European Review for Medical and Pharmacological Sciences 2020; 24: 10346-10351

MiR-655-3p inhibits growth and invasiveness of trophoblasts *via* targeting PBX3 and thus deteriorates preeclampsia

H. SONG[1], X. WANG[2], J.-C. LI[3], Y.-H. LV[4]

[1]Department of Obstetrics, Binzhou Central Hospital, Binzhou, China
[2]Department of Laboratory, Zibo Fourth People's Hospital, Zibo, China
[3]Department of Laboratory, Jining Center for Disease Control and Prevention, Jining, China
[4]Department of Blood Transfusion, Jinan Maternal and Child Health Hospital, Jinan, China

Hong Song and Xin Wang contributed equally to this work

Abstract. – OBJECTIVE: The purpose of this study was to elucidate the regulatory effects of microRNA-655-3p (miR-655-3p) on growth and invasiveness of trophoblasts to influence the development of preeclampsia (PE).

PATIENTS AND METHODS: Relative levels of miR-655-3p and PBX3 in 24 PE pregnant women and 24 healthy ones were examined. Then, the regulatory effects of miR-655-3p and PBX3 on viability and invasiveness in HTR-8/SVneo cells were assessed by Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. Next, the expression levels of apoptosis-associated genes were determined. Finally, the interaction between miR-655-3p and PBX3 was tested by Dual-Luciferase reporter assay.

RESULTS: MiR-655-3p was upregulated in placental tissues of PE pregnant women than those of healthy ones. The overexpression of miR-655-3p suppressed viability and invasiveness, and induced apoptosis in HTR-8/SVneo cells. Besides, PBX3 was the target gene binding to miR-655-3p, and more importantly, the overexpression of PBX3 partially reversed the regulatory effects of miR-655-3p on viability and invasiveness in HTR-8/SVneo cells.

CONCLUSIONS: MiR-655-3p is involved in the development of PE by regulating biological features of trophoblasts by targeting PBX3.

Key Words:
MiR-655-3p, PBX3, Trophoblasts, Preeclampsia.

Introduction

The incidence of preeclampsia (PE) is about 4.6% (2.7-8.2%)[1]. PE is a severe complication during pregnancy, which is manifested as hypertension and proteinuria after 20 weeks of gestation[2]. With the development of gestation, the symptoms of PE can gradually deteriorate into severe PE and eclampsia, which seriously affect maternal and child health[3]. The pathogenesis of PE is complicated, involving abnormal invasiveness of placental trophoblasts, oxidative stress, inflammatory immunity, endothelial cell activation, and genetic susceptibility[4]. MiRNAs expressed in placental tissues are of significance in the early stage of PE and have been identified to affect the development of PE by regulating multiple relevant pathways[5].

MicroRNAs (miRNAs) are vital non-coding RNAs capable of regulating 30% protein-encoding genes. By recognizing and binding 3′-untranslated region (3′-UTR) of target mRNAs, miRNAs specifically inhibit the target gene expressions[6]. MiRNAs are extensively involved in life activities and disease progression[7]. Abnormally expressed miRNAs in tumors have been considered as effective tumor markers[8,9]. Chang et al[10] reported the anti-tumor effect of miR-655-3p. Its potential function in PE, however, has been rarely reported.

Trophoblasts are the major cells constituting placenta tissues, which participate in the formation of placenta[11] and maintain pregnancy in the early phase. Furthermore, abnormally functioned trophoblasts lead to insufficient reconstruction of spiral artery, as well as ischemia and hypoxia of the placenta. Invasiveness of trophoblasts can be mediated by several miRNAs[12].

PBX3, well studied in leukemia, is a member of the PBX family. It exerts a vital function in cell development and evolution. Notably, PBX3 serves as a protooncogene inducing tumorigenesis[13,14].

Corresponding Author: Yihua Lv, MM; e-mail: fyx@sdaeu.edu.cn

In this paper, miR-655-3p level in PE pregnant women and healthy ones was determined. Through a series of *in vitro* experiments, the potential influence of miR-655-3p on trophoblasts to influence the development of PE was analyzed.

