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Original Article

Expression and significance of miR155 and vascular endothelial growth factor in placenta of rats with preeclampsia

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Abstract: Objective: This study is to investigate the expression and significance of miR155 and vascular endothelial growth factor (VEGF) in placenta of preeclampsia (PE) model rats. Methods: A total of 20 SD rats were randomly divided into normal pregnant group and PE model group. PE model was established by subcutaneous injection of L nitro arginine methyl ester continuously for 4 days with a dosage of 200 mg/Kg·d from the 13th day of pregnancy. Blood pressure, urinary protein and renal function were detected to evaluate fetal development. Real-time fluorescent quantitative polymerase chain reaction was used to test the expression of placental miR155. Western-blot assay and ELISA were performed to detect the expression of placental VEGF protein. Results: Blood pressure, urine protein, blood urea nitrogen and creatinine in PE model group were higher than in normal pregnant group, and there was statistically significant ($P < 0.05$). Expression of miR155 in PE group was higher than that of normal pregnant group and VEGF protein expression was lower than that in normal pregnant group, both were statistically significant ($P < 0.01$). Conclusion: In PE model group, miR155 expression significantly increases whereas the expression of VEGF decreases. PE might be correlated with the down regulation of VEGF by miR155.

Keywords: MiR155, vascular endothelial growth factor, preeclampsia, rat model

Introduction

Preeclampsia (PE) refers to a series of diseases manifested as hypertension and proteinuria after 20 weeks of pregnancy while blood pressure was normal and no proteinuria appeared previously in women. PE is one of the main causes of maternal and perinatal death, and it is a serious disease which threats maternal and infant health [1, 2]. The incidence of PE in China is about 12-21% and to date, the mechanism of PE is unknown. In recent years, microRNA (miRNA) has attracted much attention in the regulation of gene expression at post transcription level. It has been reported that miRNA plays important roles in development, cell proliferation, apoptosis, differentiation and tumorigenesis [3].

The role of miRNA in PE patients is noticed as well. Differential expression of miRNA is found in placenta in both mouse and human. For example, the expressions of miR-210, miR-152 and miR-518b increase while the expressions of miR-411, miR-377, miR-18a and miR-363 decrease in PE placenta [4]. This situation suggests that miRNA may play a certain role in the process of placental growth and fertility. Following early indications, when fetal membrane inflammation occurs or the body is in hypoxic conditions, these pathological changes related miRNA distribution changes will occur. As an inflammation related miRNA, differential expression of miR155 is common in PE placenta [5]. Vascular endothelial growth factor (VEGF) is associated with placental trophoblastic cells. Reduced expression of VEGF will lead to too shallow invasion of trophoblast cells to uterine grassroots, which, will cause placental ischemia thus leading to PE [6]. The miR155 and VEGF may play an important role in the occurrence and development of PE, in this study, methods like real-time fluorescent quantitative polymerase chain reaction were used so as to investigate the expression significance of miR155 and VEGF in PE.

