Diagnostic value of serum miR-25-3p in hypertensive disorders in pregnancy

Dexia Zhou MM, Bin Qu BD, and Xuan Zhang BD

Department of Internal Medicine, Nantong Maternity and Child Health Hospital, Nantong, Jiangsu, China

ABSTRACT
Hypertensive disorders in pregnancy (HDIP) represent one of the leading causes of maternal and perinatal mortality. MicroRNA (miR)-25-3p plays roles in HDIP diagnosis. We explored miR-25-3p clinical roles in HDIP. HDIP patients [gestation hypertension (GH), mild preeclampsia (mPE), and severe preeclampsia (sPEz)], and normal pregnant women serving as the control were enrolled. Serum miR-25-3p expression patterns were detected by RT-qPCR. The diagnostic efficacy of miR-25-3p on HDIP was analyzed with a ROC curve. Patients were assigned to the high/low miR-25-3p expression groups according to the median value of miR-25-3p expression. All patients were followed up until delivery, and gestational weeks and pregnancy outcomes were recorded at delivery. The effects of miR-25-3p expression on pregnancy outcomes of GH, mPE, and sPEz patients were analyzed by Kaplan-Meier. miR-25-3p expression in GH, mPE, and sPEz patients was up-regulated. In sPEz patients, systolic and diastolic blood pressure, 24-h urine protein, AST, ALT, GGT, and SCr were increased, and PLT was decreased in the high expression group. High miR-25-3p expression was associated with an increased risk of adverse pregnancy outcomes in PE patients. Collectively, high miR-25-3p expression could aid HDIP diagnosis, and associated with an increased risk of adverse pregnancy outcomes in PE patients.

Introduction
Hypertensive disorders in pregnancy (HDIP) is a multiple organ syndrome that occurs in pregnant women, associated with chief clinical symptoms of hypertension and proteinuria in previously normotensive and non-proteinuric women after 20 weeks of pregnancy, leading to heightened risks of maternal and infant death (Sutton, Harper, and Tita 2018; Zhong et al. 2015). According to severity, HDIP can be divided into gestational hypertension (GH), preeclampsia (mild and severe) (PE, mPE, sPEz), eclampsia, pregnancy complicated with chronic hypertension, and chronic hypertension complicated with PE, among which GH and PE are the most prevalent (Umesawa and Kobashi 2017). Unfortunately, the incidence of HDIP has been increasing over the last decade, with the incidence rate abroad at present of 6.4 percent-7.0 percent and in China of about 9.4 percent, which seriously threatens the health of pregnant women and perinatal infants (Hauspurg, Countouris, and Catov 2019; Shah and Gupta 2019). Therefore, it is prudent to advance the search for reliable and accurate HDIP biomarkers for the early prediction and effective prevention of HDIP.

MicroRNAs (miRNAs) represent small non-coding RNAs, possessing the ability to regulate gene expression at a post-transcriptional level by promoting messenger RNA (mRNA) degradation or promoting mRNA translation (Correia de Sousa et al. 2019). In addition, miRNAs are widely expressed in reproductive tissues, while deregulation of miRNAs is implicated in the placental

CONTACT Dexia Zhou dexz0126@163.com Department of Internal Medicine, Nantong Maternity and Child Health Hospital, 399 Century Avenue, Nantong, Jiangsu 226000, China Supplemental data for this article can be accessed online at https://doi.org/10.1080/03630242.2022.2108193. © 2022 Taylor & Francis Group, LLC
formation and pregnancy-related pathological processes (Morales Prieto and Markert 2011). What's more, miRNAs have been previously shown to influence the progression of HDIP (Bounds et al. 2017; Jin et al. 2021; Lv et al. 2019; Skalis et al. 2019). Recent investigations have further indicated vascular endothelium that functions as an endocrine organ and can preserve the key balance of homeostasis by responding to the metabolic status changes, as a key factor in the initiation and the development of disorders in pregnancy (Echeverria et al. 2020). Meanwhile, PE is established as a pregnancy-specific disease with endothelial cell dysfunction, characterized by high blood pressure, proteinuria, and edema, affecting 3–5 percent of pregnant women all over the world (Kim et al. 2017). Notably, one miRNA, namely miR-25, is known to be significantly up-regulated in the placenta of PE patients (Choi et al. 2013). On the other hand, over-expression of lncRNA SNHG12 exerts an attenuating effect on endothelial injury in hypertensive mice by inhibiting the expression of miR-25-3p (Qian et al. 2021). However, there is a scarcity of domestic and foreign reports on miR-25-3p expression and its clinical applicability in serum of patients with HDIP to the best-of-our-knowledge. In lieu of the same, the current study sought to investigate the expression of miR-25-3p in the serum of HDIP patients, and its clinical value in the diagnosis, severity evaluation, and pregnancy outcome prediction of HDIP patients, in an effort to provide a novel reference value for the early prediction, diagnosis, and prognosis of HDIP.

