Potential Diagnostic Value of Circulating miR-1183 and CHURC1 Biomarkers in Early-Onset Preeclampsia

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Abstract

Background

Preeclampsia (PE) is a multi-systemic disease of pregnancy and leading cause of maternal and fetal mortality and morbidity worldwide. The differential expression of circulating microRNAs (miRs) were reported in maternal plasma of pregnant women and they could be stabilized in plasma. The aim of this study was to characterize the molecular mechanism of PE development through miR-1183.

Methods and Results

Plasma samples were obtained between 26 and 32 weeks of gestation from early-onset PE cases (n=31) and women with normotensive pregnancies (controls, n=22). The expressions of miR-1183 and Churchill containing domain 1 (CHURC1) were measured in maternal plasma. CHURC1 3’ untranslated region was determined as the target of miR-1183 using in silico analysis. miR-1183 and CHURC1 expression levels were assessed by real-time polymerase chain reaction (RT-PCR).

An inverse correlation was found between circulating miR-1183 and CHURC1 expression levels with, miR-1183 up-regulated (p=0.002) and CHURC1 down-regulated (p<0.001) in maternal plasma of preeclamptic women compared to controls. The accuracy of levels of miR-1183 and CHURC1 according to area under receiver operating curve (ROC) analysis were characteristic curve analysis: the area under the curve (AUC) were: 0.79; (CI: 0.62-0.91) and 0.96; (CI: 0.78-0.99), respectively.

Conclusion

These findings not only characterize a new mechanism for the disease, but also provide potential therapeutic targets.

Introduction

Preeclampsia (PE) is a multisystemic disorder of pregnancy, defined as hypertension accompanied by proteinuria, and complicates 5%-8% of all gestations and develops after 20 weeks of gestation (1). One-quarter of stillbirths and neonatal deaths in developing countries are associated with preeclampsia and eclampsia complications (2). Even though the exact mechanism of PE remains largely elusive, it is confirmed that PE results from abnormal placentation which in turn leads to insufficient placental perfusion and ischemia (3). Subclassification of preeclampsia is controversial and defined as mild, moderate and severe, as well as early and late. The concept of early and late is more modern and it has been suggested that these are two entities with different pathophysologies (4). Early-onset PE (before 34 weeks) comprises 5%-20% of all PE cases worldwide. It is commonly associated with inadequate and incomplete trophoblastic invasion of maternal spiral arteries, fetal growth restriction and considerable additional maternal morbidity & mortality. Late-onset PE (after 34 weeks) constitutes more than 80% of all preeclampsia cases worldwide and is associated with a normally grown baby with no signs of growth
restriction and, normal or only slightly altered behavior of the uterine spiral arteries (5). Despite the lack of therapeutic treatment, predicting preeclampsia is an urgent and essential issue for management of maternal and fetal complications both mother and fetus. Since the subgroup of early-onset PE has more devastating outcomes, basic and clinical research is more focused on this entity.

Biochemical and ultrasound markers are being investigated as additional predictors for preeclampsia. There is a large body of work indicating a role of circulating angiogenic factors, such as soluble fms-like tyrosine kinase (sFlt-1) and the proangiogenic placental growth factor (PIGF), in the pathogenesis of preeclampsia. Women with preeclampsia have higher circulating levels of sFlt-1 and lower levels of PIGF were shown in some studies but clinicians and researchers still on the hunt for more definitive tests.

MicroRNAs (miRNAs) are small noncoding RNAs that act at a posttranscriptional level to degrade or rarely activate the expression of target mRNA molecule by complementary base pairing in the 3’ untranslated region (3’UTR) of the mRNA. Different cells/tissues/organs possess different miRNA expression profiles (6). miRNAs also exist in circulation, and a number of extracellular fluids, which are called circulating miRNA or cell-free miRNA. They may serve as non-invasive biomarkers for cancer, diabetes, central nervous system disorders, and pregnancy associated disorders (7, 8). In pregnancy, miRNAs which are synthesized by human trophoblast cells, can be secreted into the maternal circulation (9). They have the potential of serving as biomarkers in diagnosis of pregnancy associated disorders. Their exact roles in the pathogenesis of PE still need further investigation.

