Comparing the Role of Placental Tissue Remodeling Biomarkers MXRA5 and TIMP2 in Predicting the Severity of Preeclampsia.

Sara Atta[1], Mona A. H. El Baz [1], Ahmed M. A. Sobh[2]*, Alshimaa H. M. Hassan[3]*, Shimaa A. A. Hafeez [1]

[1] *Medical Biochemistry Department, Faculty of Medicine, Assiut University, Assiut, Egypt.*
[2] *Department of Obstetrics and Gynecology, Faculty of Medicine, Assiut University, Assiut, Egypt.*
[3] *Fourth-year medical student, Faculty of Medicine, Assiut University, Assiut, Egypt.*
***Corresponding author:** Dr. Sara Abdel-Reheem Atta, **E-mail:** saraatta@aun.edu.eg*

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ABSTRACT
To evaluate the role of matrix-remodeling associated 5 (MXRA5), and tissue inhibitor metalloproteinase 2 (TIMP2) levels in predicting the severity of preeclampsia. Forty-eight pregnant women complicated by preeclampsia, whose ages ranged from 20 to 40 years, in addition to 48 age- and sex-matched normotensive pregnant women, served as the control group included in the study. The placental samples in this study were obtained via cesarean section, frozen immediately in liquid nitrogen, and stored at − 80 °C until the time of assay for subsequent RNA and protein biochemical measurements. There was a statistically significant decrease in the mean MXRA5 level among cases compared with controls ($p < 0.001$ for both). However, there was no statistically significant difference in the mean TIMP2 level between the cases and controls. There was a statistically significant lower mean difference in pAKT-ELIZA among PE patients (0.29±0.04) than among controls (0.56±0.14, $p<0.001$), with a positive correlation between pAKT and MXRA5. MXRA5 performed high accuracy (sensitivity: 96.7%, specificity: 94.4%) in excluding cases and controls with PE. Furthermore, MXRA5 exhibited greater sensitivity in distinguishing between severe and mild cases (95.5%, sensitivity 96.7%, specificity), whereas the role of TIMP2 was insignificant.
In conclusion: in pre-eclampsia patients, reduced MXRA5 expression is a highly sensitive biomarker for detecting and predicting the severity of PE.

Introduction

Preeclampsia (PE) is a severe pregnancy-associated illness that manifests after the twentieth week of pregnancy and is characterized by newly developed hypertension and proteinuria [1]. Approximately nine percent of all pregnancies are affected by preeclampsia [2]. PE is a leading factor in maternal and fetal morbidity and mortality, especially in underdeveloped nations, which experience PE seven times more frequently than developed nations do [3]

Although the exact mechanism of PE is still unknown, defective trophoblast invasion, apoptosis, and impaired remodeling of spiral arteries[4] are the major causes of PE-associated impaired placentation [5, 6].

MXRA5 (matrix-remodeling associated 5) is a member of the MXRA (matrix-remodeling associated) family that is made up of three genes (MXRA5, MXRA7, and MXRA8). MXRA5 is an adhesion protein with leucine-rich repeats that contributes to matrix remodeling and cell adhesion [7]. Consequently, it has been demonstrated that preeclamptic patients' placentas have downregulated MXRA5 expression[5].The involvement of MXRA5 mutation in matrix remodeling and cell invasion has been linked to non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) [8, 9]. Furthermore, MXRA5 reacts to transforming growth factor-1 (TGF-1) therapy in chronic renal disease by exerting anti-inflammatory and antifibrotic effects[10]. However, the precise function of MXRA5 in the pathophysiology of preeclampsia is still unclear.

Much attention has been focused in recent years on the role of matrix metalloproteinases (MMPs) in the pathophysiology of preeclampsia. It includes at least 28 different enzymes of zinc-dependent endopeptidases that hydrolyze the extracellular matrix [11, 12]. Particularly important for effective cytotrophoblast invasion during the early stages of pregnancy are MMP-2 and MMP-9, which are regarded as essential enzymes for

basement membrane degradation[13]. Extracellular matrix remodeling enzymes are essential effectors of developmental processes such as cell migration, proliferation, apoptosis, tissue morphogenesis, and tumor progression, which are counterbalanced by their tissue inhibitors (TIPs)[14, 15].

