Original Article
miR-30b facilitates preeclampsia through targeting MXRA5 to inhibit the viability, invasion and apoptosis of placental trophoblast cells

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Abstract: Preeclampsia (PE) may induce gestational failure threatening a significant number of pregnant women. Dysfunctional placental trophoblast cells have an important impact on PE progression. microRNAs (miRNAs) have been reported to participate in PE progression, whereas the mechanism that underlies miR-30b involved in PE progression and function of placental trophoblast cells remains poorly understood. Cell viability was investigated by cell counting kit-8 (CCK-8) assay. Cell apoptosis was detected by flow cytometry using Annexin V-FITC/propidium iodide (PI) staining. Cell invasion was analyzed by trans-well assay. The expression of miR-30b was measured by quantitative real-time polymerase chain reaction (qRT-PCR). The abundance of matrix-remodeling associated 5 (MXRA5) protein was detected by western blots (WB). The interaction between miR-30b and MXRA5 was investigated by bioinformatics analysis and luciferase activity assay. The effect of miR-30b and MXRA5 on mitogen-activated protein kinases (MAPK) pathway and invasion was evaluated by WB. Then we found miR-30b was highly expressed in PE and its overexpression inhibited cell viability and invasion while enhanced apoptosis in JEG-3 and HTR8/SVneo cells. Moreover, MXRA5 was targeted by miR-30b and MXRA5 restoration attenuated the effect of miR-30b on cell processes in HTR8/SVneo cells. Besides, both of miR-30b and MXRA5 were associated with MAPK pathway in HTR8/SVneo cells. Our data suggested miR-30b might contribute to PE through inhibiting cell viability, invasion while inducing apoptosis of placental trophoblast cells via MAPK pathway by targeting MXRA5. These indicated that miR-30b might be a novel biomarker for PE treatment.

Keywords: Preeclampsia, placental trophoblast cells, miR-30b, MXRA5

Introduction
Preeclampsia (PE) is regarded as a serious pregnancy syndrome, which may induce gestational failure with an incidence of 3-6% [1]. Placental abnormal has been suggested to be associated with PE progression [2]. Inhibition of trophoblast cell invasion may contribute to PE via different pathway [3]. PE has been reported to trigger the risk of pregnant women and fetuses and many biomarkers of pregnancy disorders have gained considerable attentions [4]. However, novel and more effective drivers are expected for PE treatment.

MicroRNAs (miRNAs) are a class of noncoding RNAs, which play essential roles in pregnancy outcomes, including PE [5]. Moreover, miRNAs have been shown to associate with PE progression and to provide biomarkers and therapeutic targets for PE [6]. For example, miR-145 has an important effect to predict PE by regulating cell proliferation and apoptosis by phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT)/mTOR pathway [7]. Moreover, miR-134 suppresses cell infiltration of placental trophoblast cells by regulating integrin subunit beta 1 (ITGB1) expression in PE [8]. miR-4221 also contributes to PE progression by mediating CYP11B2 [9]. Besides, miR-30a-3p, one of the miR-30 family, promotes PE progression and regulates trophoblast invasion and apoptosis by targeting insulin-like growth factor 1 (IGF-1) [10]. Similarly, miR-30b is another of miR-30 family, which has essential roles in proliferation, invasion, and autophagy in osteosarcoma cells [11]. However, there is no direct evidence in support of miR-30b being involved in PE pro-
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Matrix-remodeling associated 5 (MXRA5), one member of MXRA protein family, may take part in cell adhesion and matrix remodeling [12]. Moreover, MXRA5 plays anti-inflammatory and anti-fibrotic properties in chronic kidney disease in response to transforming growth factor-β1 (TGFβ1) treatment [13]. Additionally, MXRA5 serves as a novel biomarker of poor prognosis by regulating tumor progression and overall survival in non-small cell lung cancer [14]. Likewise, MXRA5 is also regarded as a new biomarker of metastasis in colorectal cancer [15]. Recently, MXRA5 has been suggested to be dysregulated in PE and associated with trophoblast cell invasion, viability, and apoptosis [16]. Intriguingly, the association of miR-30b and MXRA5 is predicted by bioinformatics analysis. Therefore, we presumed MXRA5 might be required for miR-30b-mediated processes in placental trophoblast cells. In this study, we measured the expression of miR-30b in PE tissues and probed the potential effect of miR-30b on cell viability, invasion and apoptosis in placental trophoblast cells. Moreover, we explored the interaction between miR-30b and MXRA5 and investigated the effect of MXRA5 on miR-30b-mediated cell processes. Besides, we probed the potential pathway involved in cell processes in this study.

