MicroRNA-155 contributes to preeclampsia by down-regulating CYR61

Yanqing Zhang, MD; Zhenyu Diao, PhD; Li Su, MD; Haixiang Sun, MD, PhD; Ruotian Li, MD; Hengmi Cui, PhD; Yali Hu, MD, PhD

OBJECTIVE: The aim of this study was to characterize the molecular mechanism of preeclampsia (PE) development through miR-155.

STUDY DESIGN: PE and normal placentas were used to measure miR-155 and cysteine-rich protein 61 (CYR61) expression. CYR61 3' untranslated region was validated as the target of miR-155 using in vitro transfections. miR-155 and CYR61 expression levels were assessed by real-time reverse transcription polymerase chain reaction or Western blot.

RESULTS: An inverse correlation was found between miR-155 and CYR61 expression levels, with miR-155 up-regulated and CYR61 down-regulated in PE tissues. Luciferase assays and CYR61 transfection assays experimentally validated that miR-155 efficiently targets the 3' untranslated region of CYR61.

CONCLUSION: This study reported for the first time that overexpression of miR-155 contributes to PE development by targeting and down-regulating angiogenic regulating factor CYR61, leading to pathological alterations. This finding not only characterizes a new mechanism for the disease but also provides a potential therapeutic target.

Key words: cysteine-rich protein 61, miR-155, placenta, preeclampsia


Preeclampsia (PE), a syndrome among pregnant women characterized by elevated maternal blood pressure and proteinuria >20 weeks of gestation, is one of the leading causes of pregnancy-related maternal and fetal morbidity and mortality.1 It has long been found that the placenta plays an important role in the development of PE, in which poor placentaation, shallow invasion, and abnormal angiogenesis are the main pathological manifestations.2 In fact, more and more studies have identified these pathological processes to the results of local oxidative stress characteristic in PE placenta.3 Among these studies, soluble fms-like tyrosine kinase 1 and endoglin have been widely accepted as one classical mechanism through which placental ischemia and oxidative stress impair normal local angiogenesis process.4,5 Other mechanisms believed to contribute to PE are also being intensively investigated. Recently, another stress gene, cysteine-rich protein 61 (CYR61), was reported to be significantly down-regulated in PE patients.6

CYR61, also known as CCN1, is a member of the CCN family,7 is a secreted matrix protein expressed by nearly all types of vascular cells and trophoblasts and implicated in diverse cellular processes such as proliferation, migration, differentiation, and adhesion.8 CYR61 has been demonstrated to be one of the important early angiogenic factors during pregnancy. Targeted knockout of CYR61 gene in mice results in embryonic death due to placental vascular insufficiency and compromised vessel integrity.9 It was also found that the expression level of CYR61 in human PE placenta was significantly lower than that of the normal control.6 Therefore, we hypothesized that the down-regulated expression of CYR61 might contribute to PE development. Since CYR61 can induce the expression of vascular endothelial growth factor (VEGF),10 it is likely that a decreased CYR61 level would cause insufficient expression of VEGF in PE placenta. The interesting issue is what is responsible for the decreased CYR61 expression in PE placenta.

MicroRNAs (miRNAs) are a class of highly conserved short noncoding RNA molecules (about 22 nucleotides) that are key players in the regulation of gene expression mainly at the posttranscrip-
A recent miRNA expression profile in PE placenta showed 7 different miRNAs in higher expression status, indicating their potential roles in PE development. Among these miRNAs, miR-155 has been defined as an inflammation-related miRNA as it can be significantly up-regulated by exogenous, eg, tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS), and it can regulate various inflammation-related nuclear factors (eg, activator protein [AP]-1, nuclear factor [NF]-kB) as a feedback antiinflammation mechanism. Here, we show that the overexpressed miR-155 specifically downregulates CYR61 in PE placenta, which may be an important pathway in the development of PE.

Materials and Methods

Placental samples and placental collection

Placental samples, delivered at 36-40 weeks, were collected from 20 severe PE women undergoing cesarean section in the Department of Obstetrics and Gynecology of Drum Tower Clinical Medical College, Nanjing Medical University, from March 2005 through April 2008. Placental tissues from 20 normotensive pregnancies with gestational age-matched groups also undergoing selective cesarean section were collected as controls. Severe PE was defined as having either severe hypertension (systolic blood pressure ≥160 mm Hg or diastolic blood pressure ≥110 mm Hg) or severe proteinuria (urinary protein excretion ≥2.5 g per 24 hours). All patients had normal platelet counts, normal functioning livers and kidneys, and normal fetal weights. The level of blood pressure of all patients returned to normal and symptoms of proteinuria disappeared postpartum 6 weeks. All placental samples were frozen within a half hour after delivery.