Cell invasion, migration, metastasis, and proliferation are all impacted by the PI3K/Akt signaling pathway [16, 17]. According to a prior study, PE placentas activate the PI3K/Akt pathway to control cell proliferation[18, 19].

To verify our theory, we tested the expression of MXRA5, and TIMP2 in human PE placentas and their role in ruling out disease severity. Additionally, we correlated their expression with pAkt protein as a member of the MAPK pathway level that could affect trophoblast cell proliferation.

Materials and methods

Study population

This case–control study was carried out at the Medical Biochemistry Department and Medical Research Center, Faculty of Medicine, Assiut University, Egypt, and the Gynecology and Obstetrics Department, Woman's Health Hospital of Assiut, Egypt. Forty-eight pregnant women complicated by PE whose ages ranged from 20-40 years, in addition to 48 aged, and sex-matched normotensive pregnant women served as the control group were enrolled in the study.

Patients were diagnosed with PE when their urine excretion exceeded 300 mg of protein in a 24-hour period and their blood pressure was greater than 140/90 mm Hg on two separate occasions. Among them, 18 patients experienced mild PE, and 30 patients experienced severe PE, defined as a systolic blood pressure (SBP) ≥160 mmHg and/or diastolic blood pressure (DBP) ≥110 mmHg along with proteinuria > 300 mg/24 h.

The study excluded women with multiple gestations, preterm labor, premature rupture of membranes, sepsis, fever, chronic renal disease, fibrillation, proteinuria, vaginal infection, major known fetal or chromosomal abnormalities, and gestational diabetes. Every woman in the research had an elective cesarean section (CS) at delivery.

Sampling

The placental samples used in this investigation were collected in a sterile manner following a cesarean section. Four-to six-centimeter tissue samples were taken from the center of the placenta, cleaned with cold phosphate-buffered saline (PBS), frozen, and stored at -80 °C until protein biochemical and qPCR analyses were performed.

All the samples were collected at the Assiut University Hospital's Department of Obstetrics and Gynecology. At the time of admission, information on the maternal medical history and anthropometric measurements was obtained. The experiment was carried out at the medical research center and Department of Medical Biochemistry at Assiut University's Faculty of Medicine.

The Ethical Committee of the Assiut University Faculty of Medicine reviewed and approved the collection and use of human tissues for experimentation (IRBno:17300355). Each research participant provided

written informed consent and agreed to the collection of samples.

Quantitative real-time PCR (qRT–PCR) analysis

The total RNA in placental tissue was isolated via a Quick-RNA™ MiniPrep kit (by ZYMO Research) (catalog No. R1055) following the manufacturer's instructions. RNA (500 ng) was reverse transcribed to complementary DNA (cDNA) via a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, USA; catalog no. 4374967) following quantitation via a Nanodrop spectrophotometer. Thermo Scientific Maxima SYBR Green qPCR Master Mix(2X) (catalog no. K0252) was then used to amplify the cDNA.

In the Applied Biosystems 7500 Real-Time PCR Detection System (Applied Biosystems, Germany), amplification was carried out via 40 PCR cycles. Two steps were performed as follows: an initial denaturation cycle at 95 °C for 10 min, followed by 40 amplification cycles at 95 °C for 15 sec and 60 °C for 1 min. GAPDH was used as an internal control to normalize gene expression. The following primers were designed for the studied genes:

	Forward	Reverse
MXRA5	5`-CCTTGTGCCTGCTACGTCC-3`	5`-TTGGTCAGTCCTGCAAATGAG-3`
TIMP2	5`-AAGCGGTCAGTGAGAAGGAGTGG-3`	5`-CCTTGGAGGCTTTTTTGCAGTTG-3`
GAPDH	5`-GAGCTACGAGCT GCCTGACG-3`	5`-CCTAGAAGCATTTGCGGTGG-3`

The data were calculated via the following formula: R = 2- (Δ Ct sample - Δ Ct control).

pAKT ELISA kit

The level of pAKT in both patients and controls was measured via a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) technique supplied by SinoGene Clon Biotech Co., Ltd. (catalog no. SG-16318) according to the manufacturer's protocol.