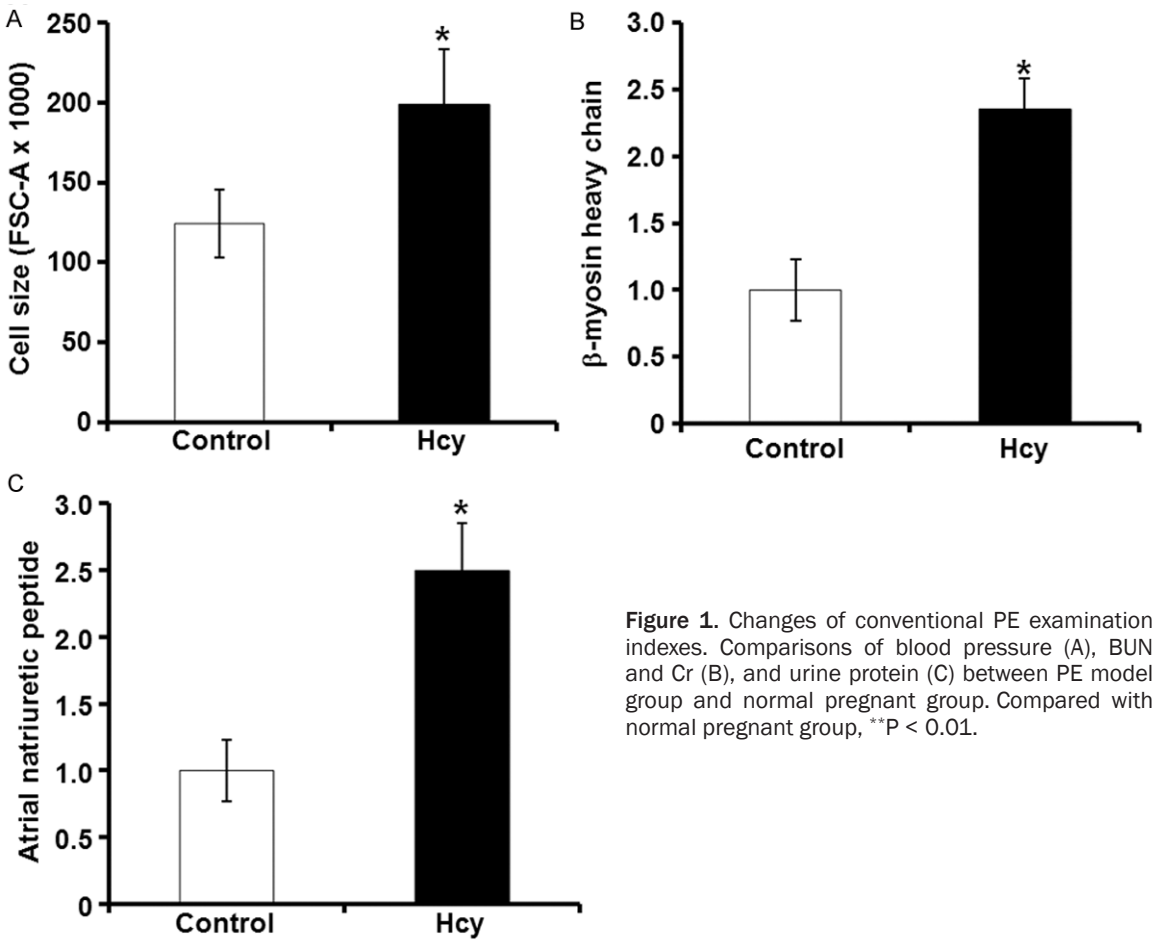

Figure 1. Changes of conventional PE examination indexes. Comparisons of blood pressure (A), BUN and Cr (B), and urine protein (C) between PE model group and normal pregnant group. Compared with normal pregnant group, $^{**}P < 0.01$.

Table 1. Fetal developmental assessment

Group	Number of normal rat fetus	Fetal weight (g)	Placental weight (g)
Normal pregnant group	9.74 ± 0.37	3.11 ± 0.24	0.58 ± 0.06
PE model group	7.41 ± 0.32**	2.32 ± 0.18**	0.41 ± 0.02*

Note: Compared with normal pregnant group, $^{*}P < 0.05$, $^{**}P < 0.01$.

Material and methods

Model preparation

A total of 20 SD rats were enrolled and divided equally into normal pregnant group (n = 10) and PE model group (n = 10) randomly. The rats were raised with the female-male ratio of 1:1 in the same rearing cage for mating and pregnancy was confirmed by checking vaginal suppository. PE model was established by subcutaneous injection of L nitro arginine methyl ester (L-NAME) continuously for 4 days with a dosage of 200 mg/Kg·d from the 13 day of pregnancy. At the 3 day of pregnancy, noninvasive blood pressure measurement was performed and rats with blood pressure of 30 mmHg or above higher than pre pregnancy indicating successful modeling. All animal experiments were conducted according to the ethical guidelines of Renmin Hospital of Wuhan University.