Trophoblast cells are specialized cells of the placenta. The proliferation, differentiation and apoptosis of trophoblasts may contribute to the development of PE. Multiple signaling pathways are involved in regulating the proliferation and apoptosis of trophoblasts during the placentation process, including the TGF-β signaling pathway (10). Our group has recently reported that miR-1183 is one of the upregulated circulating microRNAs in PE cases compared to controls with microarray analysis (11). Herewith the aims of this study were to 1) compare the circulating levels of miR-1183 by qPCR 2) investigate the putative target mRNA molecule of miR-1183 via in silico target analysis tools and detect expression levels of the target gene in the maternal plasma 3) Evaluate the miRNA and the target gene’s expression profiles as biomarkers and their potential pathological role in the development of early-onset PE.

Material And Methods

Subjects

Maternal peripheral blood samples were collected (n = 53) from the early-onset preeclamptic patients between 26 and 32 weeks of gestation (n = 31) for study group and women with normotensive pregnancies (NP) for control group (n = 22). Preeclamptic patients were diagnosed between years 2013–2015 at the departments in Obstetrics and Gynecology departments in Istanbul-Cerrahpasa University and Medicus Health Center, Istanbul. Diabetes mellitus, chronic hypertension, acute or chronic infectious diseases or other chronic illnesses are our exclusion criteria and continual drug use was not reported.
Low-dose aspirin (81 mg/day) prophylaxis is recommended in high-risk patients, mainly those with a history of preeclampsia. Our patients did not have previous preeclampsia history and aspirin usage did not reported.

**Plasma miRNA Quantification**

Blood samples were collected in EDTA tubes (Becton Dickinson, Cat. No.366643). Each peripheral blood sample was centrifuged within 4h of collection at 1600g for 15 min and plasma samples stored at -80°C until the RNA isolation step.

Total RNA that contained circulating miRNAs were isolated from maternal plasma (500µl) using the mirVana miRNA Isolation Kit (#AM1560, Life Technologies, Carlsbad, CA, USA). During the miRNA isolation, 1.6x10^8 copies of a synthetic miRNA mimic that is only expressed in *Caenorhabditis elegans* and not in mammals is spiked into each plasma sample prior to the RNA isolation for precise quantification (*cel-mir-39*, Qiagen, Valencia, CA, USA). The circulating miRNA concentration was determined by the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). 5x Taqman RT primers (Thermo Scientific) and other kit ingredients were added according to the manufacturer's protocol. Real time PCR for miRNA expression profiling was done using Taqman MicroRNA Assay (Thermo Scientific) according to the manufacturer's protocol. Each sample was run in duplicate for analyses.

**Plasma mRNA Quantification**

Plasma samples were thawed on ice and further centrifuged 2000g for 5 min at 8°C in order to remove cellular debris. RNeasy Mini Kit (Qiagen) was used to extract total RNA from 500µl of plasma from each sample, by adding 1000µl of Trizol LS (Life Technologies), and finally the samples eluted in 20µl of nuclease-free water. cDNA was synthesized by the Random Hexamers (pdN₆) (Roche Diagnostics) and M-MLV Reverse Transcriptase (Life Technologies) from 500 ng of total RNA. cDNA samples were stored at -20°C until further steps.

mRNA expression was performed using LightCycler 480 instrument (Roche Applied Sciences, Germany). The specific primer-probe sets were designed using the Universal ProbeLibrary System Assay Design for human (Roche Molecular Systems, Inc.). *GAPDH* was used as reference gene for expression analysis.

**Statistical and Data Analysis**

Continuously distributed demographic and clinical variables are presented as mean with SD and were compared using an unpaired *t*-test. Plasma miRNA levels among different groups of subjects were normalized by exogenous control *cel-mir-39*. The relative quantification method (2^ΔΔCt) was used to evaluate quantitative variations based on the mathematical model described by Livak et.al (12). Statistical analyses were carried out by Mann-Whitney test. Spearman correlation coefficients were calculated to examine the correlation between the circulating miRNA and the target gene expressions. The receiver-operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to
evaluate the sensitivity and specificity of miRNA and mRNA biomarkers for the diagnosis of PE. ROC analyses were done by the MedCalc Statistical Software version 18 (MedCalc Software bvba, Ostend, Belgium). All statistical evaluations and graphs, except for the ROC analysis, were performed using the GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). p values of < 0.05 were considered statistically significant.

