ORIGINAL ARTICLE

MICRO RNA 182-3-P, 519-D-5P, 378-3P AS NON-INVASIVE PREDICTORS OF PREECLAMPSIA

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Background: MicroRNAs (miRNAs) are an emerging field of interest in many diseases. Some of the miRNAs have been reported to be expressed differentially in diseased states of pregnancy. The current study was designed to measure and compare the levels of microRNA 182-3-p, 519-d-5p, and 378-3p and it was hypothesized that the microRNA 182-3-p, 519-d-5p, and 378-3p can be used as a non-invasive predictor of preeclampsia. **Methods:** Expression level of the miRNAs 182-3-p, 519-d-5p, and 378-3p was measured in the serum of preeclamptic and normal pregnancies by real-time PCR. Data was entered and analysed by Statistical Package for the Social Sciences 22 (SPSS). **Results:** Significantly high expression levels of MiRNA 182-3p, 519-d-5p and low levels of miR-378-3p were associated with preeclampsia (PE). **Conclusion:** The results revealed that miR-182-3p is a powerful predictor of PE with an Odds Ratio of 5.9 and can be used as a noninvasive, reliable predictor of PE to screen these patients at an early stage. Screening at early gestation with follow-up studies can emphasize the results.

Keywords: Hypertension; AicroRNA; Predictors; Pregnancy; Real time PCR

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INTRODUCTION

MicroRNAs (miRNAs) are the emerging field of interest in many diseases, as these small noncoding RNAs can regulate and alter gene expression at the post transcriptional level.[1] They are known to actively participate in numerous cellular activities like apoptosis, cell proliferation, differentiation, etc.[2] Some of the miRNAs are placental specific and have been reported to be expressed differentially in diseased states of pregnancy.[3] Balanced proliferation, differentiation, and invasion of the maternal tissues by the trophoblasts is the key to a healthy pregnancy. In the due course of these processes, trophoblasts encounter different immune cells of the mother e.g., T cells, macrophages, and natural killer cells of the decidua.[4] A complex dialogue is established between the maternal and foetal cells to reciprocally control the aggregation of immune cells at the site of implantation, and trophoblastic proliferation.[5] Several environmental and genetic factors can influence and disrupt these processes resulting in various pathologies e.g., preeclampsia, placenta accreta, intra uterine growth restriction etc.[6] Preeclampsia (PE) is a placental specific hypertensive disease of gestation with threatening consequences for both foetus and mother. The pathogenesis of PE is multiplex with abnormal placentation playing a central role due to altered angiogenesis, apoptosis and trophoblastic differentiation.[7] Although several theories have been proposed to elaborate the complex pathogenesis of this multisystem disease, the challenge to prevent and diagnose the disease at an early stage remains there.

In recent years, miRNAs have emerged as novel potential markers to diagnose as well as screen pregnancies at risk of being complicated with PE. Variable reports have been published regarding the expression of various miRNAs in PE, with upregulation of the expression of a few miRNAs while decreased expression of others.[8,9] Initially, most of the reported miRNAs were studied in the placental tissue but recently a number of researchers have studied the expression of different miRNAs in the sera of preeclamptic cases.[8,10] Detection of aberrant expression of miRNAs in blood samples from preeclamptic pregnancies and their involvement in different cellular pathways has deciphered the pathogenesis of such a complex disorder e.g. miR-155 is reported as a risk factor for PE.[11] Numerous studies have reported differential expression of different miRNAs and their involvement in the development of PE. MiRNA-182-3p, miR-519-d-5p, and miR-378-3p can alter the process of placentation by affecting angiogenesis and trophoblastic differentiation, the key components of normal placental development.[12,13] Early detection of the evidence for the disruptions of these processes can help in screening out susceptible pregnancies for prompt treatment and care. The current study was planned to measure and compare the levels of microRNA 182-3-p, 519-d-5p, and 378-3p in

the serum of preeclamptic and normal pregnancies and it was hypothesized that the microRNA 182-3-p, 519-d-5p, and 378-3p can be used as a non-invasive predictor of preeclampsia.