**Materials and methods**

**Ethics statement**

The current study was authorized by the academic ethics committee of Nantong Maternity and Child Health Hospital (Approval No. Y2018030). All experimental procedures were performed in strict accordance with the code of ethics. All the subjects involved were fully informed of the objective of the study and signed informed consent prior to sampling.

**Study subjects**

A total of 190 patients with HDIP at 24 weeks of gestation [calculated mean age: 31.29 ± 3.71; calculated body mass index (BMI): 25.39 ± 1.95; calculated gestational weeks: 38.23 ± 2.88] who were treated at the outpatient department of Nantong Maternity and Child Health Hospital from January 2018 to December 2020 were enrolled in the study. The patients comprised 65 cases of GH, 67 cases of mild preeclampsia (mPE group), and 58 cases of severe preeclampsia (sPEz group). Additionally, 60 normal pregnant women at 24 weeks of gestation (calculated mean age: 30.65 ± 3.45; calculated BMI: 25.16 ± 1.74; calculated gestational weeks: 38.84 ± 1.53) were included in the control group. The age, BMI, and gestational age of healthy subjects in the same period were matched with those of the HDIP patients. Whole blood samples and 24-h urine samples were collected from all subjects and stored for further experimentation.

Inclusion criteria were as follows: singleton pregnancy, age 20–40 years, with no cardiovascular disease, kidney disease, endocrine disease, or other chronic diseases, and no history of special medication. Exclusion criteria were as follows: hypertension history, pathological changes in main organs such as liver, kidney, and lung, autoimmune diseases, and pregnancy with anemia, diabetes, or heart disease.

**Diagnostic criteria**

The diagnosis of HDIP was based on the relevant diagnostic and grading standards of HDIP in the American College of Obstetricians and Gynecologists (ACOG) Practice Bulletin No. 202: Gestational Hypertension and Preeclampsia in 2019 (2019). (1) Diagnostic criteria of GH: after 20 weeks of gestation, systolic blood pressure ≥140 mmHg or diastolic blood pressure mH90 mmHg or both
occurring twice at an interval of at least 4 h without proteinuria. (2) Diagnostic criteria of PE: after 20 weeks of gestation, systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or both occurring twice at an interval of at least 4 h, in addition to 24-h proteinuria ≥300 mg or urinary protein/creatinine ≥0.3 mg/dL. (3) Diagnostic criteria of sPEz: on the basis of the diagnostic criteria of PE, the occurrence of any of the following adverse conditions: (a) platelet count <100 × 10⁹/L; (b) impaired liver function, manifested as liver enzyme concentration in blood twice the upper limit of normal concentration; (c) persistent severe epigastric pain; (d) renal insufficiency [(serum creatinine (SCr) concentration more than 1.1 mg/dL or doubled in the absence of other renal diseases); (e) pulmonary edema; (f) new-onset headache unresponsive to drugs, excluding other diagnoses. (4) Diagnostic criteria of mPE: The patients met the diagnostic criteria of PE but did not meet the diagnostic criteria of sPEz.