The absorbance of the ELISA plate was measured at 450 nm via an ELISA plate reader. Next, by comparing the optical density of the samples to the standard curve, the concentration of pAKT in the samples was determined. The detection range was 0.05 ng/ml to 4 ng/ml.

Statistical analysis

The Statistical Package for Social Science (SPSS), version 26.0 for Windows, was used to analyze the data. The Shapiro–Wilk test was used to check the normality of the quantitative data, which were then expressed as the mean ± standard deviation (SD) or median and range depending on the normality of the data. The qualitative data are expressed as frequencies and percentages.

Independent sample t tests/Mann–Whitney U tests were used to compare the means/median differences between two independent groups. To compare the means/median differences between more than two groups, one-way ANOVA and the Kruskal–Wallis tests were utilized. Chi-square or Fisher’s exact tests were used to compare proportions between groups.

For investigating the correlation between the three groups and other variables, Spearman correlation was employed. A ROC curve analysis was conducted to determine the diagnostic potential of biomarkers for preeclampsia prediction. The results included the calculation of the area under the curve, sensitivity, specificity, positive predictive value, and negative predictive value. The significance level was set at a P value < 0.05.

Results

Table 1 shows the demographic criteria of the study participants. There was no statistically significant difference between the groups.

Additionally, there was a statistically significant decrease in the mean MXRA5 among cases compared with controls (p <0.001 for both). However, there was no statistically significant difference in the mean TIMP2 between the cases and controls **(Table 2).**

There was a statistically significant decrease in the mean MXRA5 among patients with severe PE compared with those with mild PE (p<0.001). On the other hand, there was no statistically significant difference in the mean TIMP2 level between mild and severe PE patients **(Table 3).**

Table 4 shows the correlations between the studied genes (MXRA5, and TIMP2) and patients' clinical and laboratory data. No statistically significant correlations between MXRA5, or TIMP2 and other characteristics of patients with preeclampsia (age, systolic BP, diastolic BP, weight, height, and BMI).

Regarding MXRA5, there were statistically significant moderate negative correlations between MXRA5, PT (r=-0.410, p=0.008), INR (r=-0.471, p=0.001), and urea (r=-0.524, p<0.001) and strong negative correlations with creatinine (r=-0.802, p<0.001), ALT (r=-0.401, p=0.006), and AST (r=-0.401, p=0.011). Moreover, there were statistically significant positive moderate correlations between MXRA5, PC (r=0.539, p<0.001), platelet count (r=0.450, p =0.001), total protein (r=0.421, p =0.003), and albumin (r=0.467, p=0.001). However, TIMP2 expression was not significantly correlated with laboratory data in patients with PE.

There was a statistically significant lower mean difference in pAKT-protein level among PE patients (0.29±0.04) than among controls (0.56±0.14, p<0.001). Moreover, there was a statistically significant positive moderate correlation among patients with PE between the pAKT and MXRA5 (r=0.430, p=0.005), with no correlation with TIMP2 **(Table 5)**

When we tested the sensitivity and specificity of MXRA 5 we found that AUC=0.964, at a cut of point ≤ 0.682, it has an accuracy of 94.0%, sensitivity of 91.7%, specificity of 95.8%, positive predictive value of 95.7% and negative predictive value 92.0% in prediction of pre-eclampsia, P value <0.001. Moreover, it has an accuracy of 95.5%, sensitivity of 96.7%, specificity of 94.4%, positive predictive value of 96.7%, and negative predictive value of 94.4% in the predicting severe cases of pre-eclampsia, P value <0.001. Whereas, for TIMP2 the sensitivity and specificity for the detection and prediction of disease severity were non-significant **(Tables 6 and 7).**

Table (1): Characteristics of patients with preeclampsia and controls

Variables	Cases (n= 48)	Controls (n =48)	P Value
Age in years			
Mean ± SD	27.29±5.77	26.79±5.76	0.672
Residence			
▪ Urban	0 (0.0%)	2 (4.2%)	0.495
▪ Rural	48 (100.0%)	46 (95.8%)	
Blood pressure			
▪ Systolic	155.83±9.63	114.17±8.20	
▪ Diastolic	96.67±4.76	75.83±7.09	
Anthropometric measures			
▪ BMI	32.90±6.65	31.79±4.44	0.341
Gravity			
▪ **Primigravida**	19 (39.6%)	16 (33.3%)	0.525
▪ **Others**	29 (60.4%)	32 (66.7%)	
Previous cesarian section	20 (41.7%)	6 (12.5%)	**0.001**
History of PE	12 (25.0%)		

The data are expressed as frequencies and percentages or means ± SDs.