Materials and methods

Tissue samples

This research was accepted by the Institutional Research Ethics Committee of Huzhou Maternal and Child Health Hospital and informed consent was obtained from every donor. In this study, a total of 32 pregnant women were involved in this study (Table 1). PE patients were diagnosed by systolic/diastolic blood pressure (> 140/90 mm Hg) and proteinuria. All patients without history of other systemic illnesses were delivered by cesarean section. Villi tissues of placentas were obtained by cesarean section.

Cell culture and transfection

The human placenta trophoblast cell lines JEG-3 and HTR8/SVneo cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in a humidified incubator (37°C, 5% CO₂) using DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA).

miR-30b mimics, miR-NC mimics, miR-30b inhibitors, miR-NC inhibitors, MXRA5 overexpression vectors (MXRA5) and vector alone were synthesized by GenePharma (Shanghai, China). Transfection was conducted into JEG-3 or HTR8/SVneo cells using Lipofectamine 2000 (Invitrogen) referring to the manufacturer’s protocol.

Cell viability

Cell viability was measured by using cell counting kit-8 (CCK-8) assay. Transfected cells were seeded into 96-well plates with 1 × 10⁴ cells per well for 6, 24, 48 or 72 h. Then cell medium was replaced with 10% CCK-8 (Sigma, St. Louis, MO, USA) in medium and incubated for 2 h at 37°C. The absorbance was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell apoptosis

Cell apoptosis was investigated by flow cytometry using Annexin V-FITC/propidium iodide (PI) cell apoptosis detection kit (Sigma). Treated trophoblast cells were collected and washed with PBS, followed by resuspended in binding buffer and incubated with Annexin V-FITC and PI for 20 min without light. The stained cells were analyzed by using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Invasion assay

Cell invasive ability was detected by trans-well assay. The upper chambers (Costar, Corning, NY, USA) were coated with Matrigel (BD, San Jose, CA, USA) and the lower chambers were added DMEM with 10% fetal bovine serum. Transfected cells were seeded in the upper chambers in serum-free medium at 37°C for 24 h and then carefully removed with a cotton swab. Invasive cells were fixed with 4% paraformaldehyde (Sigma) for 10 min, stained with crystal violet (Sigma) and then observed under a microscope (Olympus, Tokyo, Japan).
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Table 1. Characteristics of pregnant women in preeclampsia and control group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group</th>
<th>Preeclampsia group</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>28.4 ± 3.1</td>
<td>29.3 ± 2.5</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>38.2 ± 0.8</td>
<td>37.4 ± 1.5</td>
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<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>113.6 ± 5.8</td>
<td>158.4 ± 13.6</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>76.6 ± 9.2</td>
<td>112.2 ± 10.6</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>None</td>
<td>+++</td>
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</table>

Figure 1. The expression of mir-30b was enhanced in PE villi compared with normal group. n = 16, ***P < 0.001.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was prepared from tissues or cells by using mirVana™ miRNA Detection Kit (Thermo Fisher, Wilmington, DE, USA) according to the manufacturer’s instructions. Subsequently, the RNA was used to synthesize first strand cDNA by TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then cDNA was diluted and used for qRT-PCR using SYBR Green following the amplification instructions. U6 small RNA was used as housekeeping gene to normalize miR-30b expression. All primers were listed as follows: miR-30b (Forward, 5’-CGCGCCTGTAACATCCTACAC-3’; Reverse, 5’-GTGCAAGGTCGAGGT-3’), U6 (Forward, 5’-GCTTCACGCACAGCAT ATACTAAAT-3’; Reverse, 5’-CGCTTCACGAATTTGCGGTCA-CAT-3’).

 Luciferase activity assay

Putative sites of miR-30b and MXRA5 were predicted by TargetScan online. Wide type or mutant type 3’-UTR of MXRA5 were amplified and cloned into pGL3 vectors (Promega, Madison, WI, USA) to obtain WT or MUT luciferase report vectors, respectively. Then the luciferase reporter vectors were co-transfected with miR-30b mimics, miR-30b inhibitors or negative controls (miR-NC mimics, inhibitors) in HTR8/SVneo cells using Lipofectamine 2000 according to the manufacturer’s protocols. Then luciferase activity was analyzed using Dual-Luciferase Assay Kit (Promega) after 48 h.