Patients and Methods

Clinical Samples

A total of 24 PE pregnant women and 24 healthy ones undergoing regular pre-natal examination in the Binzhou Central Hospital from January 2016 to December 2018 were enrolled. Placental tissues were collected after delivery. This study was approved by the Ethics Committee of the Binzhou Central Hospital and conducted in accordance with the Declaration of Helsinki. Signed written informed consents were obtained from all participants before the study.

Cell Culture and Transfection

HTR-8/SVneo cells were provided by American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted in ethylenediaminetetraacetic acid containing 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) at 80-90% confluence. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from cells or placental tissues, followed by determination of RNA purity and concentration (Invitrogen, Carlsbad, CA, USA). Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) and then applied for qRT-PCR using the SYBR Green kit (TaKaRa, Otsu, Shiga, Japan). The primers are listed below: miR-655-3p, forward: 5'-ATAATACATGGTTAACCTCTTT-3', reverse: 5'-AGAGGTTAACCATGTATTATTT-3'; PBX3, forward: 5'-CGGTTCAGGTACTCAGTCATCC-3', reverse: 5'-TCTCGGAGAAGGTTCATCACAT-3', Bax, forward: 5'-CCCGAGAGGTCTTTTTCCGAG-3', reverse: 5'-CCAGCCCATGATGGTTCTGAT-3', Bcl-2, forward: 5'-GGTGGGGTCATGTGTGTGG-3', reverse: 5'-CGGTTCAGGTACTCAGTCATCC-3', glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-GCAAGGATACTGAGAGCAAGAG-3', reverse: 5'-GGATGGAATTGTGAGGGAGATG-3'. U6, forward: 5'-GCGCGTCGTGAAGCGTTC-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'.

Western Blot

The cells were lysed in radioimmunoprecipitation assay (RIPA, Beyotime, Shanghai, China) for extracting proteins. After concentration determination, the protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. The membranes were then incubated with primary and secondary antibodies. Finally, band exposure and grey value analysis were finally conducted.

Transwell

2.5×10^4 cells were applied on the upper of a transwell insert pre-coated with Matrigel, and 750 μL of complete medium was added in the bottom. After 36-h cell culture, the transwell insert was taken out and fixed in 95% methanol for 20 min. Through 10-min violet crystal staining and phosphate buffered saline (PBS) washing, the cells retained on the upper chamber were wiped off. After that, cells invading to the bottom were captured and counted in 6 randomly selected fields (200×) (Carl Zeiss, Jena, Germany).

Cell Counting Kit-8 (CCK-8) Assay

The cells were inoculated into a 96-well plate. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was recorded.

Dual-Luciferase Reporter Assay

The cells were inoculated in a 96-well plate with 5×10^3 cells per well and co-transfected with NC/miR-655-3p mimics and PBX3 WT/PBX3 MT for 48 h. Subsequently, the cells were lysed, and the supernatant was collected for measuring the relative Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM Corp., Armonk, NY, USA) was used for data analysis. All data were expressed as mean ± SD (standard deviation).

The paired two-tailed *t*-test was used for comparing the differences between two groups. $p<0.05$ was considered to be statistically significant.

Results

Expression Levels of MiR-655-3p and PBX3 in Placenta Tissues

Compared with that in healthy pregnant women, miR-655-3p was highly expressed in placenta tissues of PE pregnant women (Figure 1A). Potential binding sequences in the 3'UTR between PBX3 and miR-655-3p were identified (Figure 1B). Subsequently, the changes in the Luciferase activities after co-transfection of miR-655-3p mimics or inhibitor and PBX3 WT verified that PBX3 was the target gene binding to miR-655-3p (Figure 1C, 1D). In placenta tissues of PE pregnancies, PBX3 was lowly expressed (Figure 1E), showing a negative correlation with miR-655-3p expression (Figure 1F).

MiR-655-3p Suppressed Viability and Invasiveness in Trophoblasts

Transfection of miR-655-3p mimics effectively upregulated miR-655-3p in HTR-8/SVneo cells (Figure 2A). Cell viability markedly decreased in HTR-8/SVneo cells overexpressing miR-655-3p (Figure 2B). In addition, invasive cell number was reduced after transfection of miR-655-3p mimics (Figure 2C, 2D). It is suggested that miR-655-3p markedly suppresses proliferative and invasive capacities of trophoblasts.