MATERIAL AND METHODS

An observational cross-sectional comparative study was designed and conducted at the Department of Physiology and Cell Biology, University of Health Sciences, Lahore, Pakistan, from 2016 to 2018. Approval was taken from the institutional ethics review board and the sample size was calculated using the World Health Organisation (WHO) calculator[15] on the basis of the formula:

$$n = \frac{2\sigma^2(z_{1-\alpha/2} + z_{1-\beta})^2}{(\mu_1 - \mu_2)^2}$$

A sample of 50 cases was recruited using purposive sampling technique from preeclamptic pregnancies (aged 18–40 years) in their third trimester (28–40 weeks). Preeclamptic patients had new onset of systolic blood pressure of more than 140mmHg or diastolic blood pressure equal to or more than 90mmHg at >20 weeks of gestation along with 24hrs proteinuria ≥300 mg (≥1+ on dipstick), in at least two samples of urine collected randomly 4–6 hrs apart.[16] Healthy Normotensive pregnancies (45 in number) with comparable age in the final trimester (28–40 weeks) were recruited as controls. All patients who presented with PE history in the previous pregnancy, multiple pregnancies and chronic hypertension were excluded, and similarly, those with other chronic diseases, like arthritis, disease of kidneys, inflammatory bowel disease, and cardiac pathology, like the ischemic disorder of heart, diabetes mellitus was not included. Smoking was also considered an exclusion criterion.

Data was collected according to Helsinki guidelines, after informed written consent from each participant. Three ml blood, collected under aseptic measures, was added to serum separator tubes. FavorPrep miRNA Isolation Kit (Favorgen, Taiwan) was utilized for the extraction of miRNA from serum. MiRNA concentration was measured by nanodrop. Reverse transcription of miRNA was performed by utilizing the miScript II RT kit (Qiagen). Primers were constructed for the specific gene sequence to estimate levels of expression of miRNA. Real-time polymerase chain reaction (PCR) (CFX 96 machine) was used with the SYBR Green /ROX q PCR Master Mix(2X), (Fermentas, USA). The cycle included initial denaturation at 94ºC for 3min and 15 cycles of denaturation at 95 ºC for 10s. Annealing was set at 58 ºC for 20s. Melt curve analysis was performed with 65–95 ºC with an increment of 0.2 ºC for 0.01s. Relative gene expression analysis was done by utilising the $2^{-\Delta\Delta ct}$ approach.[17] Primer sequence for PCR was noted (Table-1).

Data was entered and analysis was done with SPSS-22. For the normalisation of miRNA, U6 was utilised as an internal control. MiRNAs were expressed as the median of fold change. *p*-value of less than 0.05 was considered statistically significant. The association of each miRNA with PE was checked by linear regression. The receiver operating characteristic (ROC) curve was plotted to find the optimal cutoff value. Overall diagnostic accuracy was estimated with the area under the ROC curve. Logistic regression was applied to check the role of miRNA as a predictor of PE by confounding the effect and calculating the Odds Ratio (OR).

RESULTS

The study population consisted of 50 women with preeclampsia and 45 healthy normotensive controls. The clinical parameters are described in Table-2. Maternal ages and gestational ages at sampling of the participants were not different between cases and controls ($p>0.05$). Expression level of miRNA 182-3p, 519-d-5p, and 378-3p was significantly different among PE and healthy pregnancies with an increase in the level of miR-182-3p and 519d-5p in PE. Contrarily a significantly decreased expression level of miR-378-3p was observed in pregnancies complicated with PE (Table-3).

To check the association of each miRNA with PE, Chi-Square test was applied, and OR was calculated along with a confidence interval. It was observed that high levels of MiR-182-3p and miR-519d-5p were associated with a 2.4 and 1.8-times high risk of PE respectively. Contrarily, higher expression of miR-378-3p was predicted to have a protective role as higher expression levels were associated with healthy pregnancies (Table-4). Receiver operating characteristic (ROC) curve analysis exhibited a value of 0.76 and 0.71 for the area under the curve (AUC) of miR-182-3p and miR-519d-5p respectively. The optimal cutoff value was 1.6 and 1.1 for miR-182-3p and miR-519d-5p respectively. Logistic regression was applied to control the confounding effect of miRNAs on each other and to check the role of individual miRNA as potential predictors of PE. Interestingly the results of logistic regression revealed miRNA 182-3p as a predictor of the disease with 5.9 times increased risk at a significant $p<0.01$. Although the values of OR for miR-519d-5p and 378-3p were significant along with miR-182-3p in the Chi-Square test, it became statistically non-significant when the confounder effect was controlled. The results revealed that when the confounder effect of miR-519-5p and 378-3p is controlled, there is still 5.9 times increased risk of developing PE with high levels of miR-182-3p (Table-5).