Blood pressure, urinary protein and renal function measurement

Non-invasive rat tail blood pressure detecting method was used to detect blood pressure before pregnancy, at 15 day and 21 day of pregnancy, respectively. Twenty-four hour urine of before pregnancy, at 15 day and 21 day of pregnancy was collected for urine protein analysis. At the 21 day of pregnancy, 3 ml peripheral blood was collected for blood urea nitrogen (BUN) and creatinine (Cr) detection.

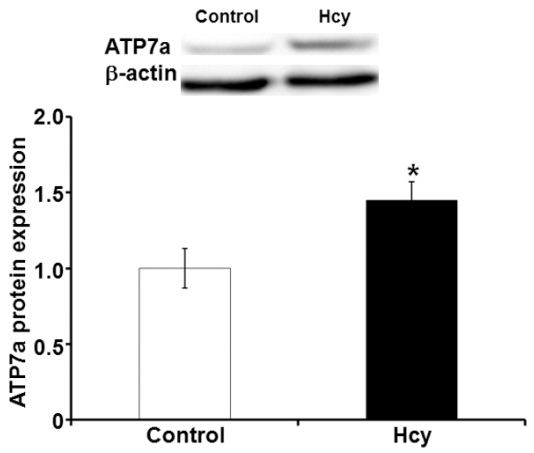

Figure 2. Expression of miR155 in the placenta. Level of miR155 in the placenta was detected by qRT-PCR. The relative expression level of miR155 gene was shown. Compared with normal pregnant group, $^{**}P < 0.01$.

Placenta collection

Caesarean section was performed at 21 day of pregnancy. Fetus number of normal rats was counted, meanwhile, rat fetal and placental weight were weighed. The placentas were clipping into about 1 cm^3 in size with sterilized instruments and were putted into freezing tubes. For each rat, 4 tubes of tissue were collected and the central part of the placenta near parent decent was clipped as far as possible to avoid calcification. After collection, the tissue was frozen overnight in liquid nitrogen and was stored at -80°C.

Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted with Trizol (sigma, St. Louis, USA) from the placenta and total mRNA reverse transcription reaction was performed using PolyA plus tail method. The following reverse transcription reaction system was taken: 0.15 μl 100 mM dNTP + 0.19 μl RNase inhibitors + 3.0 μl reverse transcription primers + 5.0 μl (10 ng) total RNA + 6.66 μl double distilled water. The synthesized cDNA was stored at -20°C. PCR amplification kit was purchased from Takara (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) and U6 RNA was used as internal reference. Primer sequences were synthesized by Sangon Biotech (Shanghai) Co., Ltd (Shanghai, China). The upstream primer of U6 was 5'-CTCGCTTCGGCAGCACA-3', the downstream primer of U6 was 5'-AACGCTTCACGAATTTGCGT-3' and the universal primer was 5'-CGCCGC CCAGTGTTCAGA-3'. The primers of miRNA-15 were 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAAGTTGAGACCCTAT-3' (upstream primer) and 5'-ACACTCCAGCTGGGT TAATGCTAATCGTCAT-3' (downstream primer), respectively. Quantitative fluorescence PCR reaction system was: 10 μl 2 × PCR buffer + 1 μl miR155 primers + 1.33 μl cDNA + 6.67 μl double distilled water. PCR products were tested by 12% polyacrylamide gel electrophoresis and the gel was observed with AlphaImager gel imaging systems, (Santa Clara, California, USA). Fluorescence was detected with ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA, USA). Relative expression rate of sample target gene (relative expression, RQ) was calculated using the ΔΔCT method, RQ = 2-ΔΔCT (CT represented the number of cycles while real time fluorescence intensity was significantly greater than background value; ΔΔCT = CT sample-CT U6 sample; Δcontrol = CT control-CT U6 control, ΔΔCT = ΔCTsample-ΔCTcontrol).