Western blots (WB)

Cells were washed with PBS, then isolated in lysis buffer with protease inhibitor and quantified by BCA assay kit (Thermo Fisher) according to the instructions. Subsequently, equal amounts of denatured samples were loaded and separated by SDS-PAGE gel, then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated in 5% BSA (Sigma) for 1 h at room temperature to block the nonspecific antigen. Following the blocking, membranes were developed with primary antibodies against MXRA5, p-p38, p38, p-ERK1/2, ERK1/2, E-cadherin, N-cadherin, MMP-2, MMP-9 or GAPDH (Abcam, Cambridge, UK) overnight at 4°C, followed with HRP-conjugated IgG secondary antibodies (Abcam) for 2 h at room temperature. The enhanced chemiluminescence (ECL) chromogenic substrate (Thermo Fisher) was used to detect protein bands.

Statistical analysis

Data were presented as the mean ± standard deviation (SD) from three independent experiments. The statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Significant differences between two groups were assayed by Student t test in this study, and *P < 0.05, **P < 0.01, or ***P < 0.001 were considered significant.

Results

miR-30b was highly expressed in PE villi

A total of 16 PE and 16 normal pregnant women were enrolled in this study, who were diagnosed by systolic/diastolic blood pressure and proteinuria. The systolic blood pressure was 113.6 ± 5.8 and 158.4 ± 13.6 mm Hg in control or PE group, respectively (Table 1). Moreover, the diastolic blood pressure was 76.6 ± 9.2 and 112.2 ± 10.6 mm Hg in two groups, respectively.
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(Table 1). In addition, women were with severe proteinuria in the PE group compared with control group (Table 1). To investigate the potential effect of miR-30b on PE, the abundance of miR-30b was first measured in the placental villi tissues. Results showed that the expression of miR-30b was significantly increased in PE tissues compared with that in normal samples (Figure 1). These data suggested that dysregulated miR-30b might be required for PE progression.

Overexpression of miR-30b inhibited cell viability, invasion and promoted cell apoptosis in placental trophoblast cells

Since miR-30b was ectopic in PE, we wondered whether miR-30b might affect cell viability, invasion and apoptosis in placental trophoblast cells. JEG-3 and HTR8/SVneo cells were transfected with miR-30b or miR-NC mimics as a result, elevated miR-30b expression was observed in JEG-3 and HTR8/SVneo cells after miR-30b transfection (Figure 2A). Addition of miR-30b effectively inhibited cell viability in JEG-3 cells after transfection for 24, 48 or 72 h (Figure 2B). Similarly, enrichment of miR-30b also suppressed cell viability in HTR8/SVneo cells compared with miR-NC treatment (Figure 2C). Moreover, a great increase of apoptosis rate was displayed in miR-30b-transfected JEG-3 or HTR8/SVneo cells, respectively (Figure 2D-F). In addition, the invasive ability of placental trophoblast cells was investigated in JEG-3 and HTR8/SVneo cells by trans-well assay.

Figure 2. Addition of miR-30b inhibited cell viability and invasion while inducing apoptosis in placental trophoblast cells. A. The expression of miR-30b was detected in JEG-3 and HTR8/SVneo cells after miR-30b or miR-NC mimics transfection. B, C. The cell viability of JEG-3 or HTR8/SVneo cells transfected with miR-30b or miR-NC mimics was measured by CCK-8, respectively. D-F. The effect of miR-30b on cell apoptosis was investigated in JEG-3 or HTR8/SVneo cells, respectively. G. The invasive ability was evaluated in miR-30b or miR-NC transfected cells. *P < 0.05, ***P < 0.001.
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Results indicated accumulation of miR-30b blocked cell invasion in JEG-3 and HTR8/SVneo cells, respectively (Figure 2G). Together, these results showed that miR-30b suppressed cell viability and invasion and induced cell apoptosis.

