, and sometimes by binding to the 5′UTR, ORF or promoters [1,2]. Furthermore, these RNAs can also upregulate the expression of certain genes. Due to incomplete binding to their targets, each miRNA can target several mRNAs and each mRNA can be targeted by several different miRNAs [3]. It has been determined that miRNAs can regulate the expression of 80% of all genes and hence, play an important role in various biological processes such as cell proliferation, apoptosis, differentiation, development and hematopoiesis [4]. Therefore, dysregulation of miRNAs expression can lead to different pathological conditions such as cardiovascular diseases, neurological disorders and cancers.

In biomedical research, miRNAs are promising biomarkers for prediction, diagnosis, prognosis, and reaction to therapy. MicroRNAs are secreted from the cells with two mechanisms including vesicle dependent (with exosomes, and apoptosomes or apoptotic body) and vesicle independent manner [4]. In vesicle independent way, miRNAs exit freely and then bind to RNA binding proteins (like NPM1 and ago2) or HDL (high-density lipoprotein cholesterol)/LDL (low-density lipoprotein cholesterol) in order to survive [5]. These tiny molecules can tolerate harsh conditions such as multiple freeze–thaw cycles, prolonged storage, and extreme changes in pH and heat. MiRNAs have also significant stability in various types of body fluids, including blood and urine [6,7]. Therefore, cfmiRNAs can be ideal biomarkers.

CfmiRNAs have been well studied. In 2008; Chim and colleagues outlined the expression of circulating miRNAs in pregnant women for the first time [8]. Preeclampsia (PE) is one of the hypertensive disorders of pregnancy which is characterized by hypertension and proteinuria [9]. There are few studies about cfmiRNAs in plasma or serum in preeclampsia, and most of them measured several miRNAs (even hundreds) by NGS (Next Generation Sequencing) methods, followed by real-time quantitative RT-PCR [10–13]. Other studies evaluated specific miRNAs related to placenta or to a known biological pathway in preeclampsia [14–16]. Further studies are needed to confirm the specificity of these miRNAs for PE and their reliability for future clinical applications. MiR-210-3p and miR-517c-3p are two miRNAs that have been associated with preeclampsia in some studies [11,13–20]. They are responsible for regulation of several processes including cell growth and proliferation, placental hypoxia, immune response, apoptosis and lipid metabolism [20–22]. Therefore, in order to clarify relationship

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Table 1
Clinical features and C_t values of miRNAs in studied groups.

T-Test	PE	NP	P Value
Maternal age (years)	29 ± 1.1	28 ± 0.92	0.44
Systolic blood pressure (mm Hg)	150 ± 2.24	104 ± 2.1	< 0.01*
Diastolic blood pressure (mm Hg)	98 ± 2.29	66 ± 1.66	< 0.01*
24-h urine protein (dipstick)	3 ± 0.13	00	< 0.01*
AST (pkat/L)	0.54 ± 0.06	–	–
ALT (pkat/L)	0.43 ± 0.08	–	–
Platelet count (x 10^9/L)	187 ± 21.05	214 ± 13.9	0.38
Gestational age at sampling (week)	34 ± 0.56	33 ± 0.64	0.49
Gestational age at delivery (week)	36 ± 0.34	38 ± 0.27	< 0.01*
New born weight (gram)	2380 ± 71.8	2650 ± 83.1	< 0.01*
Normalized C_t (fold change)			
C_t miR-517c	33.7 ± 0.42 (2.27)	34.7 ± 0.34 (1.0)	0.047*
C_t miR-210	33 ± 0.46 (3.34)	34.6 ± 0.26 (1.0)	0.002*
C_t RNA-spike in	21.5 ± 0.07	21.4 ± 0.05	0.22

PE, Preeclampsia; NP, Normal Pregnancy; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase. Values are the ± SE.
* $P < 0.05$ vs. NP group.

between these miRs and preeclampsia, more analysis by gold standard methods like real-time quantitative RT-PCR are required.

Here, we compare plasma levels of miR-210-3p and miR-517c-3p as cfmiRNAs in preeclamptic and healthy women.