Western blot assay

Placenta in normal pregnant group and PE model group was taken and placed on ice box, and then 200 μl cell lysate buffer containing 2 μl PMSF and protease inhibitor was added. After incubated for 10 min, the lysate was centrifuged at 12000 g for 10 min at 4°C and the supernatant was kept for further analysis. BCA method was used for protein concentration detection. The samples were then applied to SDS-PAGE and were transferred to PVDF membrane. Primary antibody against VEGF and β-actin was added and incubated overnight. After extensive washing, secondary antibody was added and incubated for two hours at room temperature. β-actin was used as internal reference. The result was analyzed using image analyzer and the protein bands absorbance value was determined by Image Pro-Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

ELISA assay

ELASA assay was used for detecting the expression of VEGF protein. Briefly, the standard was reconstituted into different concentrations by

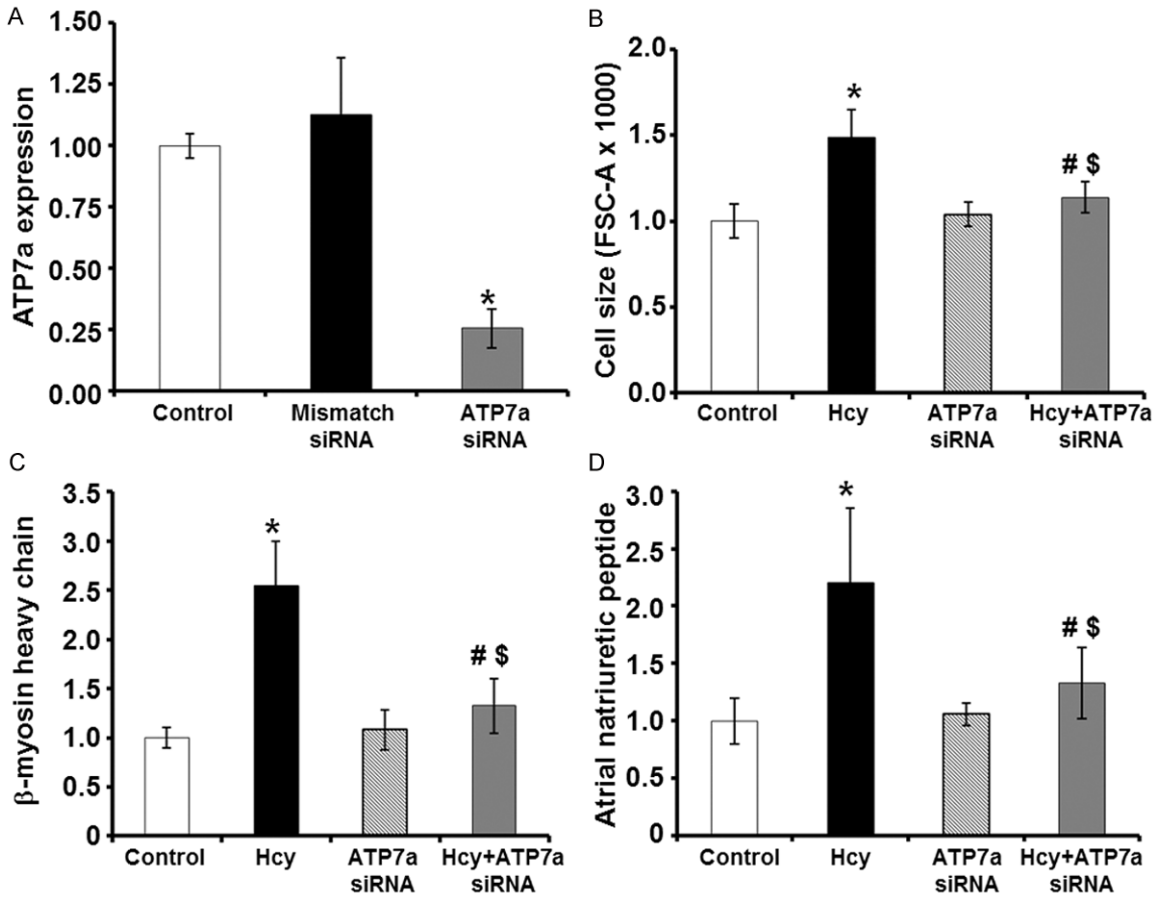

Figure 3. Expression of VEGF in placenta detected by Western blot assay and ELASA method. A. Representative (left panel) and quantitative (right panel) Western blot results. B. Level of VEGF in placenta detected by ELISA. Compared with normal pregnant group, $^{**}P < 0.01$.

addition of distilled water, the Standard Concentration was considered as horizontal axis, and OD Values were taken as the vertical axis. The regressed data were used to create a standard curve using computer software. The OD was detected at the wavelength of 450 nm with xMark microplate reader (Bio-Rad, Hercules, CA, USA) and the concentrations of placental tissue homogenate was calculated according to the standard curve.

Statistical analysis

All the statistical analyses were performed using SPSS version 17.5 (SPSS Inc, Chicago, IL, USA) for Windows. The results were expressed as Mean ± SD. One-Way ANOVA was carried for analysis of differences among multi groups and SNK method was used for comparison between groups. $P < 0.05$ was considered as statistically significant.

Results

The changes of blood pressure, urinary protein and renal function in PE model rats