Table-1: Sequences of primers for MicroRNAs

Name	Sequence
MiRNA-182-3P	5`GTGGTGGTTCTAGACTTGC`3
MiRNA-378-5P	5`GTTTCTCCTGACTCCAGGT`3
MiRNA-519-5P	5`GTTTCCTCCAAAGGGAAGC`3
Uni-R	5`GTGCAGGGTCCGAGGT`3
U6-F	5' –CTCGCTTCGGCAGCACA- 3'
U6-R	5' –AACGCTTCACGAATTTGCGT- 3'

Table-2: Demographic characteristics of the participants

	Preeclampsia n = 50	Normotensive group n = 45	*p*-value
Maternal Age (years)	26.5 (18-40)	25 (20-35)	0.271
Body Mass Index (kg/m2)	28.7 (22.9-37.8)	26.1 (19.1-35.4)	*0.000
Gestational Age	32 (28-39)	32 (28-38)	0.431
Systolic BP	150 (130-200)	100 (90-120)	*0.000
Diastolic BP	100 (90-110)	70 (60-80)	*0.000
Parity	1 (0-7)	1 (0-6)	0.767

Values presented as Median with Inter Quartile Range (IQR)

*Significant difference between preeclampsia and normotensive group, calculated by Wilcoxon Rank Sum test. BP: Blood pressure.

Table-3: MiRNA expression levels in preeclamptic and normotensive pregnancies

	Preeclampsia n = 50	Normotensive group n = 45	*p*-value
MiRNA 182-3p	2.15 (1.2-9.3)	0.46 (0.2-1.5)	*0.000
MiRNA 519d-3p	2.53 (1.1-4.9)	1.16 (0.3-2.5)	*0.000
MiRNA 378-3p	0.17 (0.02-0.4)	0.44 (0.1-0.7)	*0.000

Values presented as Median with Inter Quartile Range (IQR)

*Significant difference between preeclampsia and normotensive group, calculated by Wilcoxon Rank Sum test, n: number of subjects

Table-4: Association of MiRNAs with preeclampsia

	Preeclampsia n = 50	Normotensive group n = 45	*p*-value	OR and CI
MiRNA 182-3p			*0.000	2.44 (1.53-3.87)
High	38	14		
Low	12	31		
MiRNA 519d-3p				
High	36	27	*0.002	1.8 (1.21-2.67)
Low	14	18		
MiRNA 378-3p				
High	23	33	*0.006	0.62 (0.44-0.88)
Low	27	12		

Chi-square was applied to calculate *p*-value, Odds ratio (OR) and confidence interval (CI). **p*-value <0.05 is statistically significant

Table-5: Potential predictor of preeclampsia by logistic regression

	B	*p*-value	OR (CI)
MiRNA 182-3p	1.77	*0.000	5.92 (1.2-7.4)
MiRNA519d-3p	0.71	0.293	2.04 (1.1-3.9)
MiRNA 378-3p	0.48	0.484	1.61 (0.2-1.1)

Binary logistic regression was applied to check the role MiRNA as a predictor of PE. OR and CI was calculated. A **p*<0.05 is statistically significant. B: Logistic regression coefficient, CI: Confidence interval, OR: Odds ratio

DISCUSSION

The role of miRNAs in the pathogenesis, diagnosis and prognosis of PE is an emerging field of research that attracts a lot of researchers. Numerous studies have studied variable miRNAs with differential results. The present study found higher levels of miR-182-3p and miR-519d-5p and lower levels of miR-378-3p in preeclamptic pregnancies and the results are in accordance with previous research.[18–20] Numerous autocrine and paracrine factors are involved in the regulation of trophoblastic invasion during normal placentation. These trophoblast cells share characteristics like tumour cells, as both are migratory as well as invasive. The major difference is the fine and strict regulation of trophoblast invasion, limited to early gestation. Abnormal trophoblastic invasion is a hallmark of abnormal placentation and PE. Angiogenesis and apoptosis are two important pathways involved in the process of normal placentation. There is evidence that disruption of these processes is a key factor in the pathogenesis of PE.[21]