Results

Clinical characteristics of PE and NP groups are shown in Table 1. The mean age of the subjects was 31.23 ± 0.75 in the case group, and 31.91 ± 0.8 in the control group. Student’s t test showed no significant difference between the two groups in terms of age (p = 0.53). Gestational age at delivery in the PE group was significantly earlier than those in the NP group (p < 0.0001). Besides, fetal birthweight in the PE group was significantly lower than the NP group (p < 0.0001) (Table 1).

Table I

Demographic and clinical characteristics of NP and PE.

| Demographic and Clinical Characteristics | NP (n = 22) | PE (n = 31) | P-value |
|-----------------------------------------|------------|------------|---------|
| Maternal age (years)                    | 31.91 ± 0.8| 31.23 ± 0.75| NS      |
| BMI (kg/m²)                             | ND         | 31.3 ± 0.68 | ND      |
| Gestational age at delivery (weeks)     | 37.91 ± 0.5| 29.39 ± 0.41| < 0.0001|
| Systolic blood pressure (mmHg)          | ND         | 159 ± 3.81  | ND      |
| Diastolic blood pressure (mmHg)         | ND         | 100 ± 2.00  | ND      |
| Fetal birthweight (g)                   | 3190 ± 128.5| 1284 ± 104.7| < 0.0001|

In this study a correlation of miR-1183 expression with systolic blood pressure in 15 patients revealed only a slight trend towards an inverse correlation (Fig. 1).

Target Gene Identification and Bioinformatic Analysis

In order to determine the target gene of miR-1183, we employed bioinformatics approaches and used in silico target prediction tools, including miRanda (13), TargetScan (14), DIANA-microT-CDS (15). Churchill domain containing 1 (CHURC1) to be a potential targeted gene of miR-1183 with a perfect match at 9 (nine) base-pair and 2 (two) G-U wobble base pair, which stabilizes the miRNA oligonucleotide-mRNA duplex and enhancing the silencing (Fig. 2A). Also, CHURC1 was selected because of the insufficiency in the number of the studies and the function of the gene is notably unknown. Primer sequences of CHURC1
are 5'-GGACATTCCCTGTTGACTGC-3' (forward) and 5'-TGCACAGCCTGTAAAGTTCAGT-3' (reverse) and UPL probe no 80.

**Circulating miR-1183 and CHURC1 were differentially regulated in plasma from women with PE**

Plasma samples collected from 18 preeclamptic and 15 normotensive women, were subjected to qPCR process. The circulating level of miR-1183 in maternal plasma significantly increased in the PE compared to controls (Fig. 2B, Mann-Whitney test, p = 0.002). Following the discovery of miR-1183 dysregulation in women who went on to develop preeclampsia, relative expression of *CHURC1* in the maternal plasma was tested. Expression level of *CHURC1* dramatically decreased in PE cases compared to controls (Fig. 2C, Mann-Whitney test, p < 0.0001). Spearman correlation coefficient examined between the circulating miR-1183 and *CHURC1*. Even though there was no significant correlation (p = 0.5289), plasma expression levels of miR-1183 and *CHURC1* represented moderate inverse correlation (r = -0.1384, number of XY pairs = 22). The results are compatible with our previous work in PE and NP group which is performed by microarray (11). The expression of miR-1183 was chosen to be the most diminishing miRNA and according to microarray results upregulation of miR-1183 was statistically significant (p < 0.05).

**Diagnostic role of miR-1183 and CHURC1**

In order to evaluate the discriminative value of the circulating miRNA and the target gene for PE, the ROC curves were established for miR-1183 and *CHURC1*’s elevated expression levels. The AUC for miR-1183 is 0.798 (p < 0.001), indicating a powerful prediction for PE. The AUC for *CHURC1* gene is 0.962 (p < 0.001), almost 1.0, indicating perfect accuracy of the data (Fig. 3, Table 2).