To identity whether PE model was successfully established, the conventional PE evaluation indexes including blood pressure, urine protein, BUN and Cr levels were measured. As shown in **Figure 1A**, blood pressure in PE group significantly increased at 15 day and 21 day of pregnancy and there was statistically significant difference ($P < 0.01$) compared with normal pregnant group. BUN and Cr levels in PE group were higher than those in normal pregnant group, with significant difference ($P < 0.05$) (**Figure 1B**). The changes in urine protein showed similar trend as blood pressure, with urine protein at 15 day and 21 day of pregnancy in PE group significantly higher ($P < 0.05$) (**Figure 1C**). Together, the results indicated that PE model was successfully established.

PE fetal developmental assessment

In order to understand whether PE had effect on fetal development, fetal in both groups were examined and the results were compared. In this study, stillbirth appeared in PE model group and fetus number of normal pregnant group was more than that of PE group, with significant difference ($P < 0.05$). Meanwhile, fetal weight and placental weight in PE model group decreased significantly, and the difference had statistical significance ($P < 0.05$) (**Table 1**). To sum up, the results argued that PE had a negative impact on fetal development.

Expression of miR155 in the placenta

To test whether there was difference in miR155 expression in the placenta of both groups, qRT-PCR was performed. The results showed that miR155 and U6 expressed in both PE model group and normal pregnant group. The expression of miR155 in PE model group was higher than that in the normal pregnant group and the difference was statistically significant ($P < 0.05$) (**Figure 2**). In all, the results showed that miR155 expression in the placenta increased in PE model group while compared with normal pregnant group.

Expression of VEGF protein in placenta

To determine whether PE affects expression of VEGF in placenta, VEGF protein expression was tested by Western blot analysis and ELASA assay. As shown in **Figure 3A**, expression of VEGF in PE model group was lower than that in normal pregnant group, with statistically significant ($P < 0.01$). This was further verified by ELISA assay. The content of VEGF in normal pregnant group and PE model group were 108.55 ± 21.07 pg/ml and 51.47 ± 17.52 pg/ml, respectively, and there was statistically significant ($P < 0.01$) (**Figure 3B**). Given all this, it is possible to assume that expression of VEGF in placenta was negatively affected by PE.