MiR-655-3p Stimulated Apoptosis in Trophoblasts

Bax and Bcl-2 are the well-known apoptosis markers[15]. Here, mRNA and protein levels of Bax were remarkably upregulated in HTR-8/SVneo cells transfected with miR-655-3p mimics (Figure 3A, 3B). Conversely, Bcl-2 was downregulated after overexpression of miR-655-3p (Figure 3C, 3D). Therefore, miR-655-3p stimulated apoptosis in trophoblasts.

MiR-655-3p Suppressed Viability and Invasiveness in Trophoblasts by Targeting PBX3

It was shown that PBX3 level was negatively regulated by miR-655-3p at both mRNA and protein levels (Figure 4A-4C). Interestingly, inhibited viability and invasiveness in HTR-8/SVneo cells transfected with miR-655-3p mimics were partially reversed after PBX3 co-overexpression (Figure 4D-4F). It is indicated that PBX3 is responsible for cellular functions of trophoblasts regulated by miR-655-3p.

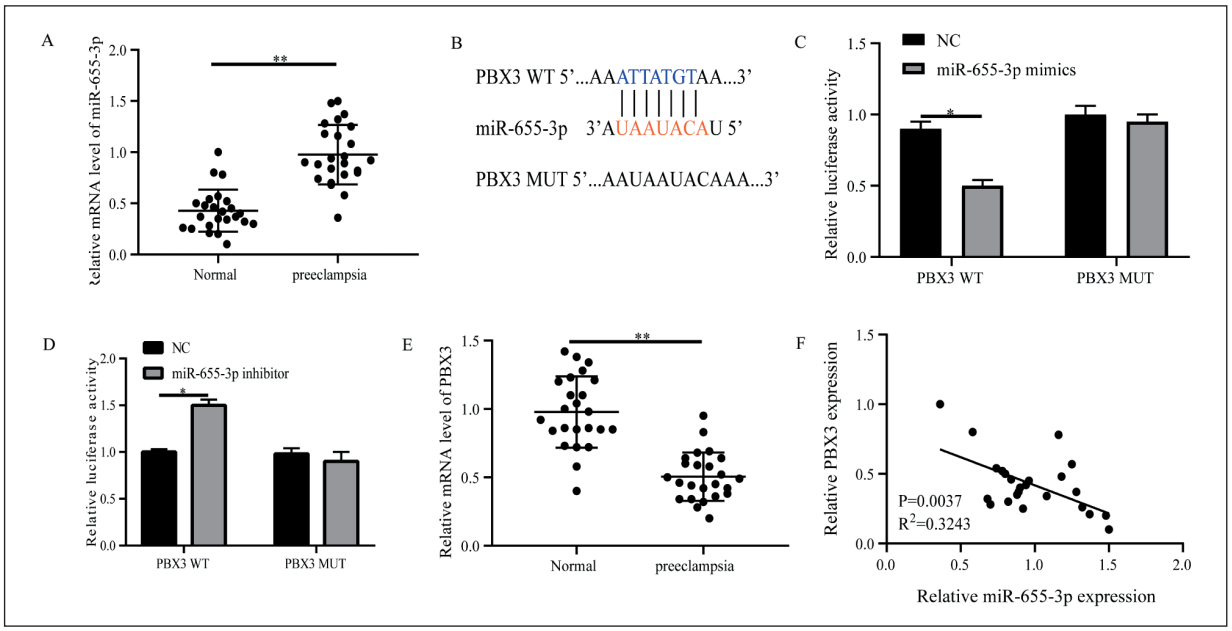

Figure 1. Expression levels of miR-655-3p and PBX3 in placenta tissues. **A,** MiR-655-3p levels in placenta tissues of healthy pregnant women and PE ones. **B,** Binding sequences in the 3'UTR between miR-655-3p and PBX3. **C, D,** Luciferase activity after co-transfection of NC/miR-655-3p mimics/miR-655-3p inhibitor and PBX3 WT/PBX3 MUT. **E,** PBX3 levels in placenta tissues of healthy pregnant women and PE ones. **F,** A negative correlation between expression levels of miR-655-3p and PBX3.