Patients with chronic hypertension, renal disease, collagen vascular disease, premature rupture of membrane, and other pregnancy complications such as fetal anomalies or chromosomal abnormalities were excluded from this study. Written consent was obtained from the patients before surgery. The Ethics Committee of Drum Tower Hospital approved the consent forms and procedures necessary to utilize the tissues. For RNA and protein isolation, only chorionic tissue from the central part of the placental maternal phase was collected. After cleansing in phosphate buffered saline, the tissues were frozen in liquid nitrogen and stored at –80°C until extraction of RNA and protein.

Cell culture and transfection

First trimester extravillous trophoblast cell line (HTR-8/SVneo) cells, human placental cell line derived from a choriocarcinoma (BeWo cells), and human embryonic kidney (HEK)-293T cells were grown in RPMI-1640, Ham’s F12, and Dulbecco’s modified Eagle media, respectively, all supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 U/mL penicillin, and 100 μg/mL (HyClone). All cells were maintained in standard culture conditions of 5% carbon dioxide at 37°C.

Real-time RT-polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. A total of 1 μg of total RNA from placental samples was used for reverse transcription.

To detect miR-155 expression, complementary DNA was synthesized using a miR-155-specific stem-loop primer: 5'-GTCGTATCCAGGTCCGAGGTTCGCAAGGATTCGCACTTGAGATCACGACCCCTA-3’. Quantitative polymerase chain reaction (PCR) analysis used the following primers: forward, 5’-CTTGTAAATGCTATCGTGAAG-3’; reverse, 5’-GAAGGTCGAGGTTGAGT-3’. Small unclear RNA U6 (U6) small RNA was used as internal control with the following primers: forward, 5’-AACGCTTCACGAATTTGCG-3’; reverse, 5’-GATTTGGTCATTG-3’. The expression of miR-155 and CYR61, relative to the control, was detected using an SYBR green-based real-time quantitative PCR assay (Toyobo Co., LTD, Osaka, Japan) as previously described.

Western blot analysis

Protein extracts were prepared from placental tissues and cells using lysis buffer supplemented with EDTA-free complete protease inhibitors (Roche, Penzberg, Germany). Tissue or cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The primary antibodies used were goat anti-human polyclonal CYR61 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit antihuman tubulin antibody (Chemicon, Hampshire, UK). Protein bands were visualized via enhanced chemiluminescence detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Plasmids and constructs

miRNA expression constructs

A 409-base pair DNA fragment encompassing the has-mir-155 gene was PCR amplified from genomic DNA using primers: 5’-CCTTTCCGATTTTACTATGCTG-3’ and 5’-CCTTTACTTTCAAACTTATTTA-3’. The PCR product was cloned into pEGFP-C1 (Clontech) by Bgl II and Hind III (Promega, Shanghai, China) digestion.

Luciferase reporter vector

Sequences of the complete 3’ untranslated region (UTR) of human CYR61 (nt 2634-3298, accession no. NM_001554) were amplified using PCR and then introduced downstream of the Luciferase reporter gene in the Xba I (a DNA restriction endonuclease) cloning sites of the pGL3 promoter vector (Promega).

CYR61 expression vector

Full-length CYR61 complementary DNAs, which encompassed the entire open reading frame with or without the 3’UTR, were amplified using PCR and the products were inserted into the eukaryotic express-
8/SVneo cells were transfected with 2.5 μg of miR-155 plasmid. Transfection was performed using Lipofectamine 2000 (Invitrogen). HEK-293T cells were cotransfected with Luciferase reporter plasmids (40 ng) and miR-155 expression vector (1-2 μg). Luciferase activities were measured via Luminometer (Promega) 36 hours after transfection.