Discussion

PE is a kind systemic syndrome behaved mainly as primary hypertension and proteinuria. The incidence rate of PE is about 3% to 5%, and PE is one of the leading causes of maternal mortality, premature delivery and neonatal death [7]. PE pathogenesis is rather complicated, however, shallow implantation of placenta is the central link in the pathogenesis of pregnant hypertensive disorder. Shallow implantation of placenta is related to proper proliferation, differentiation, migration, invasion and apoptosis of trophocyte during pregnancy. In this process, angiogenesis and angiogenesis associated factors play a regulating role. If angiogenesis was abnormal, it will lead to disorders of placental vascular network construction, cause uterine and placental circulation anomalies, result in abnormal pregnancy trophoblast cells, and eventually induce eclampsia [8]. VEGF is secreted by endothelial cells, which, plays a key role in endothelial cell proliferation and vascular lumen formation during angiogenesis. Vascular construction function that VEGF plays is rather important during pregnancy, this situation is associated with placental trophoblastic cells. VEGF specific receptor expression exists in extravillous trophoblast cell surface. After interaction of VEGF specific receptor with VEGF, extravillous trophoblast cell proliferation will be promoted and the invasion function will be fully played [9, 10]. Stimulated by cytokines like leptin, expression of VEGF via trophoblast cells will be upregulated and at last invasion and angiogenic function of trophoblast cells are promoted [11].

Two periods named vasculogenesis and angiogenesis are included in placental vascular network construction. In early pregnancy, hemangioblasts differentiate into vascular endothelial cells thus build primitive vascular. And then, placental capillaries form spiral, expand and protrude into trophoblastic cells. At the same time, for part of the capillary, angiogenesis is achieved in budding way and participates in the formation of pregnant placenta [12]. VEGF can regulate angiogenesis of villi in early pregnancy, and decrease of VEGF will lead to the shallow invasion of trophoblast cells to uterine grassroots, causing placental ischemia and leading to PE [13].

miRNA has gained more importance in PE. miRNA is a kind of endogenous single non coding RNA with the length of about 22 nucleotides. miR155 is a kind of inflammation related miRNAs, and differentially expression of miR-155 in PE in the placenta has been reported. miR155 is expressed highly in placenta in severe PE patients, however, the relationship between miR155 and PE is not clear [14]. Mature miR155 is 23 nt, locating on chromo-

some 21 in the 3rd exon, and is related to immune function and cell apoptosis [15]. Activated T cells, B cells and macrophages can induce miR155 expression, and in miR155 knockout mice, impaired immune function appeared [16]. In pancreatic cancer cells, miR155 can down regulate nuclear protein induced by tumor, thus plays an anti-tumor generating function by caspase3 mediated apoptosis [5]. To date, the relationship between miR155 and PE angiogenesis has not been reported. We hypothesized that miR155 may lead to the incidence of PE by affecting apoptosis and angiogenesis in placenta cells, as a result, we investigated miR155 and VEGF expression in placenta of severe PE rats.

As the length of mature miR155 is too short, miR155 expression could not be detected by conventional RT-PCR. Therefore, special designed stem loop structure of the reverse transcription primers which could specifically combine with the mature miR were used in this study. Once combined, a reverse transcription primer/mature miR complex was formed, and the content of miR155 was detected in accordance with the quantitative PCR method.

In this study, the results showed that the expression of miR155 in PE model group was higher than normal pregnant group and placental miR155 expression difference was statistically significant ($P < 0.05$). At the same time, VEGF content in PE model group was lower than normal pregnant group, with statistically significant ($P < 0.01$). Therefore, this study confirmed that the expression of miR155 in PE rat model was negatively related to VEGF expression. It is possible to assume that PE occurrence might be linked to trophoblast cells angiogenesis, inflammation regulation disorder and cell apoptosis caused by miR155 induced down-regulation of VEGF expression. The mechanism through which miR155 down regulates VEGF expression and the relationship between them in the formation of increased blood pressure still need further research.

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Disclosure of conflict of interest

None.

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