MXRA5 was directly targeted by miR-30b

Seeing that miR-30b was required for processes of placental trophoblast cells, we next desired to explore a putative target gene. Bioinformatics analysis mapped the potential binding sites of miR-30b and MXRA5, suggesting that MXRA5 might be a target of miR-30b in our study (Figure 3A). Hence, luciferase activity assay was conducted to validate the prediction. Results showed that miR-30b overexpression led to a great loss of the luciferase activity in HTR8/SVneo cells upon the present of MXRA5-WT, whereas the efficacy was lost in response to MXRA5-MUT transfection (Figure 3B). Conversely, an elevated activity was observed in HTR8/SVneo cells co-transfected with miR-30b inhibitors and MXRA5-WT (Figure 3C). Moreover, the effect of miR-30b on MXRA5 protein abundance was measured in HTR8/SVneo cells by overexpression or knockdown of miR-30b. Addition of miR-30b impaired the expression of MXRA5 protein, while miR-30b inhibition played an opposite effect in HTR8/SVneo cells (Figure 3D and 3E). These findings demonstrated MXRA5 was negatively regulated by miR-30b in placental trophoblast cells.

MXRA5 was required for miR-30b-mediated viability, invasion and apoptosis in placental trophoblast cells

Having established that MXRA5 was targeted by miR-30b, we next explored whether MXRA5 was involved in miR-30b regulating viability, invasion and apoptosis in placental trophoblast cells. The abundance of MXRA5 was first measured in HTR8/SVneo cells with miR-30b mimics or (and) MXRA5 overexpression vector transfection. Results reflected that miR-30b overexpression markedly decreased MXRA5 protein expression compared with mock transfection, whereas MXRA5 restoration protected the level of MXRA5 protein in HTR8/SVneo cells (Figure 4A and 4B). Moreover, addition of MXRA5 reversed the limited capacity of miR-30b for cell viability (Figure 4C). Similarly, MXRA5 overturned the miR-30b-mediated inhibitory effect on cell invasive ability (Figure 4D). In addition, introduction of MXRA5 atten-
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**Figure 4.** MXRA5 attenuated the effect of miR-30b on cell viability, invasion, and apoptosis in placental trophoblast cells. A, B. The abundance of MXRA5 protein was measured in HTR8/SVneo cells with miR-30b mimics or (and) MXRA5 transfection. C. The effect of MXRA5 on cell viability in miR-30b-transfected cells. D. Cell invasion was detected in HTR8/SVneo cells after MXAR5 treatment. E, F. The effect of MXRA5 on miR-30b-mediated apoptosis was investigated in HTR8/SVneo cells. **P < 0.01, ***P < 0.001.

ated miR-30b-induced apoptosis, uncovered by reduction of apoptosis rate in HTR8/SVneo cells (Figure 4E and 4F). In sum, MXRA5 weakened the effect of miR-30b on cell viability, invasion, and apoptosis in placental trophoblast cells.

**miR-30b and MXRA5 were involved in MAPK pathway in placental trophoblast cells**

To probe the potential pathway, the effect of miR-30b and MXRA5 on MAPK pathway was investigated in HTR8/SVneo cells. The results showed that the levels of phosphorylated p38 and ERK1/2 were obviously reduced in HTR8/SVneo cells following miR-30b transfection, whereas the phosphorylation was supported by MXRA5 restoration (Figure 5A and 5B). However, the abundances of total p38 and ERK1/2 showed little change suffering from the transfection of either miR-30b or MXRA5 in HTR8/SVneo cells (Figure 5A and 5B). Moreover, the hallmarks of cell invasion was
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miR-30b induced E-cadherin expression and impaired the levels of N-cadherin, MMP-2 and MMP-9, while introduction of MXRA5 ablated the regulatory effect of miR-30b (Figure 5C and 5D). Collectively, MAPK pathway was regulated by miR-30b and MXRA5 in placental trophoblast cells.

**Discussion**

PE is an adverse pregnancy syndrome, characterized by the occurrence of hypertension and proteinuria in pregnant women [17]. Similarly, the PE was also diagnosed in this study according to guidelines. With the advances in understanding of biomarkers in PE, miRNAs have gained rising attention [18]. Here we measured the expression of miR-30b in villi tissues and found elevated miR-30b in PE pregnancies [19]. However, the mechanism allowing miR-30b regulating PE remains poorly understood.