Luciferase assays
Luciferase (an oxidative enzyme used for reporter) assays were carried out with the Dual-Luciferase Reporter Assay System (Promega). To check for transfection efficiency, pRL-SV40 (Promega), a control plasmid, was used. Transfection was performed using Lipofectamine 2000 (Invitrogen). HEK-293T cells were cotransfected with Luciferase reporter plasmids (40 ng) and miR-155 expression vector (1-2 μg). Luciferase activities were measured via Luminometer (Promega) 36 hours after transfection.

Cell migration assay
Wound-healing experiments were performed as described previously. HTR-8/SVneo cells were transfected with 2.5 μg of miR-155 plasmid. When the cells grew up to 90% confluence, a single wound was made in the center of the cellular monolayer and cell debris was removed by washing and a reticule mark was made on the bottom of every dish. After 9, 24, and 32 hours of incubation, the wound closure areas were visualized under an inverted microscope. The widths of the wounds were measured in 5 different locations averaged over 3 fields of view per well from a 3-4 replicate set of samples.

Measurement of free VEGF
Conditioned media were collected and centrifuged to remove cellular debris. VEGF165 secretion was detected by a human VEGF colorimetric enzyme-linked immunosorbent assay kit (Sheng Gong Biotech Co., Ltd, Shanghai, China) according to the manufacturer’s instructions. HTR-8/SVneo cells were plated in 24-well plates (5×10^3 cells/well) in culture medium with 1% FBS containing various concentrations of CYR61 (20-80 ng/mL) and incubated for 24 hours at 37°C with 5% carbon dioxide atmosphere. Sample or standard VEGF165 was added to each well, previously coated with human monoclonal anti-VEGF antibody. After 1.5 hours of incubation, wells were washed and incubated with an enzyme-linked polyclonal anti-VEGF antibody. Tetramethylbenzidine substrate solution was added to each well and the color developed in proportion to the amount of VEGF bound in the initial step. The plate was scanned at a wavelength of 450 nm.

Statistical analysis
All experiments were repeated at least 3 times. Results were expressed as mean ± SEM or mean ± SD. For nonparametric independent 2-group comparisons, the data were analyzed for statistical significance by 2-tailed Student t test or the Mann-Whitney test when data did not follow the Gaussian distribution with the program SPSS 11 (SPSS, Inc, Chicago, IL). Analysis of variance was used for comparing the data from 3 groups. Differences with a P value < .05 were considered statistically significant.

FIGURE 1
Expression of miR-155 and CYR61 in PE and normal placentas

A. Schematic representation of miR-155 and CYR61 expression levels. miR-155 and CYR61 were analyzed with real-time reverse transcription polymerase chain reaction, normalized with U6 and GAPDH in PE (n = 20) and normal (n = 20) placentas. Error bars = SD; **P < .01; ***P < .001. B. Representatives of protein levels of CYR61 in PE and normal placentas. Detection of CYR61 protein from placentas was done by Western blots using antibodies against CYR61 and tubulin. First 3 lanes show normal placentas and other lanes show PE placentas.

**Results**
miR-155 was up-regulated whereas CYR61 was down-regulated in severe PE placentas

We examined the differences in miR-155 and CYR61 expression levels between the control and PE placentas by quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) (Figure 1, A). The expression of miR-155 in severe PE placenta is 2.58-fold higher in comparison to the control placentas (P < .001). The transcript level of CYR61 was significantly lower in severe PE placenta compared to the control group (Figure 1, A) (P < .01). Expression of miR-155 and CYR61 was negatively correlated (r =
Western blots were also used to investigate the expression of CYR61 protein in severe PE and normal placenta. Protein level of CYR61 decreased in PE placenta, similar to the results obtained from quantitative RT-PCR. These findings suggest an inverse relationship between miR-155 and CYR61 expression, and an increased miR-155 expression associated with the down-regulation of CYR61 in PE placenta.