2. Materials and methods

2.1. Subjects

This study was approved by the Institutional Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC.1395.486). Forty pregnant women [20 healthy controls and 20 with preeclampsia) who were referred by the Department of Obstetrics and Gynecology participated in this study after signing the written informed consent. All subjects were matched for BMI (body mass index, 29–39 kg/m^2), ethnicity (Iranian), smoking (non-smoker). None of them had other background disease such as diabetes. Based on American College of Obstetricians and Gynecologists guidelines, preeclampsia is defined as increased blood pressure (≥140 mmHg systolic, or ≥90 mmHg diastolic on 2 or occasions at least 4 h apart) in addition to any of following features: significant proteinuria (≥0.3 g/L in 24 or ≥1 +), thrombocytopenia (low blood platelet count, platelet count ≤ 10^5/microliter), renal insufficiency (serum creatinine concentrations ≥ 97.24 μmol/L), and impaired liver function (doubling of blood transaminases concentration) after 20 weeks of gestation in a woman with a previously normal blood pressure [9]. Our subjects were selected in accordance with above guidelines and women with pre-existing preeclampsia and hypertension, twin pregnancy or history of any other disease were excluded from the study.

7 ml of maternal venous blood was collected in standard vacutainer tubes (EDTA, BD BioSciences, USA). The tubes were centrifuged immediately at room temperature (10^3 RPM/10 min) and plasma samples were kept at −70 °C before RNA extraction.

2.2. Quantification of circulating miRNAs

Total miRNAs were isolated from plasma (600 μL) using the miRCURY™ RNA Isolation Kit (Exiqon, Vedbaek, Denmark) using the manufacturer's protocol. To improve the efficiency of purification, carrier RNA (MS2 RNA; Roche, Basel, Switzerland) was added at 3 μg per each plasma sample to the lysis Solution BF. CDNA was synthesized by universal cDNA synthesis kit II (Exiqon, Vedbaek, Denmark) and miRNAs quantified by ExiLENT SYBR Green master mix and microRNA LNA PCR primer set (Exiqon, Denmark). We used miR-103 as reference gene, as recommended by the manufacturer and also used in other studies as a suitable reference gene in serum/plasma samples [23,24]. By automated default setting in real time PCR instrument (ABI step on), C_t was determined and average C_t of three replicate was calculated for each miRNA. Real-time qRT-PCR data was analyzed and normalized by REST software. To provide quality controls of the RNA isolation, the cDNA synthesis reaction and the PCR, synthetic RNA Spike-in, UniSp6 (an exogenous small RNA), was used (Exiqon, Denmark).

2.3. Statistical analysis

Data are presented as the mean ± S.E. Student's *t*-test was used to compare the clinical characteristics between the groups under study. Additionally, Pearson comparisons test was used to investigate the correlations between miRNA expression and clinical features. P-value < 0.05 was considered statistically significant. Statistical analysis was carried out by SPSS 22 software.

3. Results

The preeclampsia group had higher systolic blood pressure (SBP), diastolic blood pressure (DBP) and proteinuria and lower newborn weight (NBW) and gestational age at delivery (GAD) (both have $p < 0.05$) in comparison with the normal pregnancy (NP) group (Table 1). The C_t values of UniSp6 as the internal control in preeclampsia and NP groups were similar which shows that the isolation process is comparable between these two groups. According to our miRNA expression data, the overexpression of miR-210-3p and miR-517c-3p were statistically significant in preeclampsia compared to normal pregnancy group (Fig. 1).

4. Discussion

The miR-210-3p was up-regulated in the preeclampsia group in comparison with the NP group; with +3.340-fold change and P-value of 0.002. The miR-210-3p is located at 17p13.1 and is a member of the hypoximiR family, miRNAs that are susceptible to hypoxia and hypoxia is one of the main cause of the preeclampsia [17]. Ectopic expression of this miRNA effects *HIF1α* and *NFKB* (two important transcription factor in response to hypoxia) and has an important role in hypoxia response pathways such as angiogenesis, mitochondrial respiration, cell survivability and DNA repair [25]. Higher levels of miR-210 in placenta tissue of preeclamptic women can lead to down-regulation of genes including *EFNA3*, *HOXA9*, *ISCU*, *KCMF1* and *THSD7A*. These genes participate in migration and invasion of trophoblast. Placental dysfunction due to their dysregulation contributes to pathogenesis of preeclampsia [25–29].