**Clinical indicator detection**

A wide array of clinical indicators were measured and recorded in the enrolled subjects, including body measurements, age, gestational weeks, gravidity, BMI, and systolic and diastolic blood pressure. In addition, 24-h urinary protein was measured from subject urine samples with the help of urine protein quantitative test kits (Wanlei bio, Liaoning, China). Additionally, biochemical index measurements were performed by detecting the platelet count (PLT) with an XS800i automatic hematology analyzer, while the contents of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamyl transpeptidase (GGT) and Scr in blood samples were quantified using a TCHG500 automatic biochemical analyzer.

**Follow-up of adverse pregnancy outcomes**

All enrolled patients were followed up until delivery. Adverse maternal and/or fetal and neonatal outcomes were recorded. Adverse maternal outcomes were defined as the presence of maternal single organ dysfunction (renal failure, heart failure, and liver failure), pulmonary edema, disseminated intravascular coagulation, or maternal death. Meanwhile, adverse fetal and neonatal outcomes were defined as the incidence of intrauterine fetal death, intrauterine growth retardation, placental abruption, respiratory distress syndrome, 5-min Apgar score <7, intrauterine or 7-d postpartum death (Antonia Droge et al. 2021).

**Total RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR) detection**

Total RNA content was extracted from subject tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The obtained RNA was reverse-transcribed into cDNA with the help of PrimeScript RT reagent kits (Takara, Dalian, China). Subsequently, qPCR was performed on an ABI7900HT fast PCR real-time system (Applied Biosystems, Foster city, CA, USA) using SYBR® Premix Ex Taq™ II (Takara). The reaction conditions were as follows: pre-denaturation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extending at 72°C for 34 s. U6 was adopted as the internal parameter and the 2−ΔΔCt method was utilized for data analyses (Schmittgen and Livak 2008). The primer sequences (synthetized by Sangon Biotech Co., Ltd., Shanghai, China) are as follows: miR-25-3p Forward: GCACGCCGGCGACAGG; miR-25-3p Reverse: AGTGCAGGGTCCGAGGTATT. U6 Forward: ATTGGAACGATACAGAGAAGATT; U6 Reverse: GGAACGCTTCACGAATTTTC.

**Statistical analysis**

SPSS 21.0 software (IBM Corp. Armonk, NY, USA), GraphPad Prism 8 software (GraphPad Software Inc) and MedCalc® version 15.0 statistical software (MedCalc Software Ltd, Ostend, Belgium) were adopted for data analyses and mapping. The G*Power program was utilized for the calculation of the
sample size in advance. Variable data were expressed as mean ± standard deviation or in counts. Non-paired t test or x² was adopted for comparison between two groups and one-way analysis of variance (ANOVA) was utilized for comparison among multi-groups. Tukey's multiple comparisons test was performed for post-hoc test. Receiver operating characteristic (ROC) curve was adopted for diagnostic analyses of miR-25-3p on HDIP. Kaplan-Meier method was utilized for analysis of the effect of miR-25-3p on pregnancy outcomes in HDIP patients. A value of P < .05 was regarded as statistically significant.

Results

Comparative analysis of clinical data between HDIP patients and healthy subjects

Firstly, 190 patients with HDIP were enrolled and classified into 65 cases of GH, 67 cases of mPE, and 58 cases of sPEz following the relevant diagnostic and grading standards of HDIP, and 60 normal pregnant women were additionally included as controls. The clinical indexes of HDIP patients and the controls were compared and analyzed. There were no significant differences in regard to age, BMI, gestational weeks, and gravidity among the 4 groups. Meanwhile, significant differences were observed in systolic blood pressure, diastolic blood pressure, 24-h urine protein, PLT, AST, ALT, GGT and Scr among the GH group, mPE group, and sPEz group. There were also significant differences between the mPE group and hypertension group, and between the sPEz group and mPE group (all P < .001) (Table S1).

miR-25-3p was highly-expressed in the serum of HDIP patients

Expression patterns of miR-25-3p in serum of HDIP patients and controls were detected by means of RT-qPCR. Compared with the control group, serum miR-25-3p expression levels were higher in the HDIP group (P < .01) (Figure 1a). In addition, compared with the control group, the expression levels of miR-25-3p in patients of the GH group, mPE group, and sPEz group were all significantly up-regulated, such that miR-25-3p levels were markedly higher in patients of mPE group compared to the GH group, while miR-25-3p expression levels were further significantly higher in patients of the sPEz group relative to the mPE group (all P < .05) (Figure 1b).