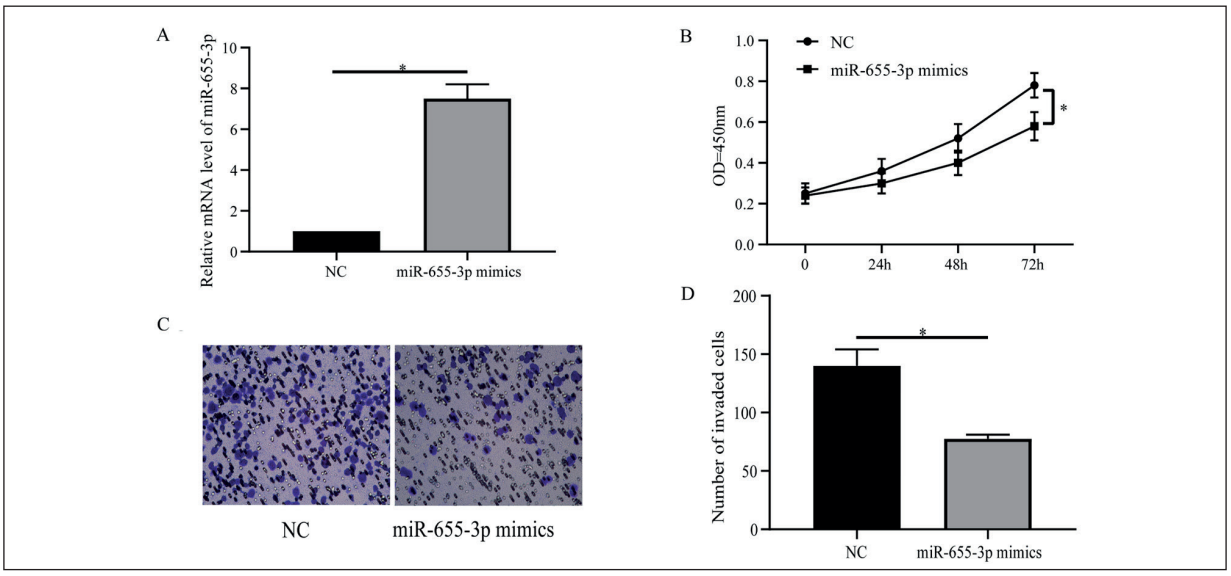

Figure 2. MiR-655-3p suppresses viability and invasiveness in trophoblasts. HTR-8/SVneo cells are transfected with NC or miR-655-3p mimics. **A,** MiR-655-3p level. **B,** Cell viability. **C,** Cell invasion (magnification: 200×). **D,** Invasive cell number.

Discussion

It is reported that miRNAs are differentially expressed in placenta and peripheral blood of PE pregnant women, posing a great impact on the development of PE[16,17]. Cellular functions of trophoblasts remarkably affect the pathogenesis of PE[18,19]. In this paper, miR-655-3p was upregulated in placental tissues of PE pregnancies, which regulated growth and invasiveness of trophoblasts through PBX3.

By mediating expressions and functions of the downstream genes, miRNAs are involved in the development of PE. Determination of miRNA levels in the circulatory system could be an effective, non-invasive approach for diagnosing,