Dysfunctional trophoblast invasion has been regarded as an essential part in PE progression [20]. miR-30b has been suggested to regulate cell proliferation, migration and invasion in esophageal cancer and osteosarcoma cells [11, 21]. Hence, we investigated the effect of miR-30b on cell viability, apoptosis and invasion in placental trophoblast cells. Results showed miR-30b inhibited cell viability and invasion but promoted cell apoptosis in JEG-3 and HTR8/SVneo cells, suggesting that miR-30b contributed to PE progression. This is also consistent with several such reports. miR-519d inhibited cell viability and invasion in PE [22]. Moreover, addition of miR-299 blocked invasion and migration in HTR8/SVneo trophoblast cells by regulating histone deacetylase 2 (HDAC2) expression in PE [23]. Besides, miR-34a induced cell apoptosis in trophoblast cells by mediating B-cell lymphoma-2 (BCL2) in PE [24].

The functions of miRNAs were realized by regulating target genes in varying conditions. For instance, miR-30b was reported to inhibit epithelial-mesenchymal transition by targeting snail in pancreatic cancer stem cells [25]. miR-30b contributed to cell function by regulating plasminogen activator inhibitor-1 level in acute myocardial ischemia [26]. Moreover, miR-30b might inhibit cell growth, migration, and invasion by mediating epidermal growth factor receptor expression in non-small cell lung cancer [27]. Besides, homeobox A1 also was targeted by miR-30b and associated with cell growth, migration and invasion in esophageal cancer [21]. Accordingly, MXRA5 is regarded as a novel cancer gene and its function remains elusive. Emerging evidence suggested MXRA5 has a vital role in development of colorectal cancer and non-small cell lung cancer [14, 15]. Moreover, MXRA5 was reported to positively correlate with cell viability and invasion in trophoblast cells [16]. Notably, there is no direct evidence suggesting the interaction of miR-30b and MXRA5. Hence, we probed the link of miR-30b and MXRA5 and elucidated MXRA5 was
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negatively regulated by miR-30b. Subsequently, we also explored the effect of MXRA5 on miR-30b-mediated cell processes in placental trophoblast cells. Our data revealed that MXRA5 attenuated the function of miR-30b in PE progression, uncovered by increased cell viability and invasion, which is similar to the former effort [16].

To further investigate the effect of miR-30b and MXRA5 on invasion, we measured E-cadherin, N-cadherin, MMP-2 and MMP-9 expression. E-cadherin and N-cadherin have been suggested to be correlated with trophoblast function [28]. Moreover, MMP-2 and MMP-9 were positively correlated with cell invasion in trophoblast cells [29]. In the present study, we also found that addition of miR-30b might augment E-cadherin expression and impede the N-cadherin level, which is in agreement with previous work [25]. In addition, miR-30b overexpression suppressed the abundances of MMP-2 and MMP-9 protein in our study consistently with former study [30]. Furthermore, MXRA5 reversed these proteins' abundances in placental trophoblast cells.

Potential pathways involved in cell viability, invasion, and apoptosis need study. The available evidence indicated MXRA5 might regulate cell viability and invasion in trophoblast cell by regulating MAPK pathway [16]. Hence, we hypothesized MAPK pathway might be involved in the cell processes. MAPK pathway has been suggested to contribute to invasion of HTR-8/SVneo cell [31]. In our study, miR-30b blocked MAPK pathway while addition of MXRA5 protected activation of MAPK pathway. This is consistent with the data in previous work [16]. These data suggested that MAPK pathway might be a key pathway participating in the regulatory effect of miR-30b on cell viability, invasion and apoptosis in placental trophoblast cells. However, there is no direct evidence in support of the MAPK pathway regulating cell viability, invasion and apoptosis in placental trophoblast cells. Moreover, we measured the effect of miR-30b on MXRA5 protein expression, while the abundance of MXRA5 was absence at transcriptional level in this study. Notably, MXRA5 is reported to be expressed in primates but not in mice or rats [16]. Therefore, an animal model of PE is absent with respect to MXRA5 in this study. The interaction of MAPK pathway with cell viability, invasion and apoptosis by addition of MAPK inhibitors or activators is expected to be studied in future efforts.

Considered together, miR-30b expression was enhanced in PE and its overexpression suppressed cell viability and invasion but promoted apoptosis in placental trophoblast cells. Moreover, MXRA5 was negatively correlated with miR-30b and abated the effect of miR-30b on cell processes in placental trophoblast cells. Besides, miR-30b was negatively associated with MAPK pathway in HTR8/SVneo cells. These findings suggested that miR-30b might be required for PE by regulating cell viability, invasion and apoptosis of placental trophoblast cells by MAPK pathway by down-regulation of MXRA5, providing a promising biomarker for PE.

Disclosure of conflict of interest

None.

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