miR-25-3p exhibits clinical diagnostic efficiency in patients with HDIP

In order to further elucidate the clinical diagnostic significance of serum miR-25-3p expression in HDIP, the diagnostic efficacy of miR-25-3p on HDIP was analyzed with a ROC curve. The area under the curve (AUC) of miR-25-3p in serum for the diagnosis of GH was 0.770, the cutoff value was 1.240, the specificity was 98.33 percent, and the sensitivity was 49.23 percent (Figure 2a); Meanwhile, the AUC for the diagnosis of mPE was 0.848, the cutoff value was 1.185, the specificity was 93.33 percent, and the sensitivity was 74.63 percent (Figure 2b); whereas the AUC for the diagnosis of sPEz was 0.946, the cutoff value was 1.235, the specificity was 98.33 percent, and the sensitivity was 87.93 percent (Figure 2c). Together, these findings that miR-25-3p exhibited diagnostic efficiency in patients with GH, mPE, and sPEz, with the highest diagnostic efficiency in patients with sPEz.

Comparison of clinical indexes in serum of HDIP patients based on different levels of miR-25-3p

According to the median value of miR-25-3p expression in the aforementioned patients, HDIP, mPE, and sPEz patients were assigned to the low expression group and high expression group, respectively, and then the clinical indicators were compared. In patients with HDIP, mPE, and sPEz, there were no significant differences in regard to age, BMI, gestational age, and gravidity between the high expression group and the low expression group. In addition, AST (P = .016) and SCr (P = .020) were both markedly
miR-25-3p was highly expressed in the serum of HDIP patients. (a-b) the expression of miR-25-3p in serum of HDIP patients and controls was detected by RT-qPCR. The data were expressed as mean ± standard deviation. One-way ANOVA was used for comparison among multi-groups. Tukey’s multiple comparisons test was used for the post hoc test. *p < .05, ** p < .01.

Diagnostic efficacy of miR-25-3p in patients with GH; (b) mPE; and (c) sPEz was analyzed using the ROC curve.

Figure 1. miR-25-3p was highly expressed in the serum of HDIP patients. (a-b) the expression of miR-25-3p in serum of HDIP patients and controls was detected by RT-qPCR. The data were expressed as mean ± standard deviation. One-way ANOVA was used for comparison among multi-groups. Tukey’s multiple comparisons test was used for the post hoc test. *p < .05, ** p < .01.

Figure 2. Diagnostic efficacy of miR-25-3p in patients with HDIP. The diagnostic efficacy of miR-25-3p in patients with (a) GH; (b) mPE; and (c) sPEz was analyzed using the ROC curve.

increased in GH patients in the high miR-25-3p expression group compared with those in the low miR-25-3p expression group, while there were no significant differences in other clinical indicators. Meanwhile, in patients with mPE, systolic blood pressure (P = .0183), 24-h urine protein (P = .0171), AST (P = .0019) and ALT (P = .0002) were all significantly higher in the miR-25-3p high expression group than those in the low expression group, while there were no significant differences in other clinical indicators. In the patients with sPEz, systolic pressure (P = .0024), diastolic pressure (P = .0084), 24-h urine protein content (P = .0068), AST (P = .0054), ALT (P = .0009), GGT (P = .0022) and SCr (P = .0118) were all significantly increased, while PLT was significantly decreased in the high miR-25-3p expression group relative to those in the low miR-25-3p expression group (all P = .0007) (Table S2).
High miR-25-3p expression increased the risk of adverse pregnancy outcomes in patients with HDIP