The miR-517c-3p was up-regulated in preeclampsia group in comparison with NP group; with +2.272-fold change and P-value of 0.047. This miRNA belongs to the C19MC family. C19MC is one of the largest gene groups that has 46 miRNA genes and located at 19q13.41. It is

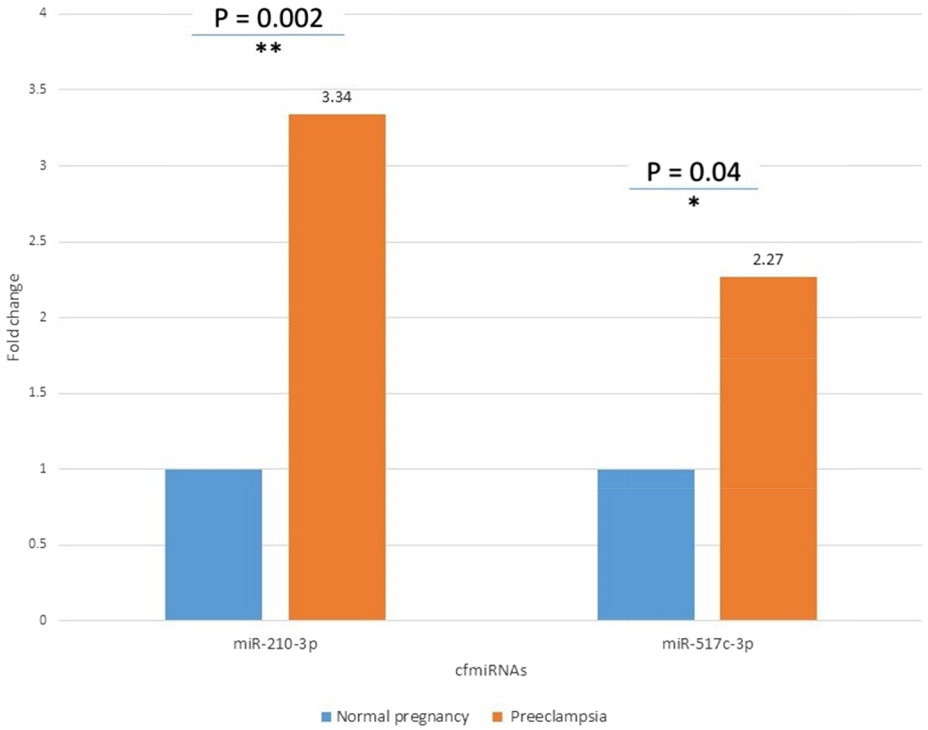

Fig. 1. Expression levels of circulating miR-210 and miR-517c in preeclamptic case-control study. The numbers above each columns are fold change. P-values are presented.

unique to primates and its genes expression is limited to the reproductive system and placenta [3]. Excess miR-517c-3p expression causes inhibition of cell proliferation in G2/S checkpoint, while lower expression of miR-517c-3p regulates the expression of protein tyrosine kinase 2 beta (*PYK2*) and promotes cell growth and proliferation [21]. Based on previous studies, plasma concentration of miR-517c-3p is higher in the placental abruption [30] and lower in spontaneous abortion [31]. Pathologic placental hypoxia can increase miR-517c-3p levels in placenta. Higher concentration of miR-517c-3p leads to decreased trophoblast invasion and increased *TNFSF15* expression. This factor changes *FLT1* splicing and produces higher amount of soluble FLT1 (sFLT1). sFLT1 is an anti-angiogenic protein (by blocking the