Table II: Receiver Operating Characteristic (ROC) parameters for plasma miR-1183 and *CHURC1*.

| ROC Curve       | miRNA-1183 | *CHURC1* |
|-----------------|------------|----------|
| Area under curve (AUC) | 0.798      | 0.962    |
| 95%CI*          | 0.622–0.917 | 0.788–0.999 |
| p - value       | 0.0002     | < 0.0001 |
| Sensitivity     | 66.67      | 92.31    |
| Specificity     | 100.00     | 90.00    |

*95%CI: 95% Confidence Interval*

The biological functions, molecular processes, and pathways of the *CHURC1* gene were determined using the online tool Panther-GO (Table 3).
Table III

CHURC1 gene ontology and pathway analysis

| Tool name        | Ontology          | Function                                   | Accession     |
|------------------|-------------------|--------------------------------------------|---------------|
| GeneOntology     | biological process| multicellular organism development         | GO:0007275    |
| GeneOntology     | biological process| positive regulation of transcription, DNA-templated | GO:0045893    |
| GeneOntology     | molecular function | zinc ion binding                           | GO:0008270    |
| Reactome Pathway | no pathway information available | -                                      | -             |
| KEGG Pathway     | no pathway information available | -                                      | -             |

Discussion

Preeclampsia remains a leading cause of maternal and fetal morbidity and mortality. Accurate prediction of PE is a significant challenge in maternal and fetal medicine. There are several studies showing that many miRNAs are selectively elevated in maternal plasma/serum of women with PE (16), suggesting a pivotal role of this epigenetic mechanism.

In the present study, the results indicate that the levels of circulating miR-1183 significantly increases in the maternal plasma of preeclamptic women. miR-1183 studies in the literature are extremely limited. One study has reported that miR-1183 overexpression may reflect pulmonary artery remodeling in rheumatic heart disease (RHD) patients with secondary pulmonary hypertension (PAH) (17). Another study has showed that ox-LDL increases the ROCK2 gene expression by reducing miR-1183 levels. A single nucleotide polymorphism at the 3'UTR of ROCK2 gene could affect the miR-1183 binding site and consequently increased ROCK2 expression is related to arterial stiffness (18). miR-1183 has also been reported to be involved in cancer progression. It has been shown that miR-1183 expression is altered in rectal cancer and Kaposi’s sarcoma samples (19, 20). Another study has reported that miR-1183 has a relationship with a functional polymorphism in the EpCAM gene in cervical cancer patients (21). This is the first study to define the role for circulating miR-1183 in pregnancy in particular, preeclampsia.

Our analysis has identified CHURC1 as a predicted target gene of miR-1183 and has shown its dowregulation in maternal plasma of preeclamptic women. Moderate inverse correlation between the circulating miR-1183 and the CHURC1 expression levels have suggested that CHURC1 could be at least one of the genes modulated by miR-1183. Since the correlation between miRNA-target gene pair is not statistically significant, expression changes of miRNA and mRNA regulated by various factors could cause the changes of correlation between them, like transcription factors and endogenous long non coding RNAs. Also, correlation coefficient could be affected due to the small sample size. CHURC1 gene
encodes a zinc finger protein that acts as a transcriptional activator. In the case of preeclampsia, only one study has evaluated the expression of \textit{CHURC1} in the peripheral blood mononuclear cells (PBMC) from preeclamptic women and demonstrated downregulation of \textit{CHURC1} \cite{22}. It is difficult to compare our results with that study since we have used the maternal plasma instead of PBMC as the source of \textit{CHURC1}. Notably, under fibroblast growth factor (FGF) control, \textit{CHURC1} represses cell migration in gastrulation and it has been shown that \textit{CHURC1} represses BMP signaling by the Sip1 and SMAD proteins \cite{23,24}. There is one study showing that BMP signaling through BMPR2 is essential during early mouse development. Deletion of BMPR2 results embryonic death \cite{25}. BMP signaling is modulated extracellularly by Noggin or intracellularly by FKB12, I-Smads, phosphatases, and microRNAs. \textit{BMPR2} encodes for a transmembrane serine/threonine kinase receptor which belongs to the transforming growth factor beta (TGF-\(\beta\)) superfamily, and initiates BMP signaling \cite{26}. TGF-\(\beta\) signaling pathway is a known regulator of placent al trophoblastic invasion and migration. TGF-\(\beta\) exerts several regulatory effects on the trophoblast cells, such as inhibition of proliferation and invasion \cite{27}, and it plays an autocrine role in regulating the gene expression in human trophoblast cell lines \cite{28}. Furthermore studies on the BMPR2 function in placental development in cKO mice, has showed that BMPR2 mediated signaling and CORIN gene has a major role on the development of preeclampsia by inducing trophoblast invasion. Nagashima et.al demonstrated that \textit{Corin} mRNA and protein levels were dramatically decreased in isolated decidual tissues of \textit{Bmpr2} cKO uteri in mice \cite{29}. These results indicate that signaling through BMPR2, directly or indirectly regulates CORIN expression and controls trophoblastic invasion. Impaired CORIN expression or function in pregnant uterus is associated with preeclampsia \cite{30}. In case of preeclampsia, where there is increased trophoblastic invasion, and placental blood flow, trophoblasts might repress \textit{CHURC1} expression and stimulate the over-expression of miR-1183. The PANTHER database revealed the results of biological processes and molecular functions of the \textit{CHURC1} gene \cite{Table 3}. As we examine these results, there is not any pathway information about the \textit{CHURC1} gene in databases and gene's function is needed to further investigate.