The above-mentioned findings revealed that miR-25-3p was highly-expressed in the serum of patients with HDIP, and closely associated with clinical indicators of patients. To further explore the value of miR-25-3p in the prognosis of GH, mPE, and sPEZ patients, the GH, mPE, and sPEZ patients were assigned to the low expression group and the high expression group respectively according to the median value of miR-25-3p expression. Both groups were followed up till delivery, and the gestational weeks and pregnancy outcomes were recorded. In GH patients, there were 4 cases of adverse pregnancy outcomes in the low expression group, and the incidence of adverse pregnancy outcomes was calculated to be 12.1 percent; there were 7 cases of adverse pregnancy outcomes in the high expression group, and the incidence of adverse pregnancy outcomes was calculated to be 21.9 percent, which was significantly higher than that in the low expression group ($x^2 = 1.099, P = .001$). In mPE patients, there were 7 cases of adverse pregnancy outcomes in the low expression group, and the incidence of adverse pregnancy outcomes was calculated to be 20.6 percent; meanwhile, there were 15 cases of adverse pregnancy outcomes in the high expression group, and the incidence of adverse pregnancy outcomes was calculated to be 45.5 percent, which was significantly higher than that in the low expression group ($x^2 = 4.695, P = .036$). In sPEZ patients, there were 8 cases of adverse pregnancy outcomes in the low expression group, and the incidence of adverse pregnancy outcomes was calculated to be 27.6 percent; there were 17 cases of adverse pregnancy outcomes in the high expression group, and the incidence of adverse pregnancy outcomes was calculated to be 58.6 percent, which was significantly higher than that in the low expression group ($x^2 = 5.695, P = .015$). Moreover, the results of Kaplan-Meier analysis showed that in GH ($P = .273$), mPE ($P = .027$) and sPEZ ($P = .027$) patients, the curve of miR-25-3p high expression group shifted to the left ($P < .05$) (Figure 3), indicating that at the same gestational age, the cumulative incidence of adverse pregnancy outcomes was higher in the high expression group. Altogether, these findings indicated that high expression of miR-25-3p was associated with poor pregnancy outcomes.

Discussion

HDIP affects over 10 percent of all pregnant women across the world, accounting for a remarkable proportion of perinatal mortality and morbidity (Sutton, Harper, and Tita 2018). Meanwhile, the hard-done work of our peers has indicated that miR-25-3p plays an essential role in HDIP (Choi et al. 2013). In an effort to expand our understanding of the same, we performed a series of experiments to explore the clinical roles of miR-25-3p in HDIP. The obtained findings revealed that high expression of miR-25-3p could assist the diagnosis of HDIP.

Accumulating evidence has shown that various miRNAs can function as potential early biomarkers for HDIP (Wu et al. 2020). However, the expression and the diagnostic value of miR-25-3p in HDIP were still unclear. Herein, our findings revealed that miR-25-3p was highly-expressed in HDIP.

![Figure 3](image-url)

**Figure 3.** High expression of miR-25-3p increased the risk of adverse pregnancy outcomes in patients with HDIP. The effect of miR-25-3p on pregnancy outcome in patients with HDIP was analyzed using Kaplan-Meier method.
patients, and further significantly up-regulated in GH, mPE, and sPEz patients compared to normal pregnant women. In addition, the expression of miR-25-3p in mPE patients was significantly higher than that of the GH patients, and that in sPEz patients was markedly higher than that of mPE patients. In line with our data, a prior study documented that miR-25 was significantly over-expressed in PE patients relative to normal pregnant women, (Choi et al. 2013). Together, these findings and evidence indicate that up-regulated expression of miR-25-3p in serum of HDIP patients may be of great value for early prediction and effective prevention of HDIP.

Furthermore, several miRNAs are abnormally elevated in PE and further exhibit strong clinical diagnostic efficacy for PE (Liao et al. 2021; Yang, Tang, and Zhao 2021). Subsequent analyses using a ROC curve in our study revealed that the AUC of miR-25-3p for the diagnosis of GH was 0.770, the cutoff value was 1.240, the specificity was 98.33, and the sensitivity was 49.23 percent. Meanwhile, the AUC of miR-25-3p for the diagnosis of mPE was 0.848, the cutoff value was 1.185, the specificity was 93.33 percent, and the sensitivity was 74.63 percent. Lastly, the AUC of miR-25-3p for the diagnosis of sPEz was 0.946, the cutoff value was 1.235, the specificity was 98.33 percent, and the sensitivity was 87.93 percent. Overall, these findings elaborate the diagnostic value of serum miR-25-3p in HDIP, such that miR-25-3p exhibits high diagnostic efficiency for GH, mPE, and sPEz patients, with the highest diagnostic efficiency for sPEz patients.