**miR-155 targets CYR61 gene**

Through computational searching, we found CYR61 to be a potential targeted gene of miR-155 with a perfect match between miR-155 and the 3'UTR of CYR61. The 3'UTR of CYR61 is highly conserved in both human beings and mice with a 95% similarity. We constructed an miR-155-expression vector (pCMV-GFP-miR-155; Clontech) and a CYR61 3'UTR-containing vector (pLuc-3'UTR; Signosis, Inc., Sunnyvale, CA) to carry out miR-155 targeting experiments. For the miR-155 expression vector, whole pri-miR-155 sequence was amplified and cloned into vector pEGPF-C1. Transfection into BeWo and 293 cell lines resulted in the overexpression of mature miR-155. Next, we generated a Luciferase reporter construct harboring a fragment of the CYR61 3'UTR target sequence of miR-155. Luciferase assays showed that miR-155 expression significantly reduces Luciferase activity 36 hours after cotransfection into 293 cells. This result suggests miR-155's role in targeting the 3'UTR sequence of CYR61, although the result remains to be confirmed in the cells derived from the PE placenta. To observe the direct effect miR-155 has on CYR61, the entire open reading frame of CYR61 with or without the 3'UTR was cloned into pCS2 vector with Myc tags (pMyc-CYR61-3'UTR; Clontech). After cotransfection, exogenous CYR61 was detected by Western blots using anti-Myc antibody. Results showed that cotransfection of the miR-155 vector and the CYR61 vector with 3'UTR produces lower levels of the exogenous.

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CYR61 protein than cotransfection of miR-155 vector and CYR61 vector without the 3’UTR as well as the control group, which were cotransfected with the CMV-EGFP vector without miR-155 (empty vector) and the pCS2-CYR61-3’UTR (+) vector. This suggests that miR-155 does specifically repress CYR61 expression by targeting 3’UTR of CYR61 (Figure 3, B). When we transfected HTR-8/SVneo cells with miR-155-containing vector, a decreased endogenous CYR61 expression was determined by Western blot probed with an anti-CYR61 antibody (Figure 3, C). Interestingly, qRT-PCR analysis of messenger RNA (mRNA) from these cells showed that CYR61 mRNA level was also influenced by miR-155 (Figure 3, D), indicating that miR-155 also affects CYR61 at the mRNA level. These results imply that the sequence of CYR61 3’UTR is a target of miR-155 and that miR-155 down-regulates CYR61 by targeting the 3’UTR sequence.

miR-155 affects trophoblast migration

After demonstrating that increased miR-155 targets the CYR61 gene leading to decreased CYR61 level in PE placenta tissues, we were interested in investigating a likely link among miR-155, CYR61, and PE disease. A normal placenta cell line, HTR-8/SVneo, was treated with various concentrations of CYR61 (20-80 ng/mL) for 24 hours. Using enzyme-linked immunosorbent assay analysis, we found that CYR61 significantly stimulates the release of VEGF in the trophoblast cells (Figure 4, A). While we found that miR-155 down-regulates CYR61 and VEGF can induce endothelial cell migration,18 we wonder if overexpressed miR-155 could interfere with trophoblast cell migration. The HTR-8/SVneo cell line transfected with the miR-155 expression plasmid (Figure 2, B) was used for a cell migration assay. Interestingly, forced miR-155 expression significantly inhibits trophoblast cell migration (Figure 4, B and C) and the effect of miR-155 on cell migration can be restored by transfection of CYR61 (data not shown), indicating that overexpression of miR-155 may mediate phenotypic alterations of trophoblasts through an miR-155-CYR61-VEGF pathway. Thus, we infer that overexpression of miR-155 may be involved in the development of PE by down-regulating CYR61 and VEGF, leading to the dysregulation of angiogenesis and inflammation of trophoblast cells in the placenta.

COMMENT

We provide the first evidence of miR-155’s involvement in PE and its possible role in placenta angiogenesis. miR-155, encoded within the only phylogenetically conserved region of B-cell integration cluster com-
posed of 3 exons located in chromosome 21q21, was found to be a common target of a broad range of inflammatory mediators. Previous studies have shown a potential association between miR-155 and PE, a syndrome associated with inflammation at the maternal-fetal interface. But little is known about the exact role miR-155 plays in the mechanism of PE. Our results suggest a previously neglected role of miR-155 in regulating placental angiogenesis by targeting the important angiogenic factor CYR61 gene. Our study confirmed the overexpression of miR-155 in PE placenta tissues as reported by Pineles et al., and using computational prediction, showed CYR61 to be a potential target gene of miR-155. Interestingly, a decreased expression of CYR61 is accompanied with an increased expression of miR-155 in PE placentas, suggesting an inverse relationship between miR-155 and CYR61 expression in PE and normal placenta tissues. Through cotransfection with miR-155 expression plasmids and plasmids containing 3’UTR of CYR61, we validated miR-155’s role in efficiently targeting 3’UTR of the CYR61 transcript, leading to the reduction of CYR61 expression. In addition, cotransfection of the miR-155 vector with a CYR61 expression vector also decreases CYR61 mRNA, suggesting that miR-155 may have a direct impact on CYR61 mRNA stability. Nevertheless, our results cannot exclude the possibility that other elements regulated by miR-155 can influence the expression level of CYR61. This remains a topic for further exploration.