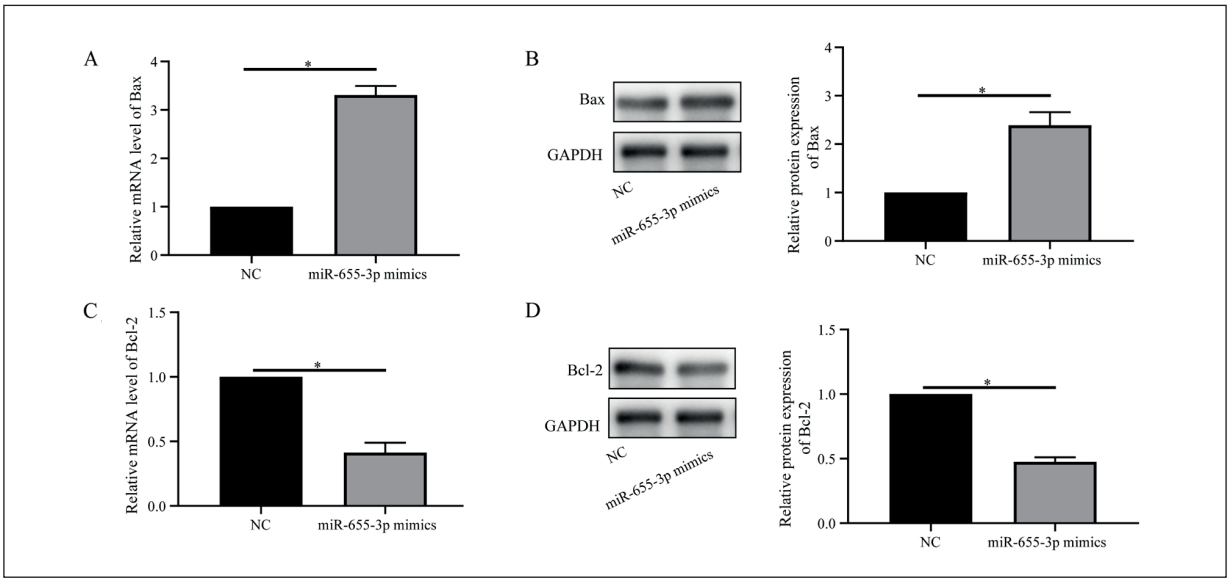

Figure 3. MiR-655-3p stimulates apoptosis in trophoblasts. **A, B,** The mRNA (**A**) and protein (**B**) levels of Bax in HTR-8/SVneo cells transfected with NC or miR-655-3p mimics. **C, D,** The mRNA (**C**) and protein (**D**) levels of Bcl-2 in HTR-8/SVneo cells transfected with NC or miR-655-3p mimics.