PE is characterized by high blood pressure and proteinuria in previously normotensive and non-proteinuric women after 20 weeks of pregnancy (Filipek and Jurewicz 2018). Moreover, PLT is known to serve as a severity marker for PE (Reddy and Rajendra Prasad 2019), while AST and ALT levels are further regarded as important indexes in PE (Tok et al. 2021). Our experimentation revealed that there were significant differences in systolic and diastolic pressure, 24-h urine protein, PLT, AST, ALT, GGT, and SCr between GH, mPE, and sPEz patients, and normal pregnant women. Consistently, elevated PLT, and systolic and diastolic pressure have been previously reported as diagnostic factors for HDIP (Leeman, Dresang, and Fontaine 2016; Yang et al. 2012). Herein, our findings indicate that GH, mPE, and sPEz patients, and normal pregnant women present with different clinical indexes, which may be useful to distinguish the normal population from HDIP patients. Furthermore, subsequent comparison of clinical indexes in the serum of HDIP patients with different levels of miR-25-3p demonstrated that in GH patients, AST and SCr levels in the high miR-25-3p expression group were higher than those in the low expression group. Meanwhile, in mPE patients, the systolic blood pressure, AST, 24-h urinary protein, and ALT levels in the high miR-25-3p expression group were higher compared to those in the low expression group. Lastly, in sPEz patients, systolic blood pressure, diastolic blood pressure, 24-hour urinary protein, AST, ALT, GGT, and SCr levels in the high miR-25-3p expression group were higher relative to the low expression group, and PLT was lower. In lieu of these results, it would be plausible to suggest that the expression of miR-25-3p was related to clinical indicators of HDIP. Moreover, in recurrent pregnancy loss, blood urea nitrogen levels decreased depending on the miR-25 allele, and creatinine levels are previously associated with the genotypes of miR-25 alleles (Trumtel et al. 1990). However, there is no domestic and foreign report at present on the clinical diagnostic efficiency of miR-25-3p expression and its correlation with clinical indexes in serum of patients with HDIP. Tackling the later, our findings highlighted that miR-25-3p exhibits close correlations with the clinical parameters of HDIP, and the change of its expression level might assist in the diagnosis of HDIP.

Furthermore, aberrant expression of miRNAs is associated with adverse pregnancy outcomes (Pfeiffer et al. 2020). For instance, abnormal expression levels of miR-346 and miR-582-3p during pregnancy were previously correlated with a variety of maternal and fetal complications, while their differential expression levels in maternal blood, umbilical cord blood, and placenta may function as potential biomarkers for adverse obstetric outcomes (Tsai et al. 2017). However, there is little known in regard to the relationship between miR-25-3p expression and adverse outcomes in HDIP. Accordingly, we analyzed the association of miR-25-3p levels with adverse pregnancy outcomes, and our findings demonstrated that in mPE and sPEz patients, the cumulative incidence of adverse pregnancy outcomes in the miR-25-
3p high expression group was higher than that in the miR-25-3p low expression group, which underscores the association between high miR-25-3p expression and adverse pregnancy outcomes in HDIP patients.

In summary, the current study supported that miR-25-3p was highly-expressed in the serum of patients with HDIP. In addition, over-expression of miR-25-3p can aid diagnose the severity of HDIP, and is further associated with an increased risk of adverse pregnancy outcomes in patients with sPEz, which provides a new reference for HDIP diagnosis, severity evaluation, and pregnancy outcome prediction. However, the number of cases and events included in our study is limited. Consequently, it is prudent to expand the sample size and carry out a multi-center study to further clarify the diagnostic and prognostic evaluation ability of miR-25-3p for HDIP.

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**ORCID**

Dexia Zhou MM http://orcid.org/0000-0002-0140-3122

**Data availability statement**

All the data generated or analyzed during this study are included in this published article.

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