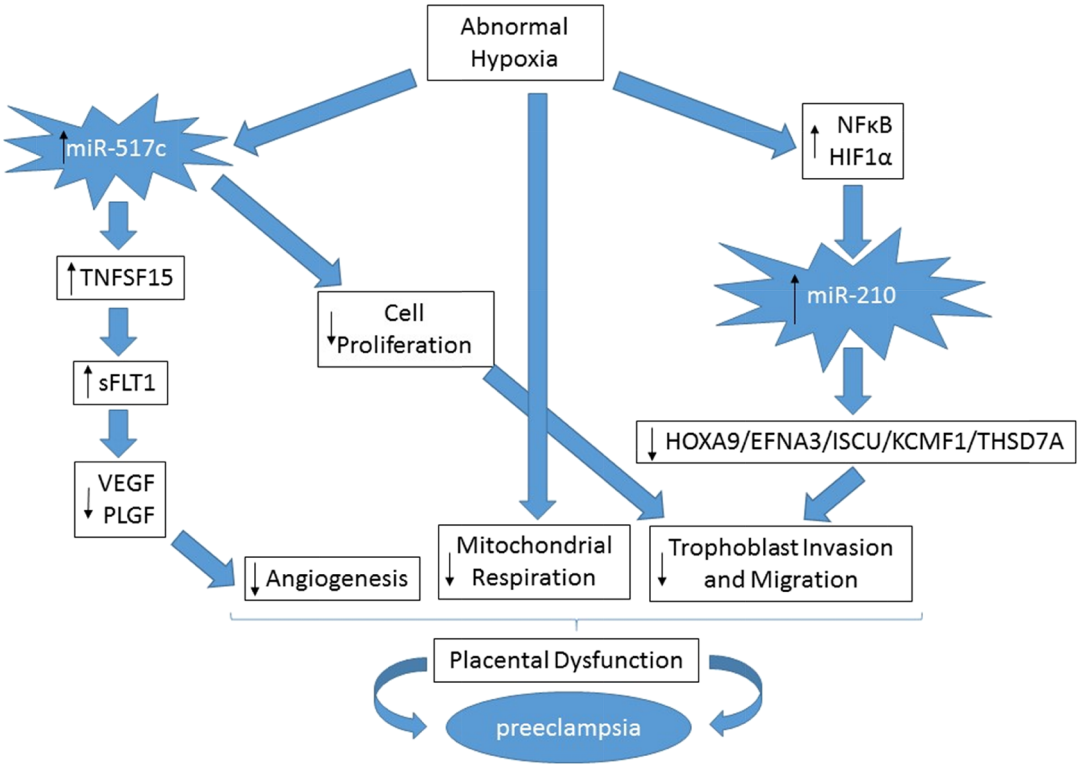

Fig. 2. Summary of the relationship between miR-210 and miR-517c functions and preeclampsia etiology.

Table 2
Correlation (Pearson) among C_t values of miR-210 and miR-517c and clinical features.

Pearson Correlation	miR-210 (r, p)		miR-517c (r, p)	
	PE	NP	PE	NP
Maternal age (years)	0.10 (0.67)	0.29 (0.22)	0.09 (0.69)	−0.09 (0.68)
Systolic blood pressure (mm Hg)	0.13 (0.57)	0.02 (0.91)	0.06 (0.79)	−0.35 (0.13)
Diastolic blood pressure (mm Hg)	−0.16 (0.50)	0.09 (0.67)	−0.15 (0.53)	−0.40 (0.78)
24-h urine protein (dipstick)	−0.04 (0.88)	–	−0.25 (0.28)	–
AST (U/L)	0.12 (0.6)	–	−0.12 (0.6)	–
ALT (U/L)	0.09 (0.94)	–	−0.29 (0.21)	–
Platelet count ($\times 10^3/\mu L$)	0.32 (0.17)	−0.27 (0.2)	0.10 (0.67)	0.11 (0.63)
Gestational age at delivery (week)	0.34 (0.14)	−0.025 (0.29)	0.12 (0.61)	0.23 (0.33)
New born weight (gram)	0.52 (0.019)*	0.24 (0.3)	0.57 (0.009)*	0.13 (0.59)

PE, Preeclampsia; NP, Normal Pregnancy; r, correlation coefficient; p, P value. Values are the ± SE.
* $P < 0.05$.

downstream signaling of *VEGF* and *PLGF*) and can reduce angiogenesis. Abnormal angiogenesis is a key factor in preeclampsia pathogenesis [10,22].

These findings suggest that degradation of the cells which are associated with hypoxic conditions in preeclampsia, the miRNAs enter the bloodstream. This may explain differential expression of our tested miRNAs in plasma samples in preeclampsia compared to normals. Fig. 2 represents miR-210 and miR-517c functions in relation to preeclampsia pathobiology.

We conducted correlation analyses between the two groups (Table 2). We found a positive correlation between miR-210 and miR-517c normalized C_t values, and NBW in preeclamptic women. This means that by increasing the expression levels of these cfmiRNAs (lower C_t values), the NBW decreases. As said, these miRs dysregulate conditions include hypoxia, angiogenesis and trophoblast invasion that result in placental dysfunction. Malfunction of placenta, as seen in severe and mild preeclampsia, is associated with significant fetal growth restriction (FGR) [32]. The FGR is defined as a failure to achieve potential for genetically determined growth [33]. Higher relative circulating levels of miR-210 and miR-517c could be a sign of more placental dysfunction and then more FGR (lower NBW) that can be an explanation for positive correlation between these miRs and NBW in preeclampsia group. There was no correlation between other characteristics and the C_t values of examined miRNAs.

Finally, we found that the expression levels of miR-210-3p and miR-517c-3p, were higher in the blood circulation of preeclamptic pregnancy. Functional analysis will be required to investigate the interaction of these miRNAs and preeclampsia-related genes. A clinical study with a larger sample size is needed to further confirm our findings.

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Declaration of interest

None.

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