**FIGURE 4**

**miR155 affects trophoblast cell phenotype through CYR61**

A. CYR61 protein stimulates VEGF protein expression. HTR-8/SVneo cells were treated with various concentrations of CYR61 (0-80 μg/mL) and continued to culture for 24 hours. Cells were then used to analyze free VEGF concentration by enzyme-linked immunosorbent assay as described in “Materials and Methods.” *Error bar = SD; *P < .05; **P < .01. B and C, miR-155 influences migration of trophoblast cells. HTR-8/SVneo cells were transfected with 2.5 μg of miR-155 plasmid (Figure 2, B) and used for wound-healing assays in vitro. Cells transfected with blank vector were used as control. B. Photographs of wound-healing assays that were taken at 0, 9, 24, and 32 hours. Compared to control, transfection of miR-155 plasmid results in bigger gaps in different time points of wound-healing assays, indicating that miR-155 inhibits migration of trophoblast cells in vitro. C. Summary histogram of cell migration of wound-healing assays. *Error bar = SEM; *P < .05; **P < .001.

CYR61, cysteine-rich protein 61; PE, preeclampsia; VEGF, vascular endothelial growth factor.

miR-155 is a common target of a broad range of inflammatory mediators and can be up-regulated by many inflammatory factors such as polyinosinoc-polycytidylic acid, a synthetic analog of double-stranded RNA, interferon-β, LPS, TNF-α, and interleukin-1β, by acting on Toll-like receptor (TLR). PE is a disorder characterized by intravascular inflammation and endothelial cell dysfunction. Activation of TLR2 and TLR4 can induce trophoblast expression of proinflammatory cytokines and trophoblast apoptosis. In this study, a higher level of miR-155 in PE placentas could contribute to trophoblast injury by down-regulating the angiogenic regulating factor CYR61.

CYR61 is normally strongly expressed in human placenta. It is prominently expressed in endothelial cells, villous stromal cells, and interstitial extravillous trophoblasts giant cells, and is involved in angiogenic processes as well as migration properties of extravillous trophoblasts cells in human placental development. Here we found the expression of CYR61 in 20 late-onset severe PE placentas was decreased at both mRNA and protein levels. The results of Gellhaus et al showed that there was only a slight, but not significant, down-regulation in the expression of CYR61 gene in 8 placentas from late-onset PE patients compared to the respective control group. The difference might be associated with the differences in the criteria of the study subjects as well as the sample amount.

TNF-α was shown to decrease the in vitro motility of juxtaglomerular epithelioid granular cells, a human cell choriocarcinoma line and HTR-8/SVneo cells and induce a negative effect on the migration and invasion of trophoblast. In addition, TNF-α can induce miR-155 in macrophages during inflammation responses via TLRs. Abrahams et al observed that in the first trimester placental tissues, TLR2 and TLR4, are highly expressed in the villous cytotrophoblast and extravillous trophoblast populations. Our findings showed that TNF-α also increased the expression of miR-155 in HTR-8/SVneo (data not shown). In addition, overexpressed miR-155 interferes with trophoblast migration through the miR-155-CYR61-VEGF pathway. Thus it is clear that miR-155 can affect the biological behavior of trophoblast cells by regulating CYR61 and hinder placenta angiogenesis leading to the pathogenesis of PE although it remains to be validated by further experimentation. On the other hand, soluble fms-like tyrosine kinase 1 was suggested to be up-regulated in an oxidative stressed microenvironment, thus it is most likely to be a result and possibly a cause of local oxidative stress. Here we identified miR-155-CYR61 as a potential alternative mechanism directly linking local ischemia and oxidative stress to its angiogenesis disorder. The cause of the overexpression of miR-155 in PE placenta will be our research focus in the future.

In conclusion, our findings provide new evidence that aberrantly expressed miR-155, through the down-regulation of CYR61, may play an important role in the development of PE. Our findings may not only characterize a new mechanism, but also provide a potential therapeutic target for this disease.

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