As the alterations identified in this study were seen at 26 to 32 weeks of gestation, miR-1183 upregulation may be a response to the trophoblastic invasion characteristic of preeclampsia. We hypothesize that after the occurrence of the disease, trophoblasts attempt to suppress \textit{CHURC1} expression to increase the trophoblast invasion in placenta. For this purpose, trophoblast cells may increase its negative regulator, miR-1183. Thus, it cannot be the factor that cause the disease, but it might have a regulating effect once PE occurs. This has led us to suggest that the increased miR-1183 might be overexpressed to reduce the \textit{CHURC1} expression, as suggested in our model presented in Figure 4.

In conclusion, the circulating miR-1183 levels were up-regulated, and \textit{CHURC1} was identified as a putative target of miR-1183 and down-regulated in maternal plasma of preeclamptic women. Since miR-1183 and \textit{CHURC1} have a high discriminative value and significant differential expression scores, they might be the potential biomarkers for the prediction of PE. To our knowledge, this is the first study dealing with these relationships in the maternal plasma of preeclamptic women. The mechanisms by which \textit{CHURC1} modulates the PE pathogenesis need to be further studied and functional cell culture studies in trophoblast cell lines are planning to be done.
Declarations

Authors' Contributions

Each author carried out manuscript drafting and editing. All authors read and approved the final version of the manuscript.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article. The datasets which contain clinical data are not publicly available regarding to ethical and legal responsibility to respect participants’ rights to privacy.

Authors' Contributions

Each author carried out manuscript drafting and editing. All authors read and approved the final version of the manuscript.

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Figures
Figure 1

Correlation of miR-1183 Expression with Systolic Blood Pressure. A slight trend to inverse correlation was observed for miR-1183 with systolic blood pressure (Number of XY pairs=15). Significance was assessed by Spearman's correlation.
Figure 2

Differences in expression of circulating miR-1183 and CHURC1 in the PE and control groups. A) The target site for miR-1183 sequences in 3'UTR of CHURC1. B) Relative expression level of circulating miR-1183, p= 0.002 by Mann-Whitney test. miR-1183 expression was normalized with cel-mir-39. C) Relative expression level of circulating CHURC1, p<0.0001 by Mann-Whitney test. CHURC1 expression was measured in comparison to GAPDH by qPCR.
Figure 3

Receiver Operating Characteristic (ROC) curves representing PE diagnostic tests by miRNA-1183 and CHURC1 in PE and control subjects. ROC curve for miRNA-1183 is a good test since the area under the curve (AUC) is 0.798 (p<0.001), with 18 samples for PE and 15 for control. The ROC curve analysis for the CHURC1 gene is an excellent test with an AUC value of 0.962 (p<0.001), number of samples for PE=13 and control=10. The diagonal line represents a reference line showing zero sensitivity and zero specificity.
Figure 4

A model suggesting that increased circulating miR-1183 expression represses target CHURC1 and CHURC1 represses its BMPR2 target genes, including CORIN.