*Chi square/Fisher exact tests were used to compare proportions between groups, and an independent sample t test was used to compare means between groups.

Table (2): Comparison of MXRA5 and TIMP2 expression among cases and controls

Variables	Cases (n= 48)	Controls (n =48)	P Value*
MXRA5			
Mean ± SD. **Range**	0.506±0.16 (0.322-1.002)	1.011±0.14 (0.530-1.276)	**<0.001**
TIMP2			
Mean ± SD. **Range**	1.091±0.22 (0.717-1.402)	1.028±0.24 (0.662-1.416)	0.186

* Independent sample t-test was used to compare the means between groups

Table (3): Comparison of MXRA5, and TIMP2 expression between mild and severe cases of preeclampsia.

Variables	Mild (n= 18)	Severe (n =30)	P Value*
MXRA5			
▪ **Mean ± SD**	0.64±0.17	0.43±0.11	**<0.001**
TIMP2			
▪ **Mean ± SD**	1.04±0.21	1.12±0.22	0.219

* Independent sample t-test was used to compare the means between groups

Table (4): Correlations between the studied genes (MXRA5, and TIMP2), clinical and laboratory data of patients with preeclampsia

Variables	MXRA5		TIMP2	
	R	P	R	P
Age	-0.027	0.856	-0.027	0.857
Systolic BP	-0.252	0.084	0.019	0.897
Diastolic BP	-0.206	0.160	-0.140	0.341
BMI	0.128	0.385	-0.047	0.754
Hb (g/dl)	-0.108	0.463	-0.187	0.203
Platelets (10	0.450	**0.001**	-0.224	0.126

3/ul)				
PT (sec)	-0.410	**0.008**	-0.039	0.791
PC (%)	0.539	**<0.001**	0.145	0.327
INR	-0.471	**0.001**	0.011	0.939
Urea (mmol/L)	-0.524	**<0.001**	0.218	0.137
Creatinine (umol/l)	-0.802	**<0.001**	0.130	0.380
ALT (U/L)	**-0.401**	**0.006**	0.288	0.057
AST (U/L)	**-0.401**	**0.011**	**0.330**	**0.022**
Total proteins (g/L)	**0.421**	**0.003**	-0.197	0.180
Albumin (g/L)	**0.467**	**0.001**	-0.062	0.674
Bilirubin (umol/l)	-0.264	0.069	0.070	0.637

*r (Spearman correction coefficient)

P (P-value significant if <0.05)

Table (5): correlation between studied genes (MXRA5 and TIMP2) and Pakt protein level among patients with pre-eclampsia.

Variables	**Pakt**	
	R	**P**
MXRA5	**0.430**	**0.002**
TIMP2	0.063	0.672

Table (6): Diagnostic criteria of MXRA5 and TIMP2 for the prediction of pre-eclampsia patients in comparison to controls.

Indices	**MXRA5**	**TIMP2**
AUC (95% CI)	0.964 (0.929-0.998)	0.585 (0.480-0.685)
Cut off	**≤ 0.682**	**> 1.096**
Accuracy	94.0%	64.5%
Sensitivity, %	91.7%	62.5%
Specificity, %	95.8%	66.7%
PPV %	95.7%	65.2%
NPP %	92.0%	64.0%
P Value	**<0.001**	0.155

PPV: positive predictive value; NPV: negative predictive value; AUC: area under curve.