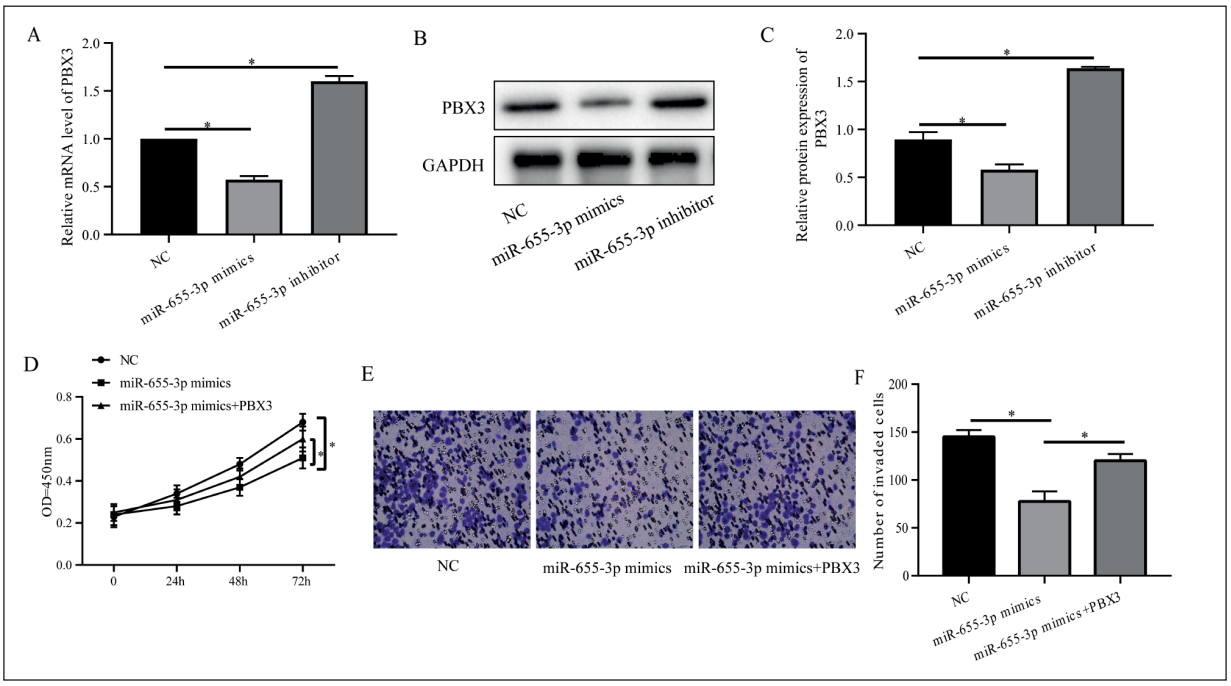

Figure 4. MiR-655-3p suppresses viability and invasiveness in trophoblasts through targeting PBX3. **A,** The mRNA level of PBX3 in HTR-8/SVneo cells transfected with NC, miR-655-3p mimics or miR-655-3p inhibitor. **B, C,** The protein level of PBX3 in HTR-8/SVneo cells transfected with NC, miR-655-3p mimics or miR-655-3p inhibitor. **D,** Cell viability in HTR-8/SVneo cells transfected with NC, miR-655-3p mimics or miR-655-3p mimics+PBX3. **E, F,** Cell invasion in HTR-8/SVneo cells transfected with NC, miR-655-3p mimics or miR-655-3p mimics+PBX3 (magnification: 200×).

treating, and monitoring PE. MiR-655-3p locates on chromosome 14q32 alongside the other three miRNAs (miR-127-5p, miR-369-3p and miR-544a)[10,20]. Previous studies have demonstrated the role of miR-655-3p in tumor development.

Inadequate invasiveness of trophoblasts induced disorders of urine spiral artery remodeling[21,22]. Similar to highly invasive tumor cells, trophoblasts maintain physiological functions of the placenta by regulating cell invasiveness. Most of relevant researchers analyzed placental tissues after delivery. Nevertheless, whether the abnormally expressed miRNAs are the cause or the result of PE could not be ascertained. *In vitro* experiments on trophoblasts revealed that the overexpression of miR-655-3p suppressed viability and invasiveness, and induced apoptosis in HTR-8/SVneo cells. Animal experiments are required in the future to validate our findings.

Through online prediction and Dual-Luciferase reporter assay, PBX3 was the target gene binding miR-655-3p. In addition, PBX3 level was negatively regulated by miR-655-3p in trophoblasts. Inhibitory effects of miR-655-3p on viability and invasiveness in trophoblasts were partially abolished by PBX3. Therefore, PBX3 was responsible for the development of PE regulated by miR-655-3p.

Conclusions

Shortly, miR-655-3p is involved in the development of PE by regulating biological features of trophoblasts by targeting PBX3.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Abalos E, Cuesta C, Grosso AL, Chou D, Say L. Global and regional estimates of preeclampsia and eclampsia: a systematic review. Eur J Obstet Gynecol Reprod Biol 2013; 170: 1-7.

2) Sircar M, Thadhani R, Karumanchi SA. Pathogenesis of preeclampsia. Curr Opin Nephrol Hypertens 2015; 24: 131-138.

3) Murphy MS, Casselman RC, Tayade C, Smith GN. Differential expression of plasma microRNA in preeclamptic patients at delivery and 1 year postpartum. Am J Obstet Gynecol 2015; 213: 361-367.

4) Ilekis JV, Reddy UM, Roberts JM. Preeclampsia--a pressing problem: an executive summary of a National Institute of Child Health and Human Development workshop. Reprod Sci 2007; 14: 508-523.

5) Zhou X, Li Q, Xu J, Zhang X, Zhang H, Xiang Y, Fang C, Wang T, Xia S, Zhang Q, Xing Q, He L, Wang L, Xu M, Zhao X. The aberrantly expressed miR-193b-3p contributes to preeclampsia through regulating transforming growth factor-beta signaling. Sci Rep 2016; 6: 19910.

6) Macfarlane LA, Murphy PR. MicroRNA: biogenesis, function and role in cancer. Curr Genomics 2010; 11: 537-561.

7) Wang ZK, Liu FF, Wang Y, Jiang XM, Yu XF. Let-7a gene knockdown protects against cerebral ischemia/reperfusion injury. Neural Regen Res 2016; 11: 262-269.