MiRNAs have been reported to be differentially expressed and modulate these processes. MiR-182-3p is one of the miRNAs involved in the regulation of both angiogenesis and apoptosis. It has been reported to modulate the process of angiogenesis by targeting both angiogenin and *VEGF*.[14] Similarly, B-cell lymphoma 2 (BCL2), an important gene involved in apoptosis, is also modulated by miR-182.[22] The current study reported that not only is the expression level of miR-182-3p elevated in PE, but it is also a risk factor (5.9 times increased risk) to complicate pregnancies with the disease. Our results are supported by another study where high expression of miR-182-3p was found in PE and it was shown to inhibit the trophoblastic invasion by targeting Rho family GTPase 3 (RND3) gene.[23]

MiR-519d-5p is another miRNA studied in the current research with a higher expression level in preeclamptic women when compared with healthy pregnancies. MiR-519d-5p is reported to be associated with pregnancy with an increase in expression levels with advancing gestation. There is evidence that it plays a role in placental development by regulating the invasive behaviour of the trophoblast. It has been reported to suppress the invasion of trophoblast. This effect of miR-519d-5p is found to be like miR-182-3p, although the targeted genes are different for both these miRNAs. MiR-519d-5p is known to target matrix metalloproteinase-2 (MMP-2) as well as Phosphatase and Tensin Homolog (PTEN) genes involved in trophoblast migration and invasion. In congruence with our findings are the results by a recent study that elucidated higher expression levels of miR519d-5p in PE along with its target gene, i.e., PTEN.[24] In addition to high levels of miR-519d-5p, it was found to be significantly associated with PE with an odds ratio of 1.8 in the present research. Contrary to miR-182-3p and miR-519d-5p, the expression of miR-378-3p was found to be lower in PE as compared to the healthy group in accordance with others. Decreased levels of miR-378-3p inhibit cell invasion and migration by targeting Nodal and Transforming growth factor beta (TGF-β), involved in the process of normal placentation. Shallow invasion of the trophoblast along with defective remodelling of spiral arteries is associated with decreased expression of miR-378-3p.[20] When checked for association, it was observed to have a protective role as the expression level was high in healthy women ($p<0.01$). Finally, on the application of logistic regression to control the confounding effect of miRNAs on each other, the association of miR-519d-5p and miR-378-3p became statistically non-significant. The result of logistic regression revealed a significant association of miR-182-3p with PE with 5.9 times increased risk of PE in pregnancies with increased expression of this miRNA. The results highlighted the role of miR-182-3p as a potential predictor of PE. Early prediction of this serious complication can help in the reduction of foetal and maternal complications. Current clinical guidelines focus on screening of all pregnant women for PE in the first trimester. Although there are several risk factors such as nulliparity, family history of PE, obesity etc., these maternal risk factors are not effective in predicting the disease. Instead, clinicians recommend a combination of various factors to screen the patients for PE at an early stage. Recently the field of molecular biology and epigenetics has immensely evolved highlighting the role of non-coding miRNAs in numerous biological processes.[25] In view of these findings and updates, we hypothesized and proved that miRNAs can be used as non-invasive predictors of PE. Although, all three studied miRNAs showed significant association with PE, the role of miR-182-3p as a predictor remained significant even when the confounding effect of the other two was controlled. The results reveal that miR-182-3p is a powerful predictor of PE as the value for AUC was 7.6 with an OR of 5.9. MiRNAs can be used as noninvasive, reliable predictors of PE to screen these patients at an early stage.

The collection of single samples of each patient in the last trimester is the limitation of our study. Sample collection in the first trimester with follow-up of the patients could emphasize the current findings.

CONCLUSION

The results of the present study conclude that miRNAs, non-invasive markers, can be used at early stages of gestation as a screening tool for PE. Early screening will help in prevention of the complications and change the disease outcome. Although few miRNAs have been reported as potential biomarkers of PE, to the best of our knowledge, MiR-182-3p is reported for the first time as a powerful predictor of PE.

Statements and Declarations:

Ethical Approval: The study was approved by the Institutional review board of the University of Health Sciences No: UHS/Education/126-16/2754/27.10.2016

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Consent to participate: Informed consent was obtained from all individual participants included in the study.

Competing interests: None

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AUTHORS' CONTRIBUTION

ZA: Concept and design of study, acquisition, analysis, accuracy, and interpretation of data. Final approval of the version to be published. UZ, AT, SZ,

SK: Study design, interpretation of data and analysis, revision, accuracy.

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