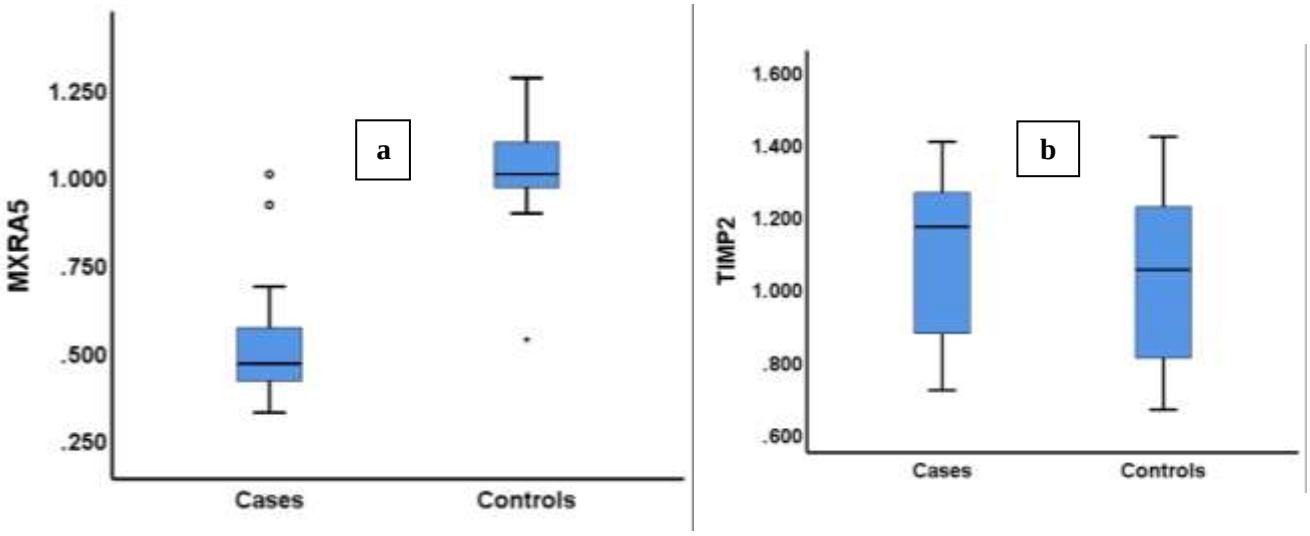
95% CI: 95% confidence interval

Table (7): Diagnostic criteria of MXRA5 and TIMP2 for prediction of severe cases of pre-eclampsia in comparison to mild cases

Indices	MXRA5	TIMP2
AUC (95% CI)	0.931 (0.819-0.984)	0.614 (0.462-0.751)
Cut off	**≤ 0.467**	**>1.159**
Accuracy	95.5%	68.0%
Sensitivity, %	96.7%	63.3%
Specificity, %	94.4%	72.2%
PPV %	96.7%	79.2%
NPP %	94.4%	54.2%
P Value	**<0.001**	0.182

PPV: positive predictive value; NPV: negative predictive value; AUC: area under curve.
95% CI: 95% confidence interval

Figures:



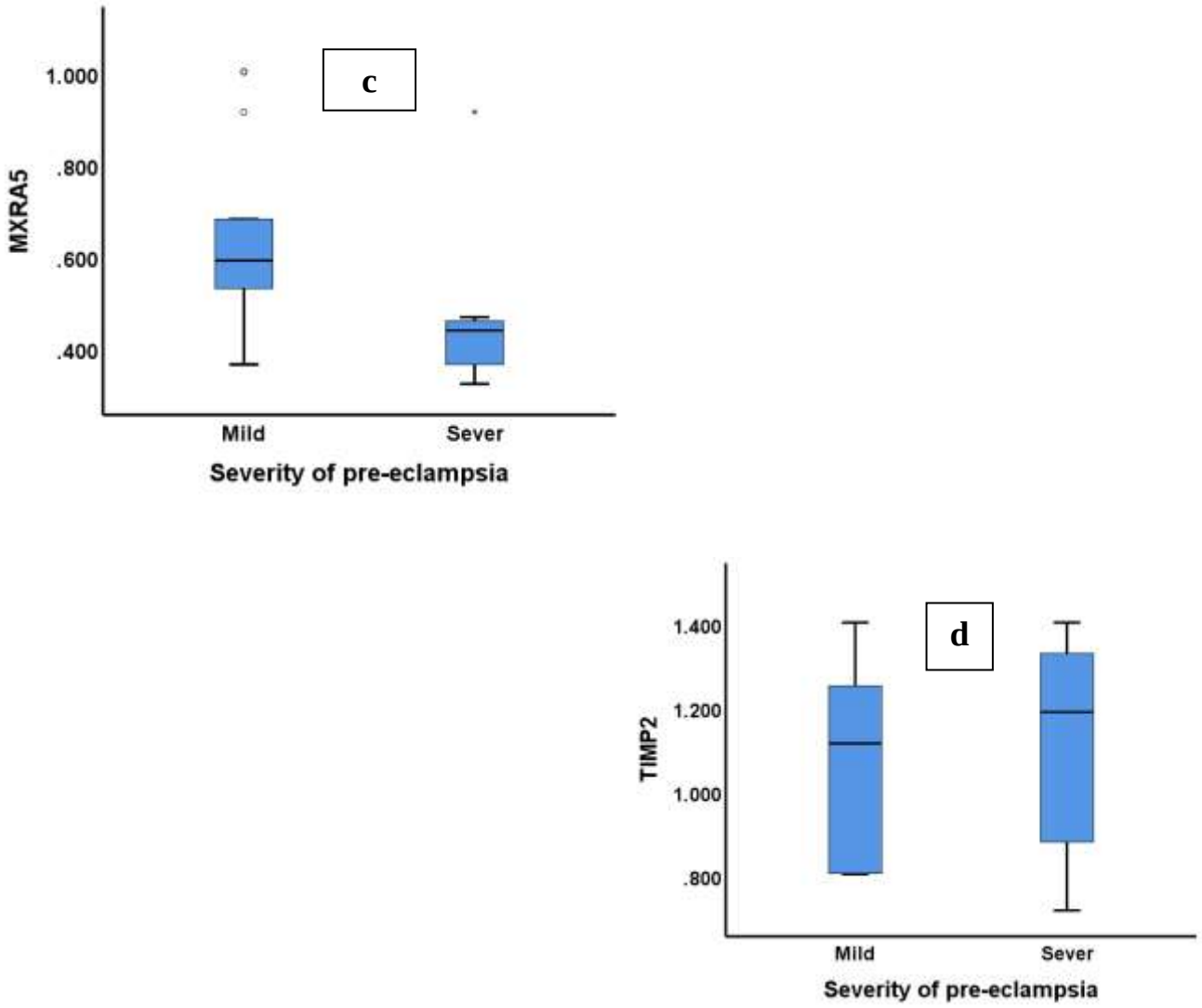


Figure (1): boxplots for comparison of (a) MXRA5 expression between cases and controls, (b) TIMP2 expression between cases and controls, (c) MXRA5 expression between mild and severe cases of pre-eclampsia, (d) TIMP2 expression between mild and severe cases of pre-eclampsia.

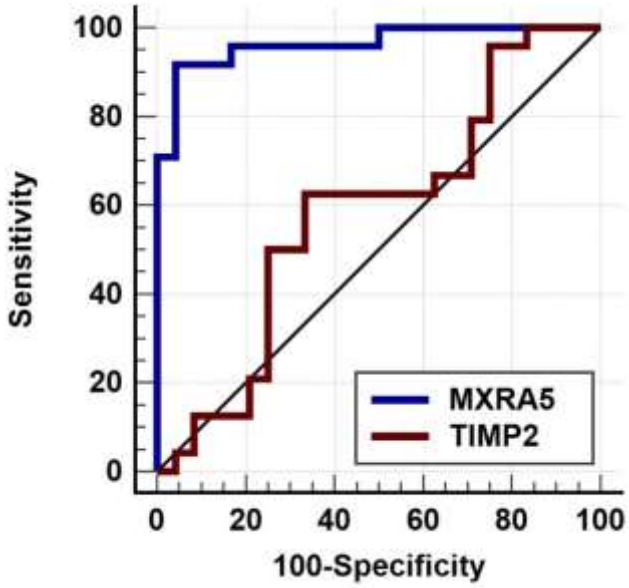


Figure (2): ROC curve for MXRA5 and TIMP2 for pre-eclampsia in comparison to controls

Discussion

PE is primarily caused by inadequate spiral artery remodeling, faulty trophoblast invasion, trophoblast apoptosis, defective matrix remodeling, and cell adhesion [7]. Our study focused on two matrix remodeling proteins that are thought to be involved in the placentation and pathogenesis of preeclampsia: MXRA5, and TIMP2.