8) Khandelwal A, Seam RK, Gupta M, Rana MK, Prakash H, Vasquez KM, Jain A. Circulating microRNA-590-5p acts as a liquid biopsy marker in non-small cell lung cancer. Cancer Sci 2019. doi: 10.1111/cas.14199. [Epub ahead of print].

9) Xin L, Liu L, Liu C, Zhou LQ, Zhou Q, Yuan YW, Li SH, Zhang HT. DNA-methylation-mediated silencing of miR-7-5p promotes gastric cancer stem cell invasion via increasing Smo and Hes1. J Cell Physiol 2019: 235; 2643-2654.

10) Chang P, Wang X, Zhou Y, Hou Y. Analysis of the correlation between the expression of miR-655 and esophageal cancer prognosis. Oncol Lett 2017; 13: 4691-4694.

11) Hod T, Cerdeira AS, Karumanchi SA. Molecular mechanisms of preeclampsia. Cold Spring Harb Perspect Med 2015; 5: 1-8.

12) Zhu XM, Han T, Sargent IL, Yin GW, Yao YQ. Differential expression profile of microRNAs in human placentas from preeclamptic pregnancies vs normal pregnancies. Am J Obstet Gynecol 2009; 200: 661.

13) Han HB, Gu J, Ji DB, Li ZW, Zhang Y, Zhao W, Wang LM, Zhang ZQ. PBX3 promotes migration and invasion of colorectal cancer cells via activation of MAPK/ERK signaling pathway. World J Gastroenterol 2014; 20: 18260-18270.

14) Han SY, Han HB, Tian XY, Sun H, Xue D, Zhao C, Jiang ST, He XR, Zheng WX, Wang J, Pang LN, Li XH, Li PP. MicroRNA-33a-3p suppresses cell migration and invasion by directly targeting PBX3 in human hepatocellular carcinoma. Oncotarget 2016; 7: 42461-42473.

15) Renault TT, Dejean LM, Manon S. A brewing understanding of the regulation of Bax function by Bcl-xL and Bcl-2. Mech Ageing Dev 2017; 161: 201-210.

16) Yang Y, Li H, Ma Y, Zhu X, Zhang S, Li J. MiR-221-3p is down-regulated in preeclampsia and affects trophoblast growth, invasion and migration partly via targeting thrombospondin 2. Biomed Pharmacother 2019; 109: 127-134.

17) Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, Lo YM. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 2008; 54: 482-490.

18) Martinez-Fierro ML, Garza-Veloz I, Gutierrez-Arteaga C, Delgado-Enciso I, Barbosa-Cisneros OY, Flores-Morales V, Hernandez-Delgadillo GP, Rocha-Pizana MR, Rodriguez-Sanchez IP, Badillo-Almaraz JI, Ortiz-Rodriguez JM, Castaneda-Miranda R, Solis-Sanchez LO, Ortiz-Castro Y. Circulating levels of specific members of chromosome 19 microRNA cluster are associated with preeclampsia development. Arch Gynecol Obstet 2018; 297: 365-371.

19) Timofeeva AV, Gusar VA, Kan NE, Prozorovskaya KN, Karapetyan AO, Bayev OR, Chagovets VV, Kliver SF, Iakovishina DY, Frankevich VE, Sukhikh GT. Identification of potential early biomarkers of preeclampsia. Placenta 2018; 61: 61-71.

20) Wu G, Zheng K, Xia S, Wang Y, Meng X, Qin X, Cheng Y. MicroRNA-655-3p functions as a tumor suppressor by regulating ADAM10 and beta-catenin pathway in hepatocellular carcinoma. J Exp Clin Cancer Res 2016; 35: 89.

21) James-Allan LB, Whitley GS, Leslie K, Wallace AE, Cartwright JE. Decidual cell regulation of trophoblast is altered in pregnancies at risk of pre-eclampsia. J Mol Endocrinol 2018; 60: 239-246.

22) Charlton F, Bobek G, Stait-Gardner T, Price WS, Mirabito CK, Xu B, Makris A, Rye KA, Hennessy A. The protective effect of apolipoprotein in models of trophoblast invasion and preeclampsia. Am J Physiol Regul Integr Comp Physiol 2017; 312: R40-R48.