MXRA5, a gene involved in modifying the extracellular matrix, the placentas of patients with preeclampsia had lower expression levels of MXRA5 than those of controls. This finding is consistent with a study by Ding, Lan, et al., who also reported lower levels of gene expression in preeclamptic patients[20]. Cytotrophoblast function may be strongly impacted by MXRA5; therefore, its downregulated expression strongly affects the function and integrity of the placenta.

There are two potential ways that MXRA5 regulates the activation of trophoblast cell migration and invasion: first, it acts as a structural matrix protein to stabilize other receptors, such as EGFR and PDGF, and to facilitate signal transduction; second, it directly affects the structure of the MAPK protein in the cytoplasm[21].

Dysregulation of MXRA5 affects the MAPK signaling pathway, which in turn weakens the invasion and survival of trophoblast cells [20, 21]. According to Trojane et al., PAKT levels are considerably lower in PE human placentas than in placentas from normal pregnancies [25]. These findings are consistent with our findings, which show that pAKT levels are considerably lower in preeclampsia patients than in healthy controls. Moreover, our study results, which revealed a positive correlation between MXRA5 expression and the serum level of p-AKT, a component of the MAPK pathway, support the above findings.

According to previous studies, activation of the PI3K/Akt/eNOS pathway is crucial for controlling angiogenesis, preventing hypertension, and regulating cell migration [22]. Therefore, the observed reduction in

pAKT levels in PE patients could be the primary cause of the inadequate placentation and trophoblast invasion necessary for a healthy pregnancy.

Owing to its role in the vasodilatation process and relevance in preeclampsia, MMP-2 is regarded as being of fundamental importance in the development of hypertension in addition to its role in migration, proliferation, apoptosis, tissue morphogenesis, and tumor progression, which are counterbalanced by their tissue inhibitors (TIMPs) [23]. Its levels were shown to be relatively high in the plasma of preeclamptic women, according to several earlier studies[24, 25].

Research has focused on the serum concentrations of MMP-9, MMP-2, and the corresponding tissue inhibitors at various stages of gestation, with some indicating a decrease and others showing an increase in their serum levels in PE[12]. Lavee et al. reported that the amniotic fluid TIMP-2 level was significantly higher in hypertensive women than in normotensive women[26]. However, our study of TIMP-2 expression in PE patients compared with controls revealed neither significant changes in TIMP-2 levels nor associations with the severity of preeclampsia. The variability between the two studies may be due to changes in sample type or the timing of sample collection.

Our findings concerning the relationships among MXRA5 and pAKT are corroborated by earlier studies by Ding et al. and Xu et al., who examined the impact of MXRA5 silencing on the inhibition of the MAPK pathway in rats [10, 21]. These findings support the hypothesis that the MAPK pathway regulates invasion degree and, in turn, PE severity and that additional use of the MAPK inhibitor MXRA5 can reduce PE severity. Moreover, our findings showed that MXRA5 performed greater role in distinguishing PE patients from controls and distinguishing between severe and mild cases.

To the best of our knowledge, this is the first study to compare the role of MXRA5 in excluding cases and controls with PE, with MXRA5 performing a high role in terms of accuracy (sensitivity: 96.7%, specificity: 94.4%). Furthermore, MXRA5 exhibited a great sensitivity in distinguishing between severe and mild cases (95.5%, sensitivity 96.7%, specificity).

To support the above findings, our study was also the first to correlate MXRA5 with the onset of PE, where MXRA5 expression was significantly greater in early-onset PE patients than in late-onset patients.

However, our study has some limitations, such as the small sample size, lack of a single time point for sample collection, and the need for additional measurements of studied gene proteins in the placenta, which should be considered in future studies.

Conclusions

Two genes, MXRA5 and TIMP2, activate the PI3K/Akt/eNOS pathway, which is an essential route for regulating angiogenesis and cell migration. MXRA5 and TIMP2 could act alone or in concert with appropriate placentation. Thus, any dysregulation of their expression level in the placenta could be a pathogenic factor for PE. Our study concluded that, in preeclampsia patients, there was a statistically significant positive correlation between the pAKT protein level and MXRA5 but not with TIMP2 compared with controls. Furthermore, reduced MXRA5 expression is a high sensitivity biomarker for predicting the severity of PE.

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