RESEARCH ARTICLE

Expression levels of *MiRNA-16, SURVIVIN* and *TP53* in Preeclamptic and Normotensive women

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Abstract

Objective: To measure and compare micro ribonucleic acid-16, survivin and tumour protein p53-regulated apoptosis-inducing protein 1 expression levels in preeclamptic and normotensive pregnancies, and to check the correlation of micro ribonucleic acid-16 with messenger ribonucleic acid expression of survivin and tumour protein p53.

Method: The observational cross-sectional comparative study was conducted at the Department of Physiology and Cell Biology, University of Health Sciences, Lahore, Pakistan, from 2016 to 2018, and comprised preeclamptic women in group A and normotensive women in group B. The preeclamptic patients were further categorised into early-onset preeclampsia subgroup A1and late-onset preeclampsia group A2. Expression of micro ribonucleic acid-16, messenger ribonucleic acid expression of survivin and tumour protein p53 in preeclamptic and normotensive pregnancies were analysed using real-time polymerase chain reaction. Data was analysed using SPSS 22.

Results: Of the 54 patients, 27(50%) were in each of the two groups. Within group A, 14(52%) patients were in group A1 and 13(48%) in group A2. The expression of micro ribonucleic acid-16 showed significant increase in group A compared to group B ($p<0.05$). The difference was not significant between the subgroups A1 and A2. The levels of messenger ribonucleic acid expression of survivin and tumour protein p53 were deregulated in group A, with a decrease in survivin and an increase in tumour protein p53. The messenger ribonucleic acid expression of survivin and tumour protein p53 showed statistically significant differences across subgroups A1 and A2 ($p<0.05$). The micro ribonucleic acid-16 expression correlated negatively with messenger ribonucleic acid expression of survivin, but exhibited a positive correlation with tumour protein p53.

Conclusion: Deregulated micro ribonucleic acid-16 along with differentially expressed apoptotic genes, survivin and tumour protein p53 might result in altered apoptosis implicated in the pathogenesis of preeclampsia.

Keywords: Survivin, TP53, miRNA, Apoptosis, Pregnancy, Preeclampsia. (JPMA 71: 2208; 2021)

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Introduction

Preeclampsia (PE) is a prevalent disorder of pregnancy marked by onset of hypertension (HTN) and excretion of protein in the urine.[1] Anomalous placental development with altered apoptosis has been observed as one of the multiple disrupted pathways involved in the pathogenesis of the disease. Tumour protein p53-regulated apoptosis-inducing protein 1 (TP53AIP1, or TP53) is a key regulatory tumour suppressor and maintains homeostasis when the cells are in a state of stress. It is triggered by numerous stimuli e.g. oxidative stress (OS), damage to deoxyribonucleic acid (DNA), etc. In the presence of these noxious stimuli, TP53 induces the transcription of downstream elements responsible for cell cycle arrest and apoptosis, such as Bax protien. Bax leads to an increase in the permeability of mitochondrial membrane with resultant release of various elements that promote stimulation of caspases.[2] Apoptosis of the cells due to TP53 usually follows the intrinsic mitochondrial pathway, but it can involve the extrinsic pathway as well by modulating the cellular death receptors. Overexpression of TP53 promotes the trafficking of Fas from the Golgi complex and raises its levels at the cell surface. The increased cell surface Fas levels result in increased susceptibility of these cells to apoptosis.[3] Increased expression of TP53 has been reported in villous trophoblast in PE.[4] Moreover, the upregulation of TP53 has been correlated with increased apoptosis in cytotrophoblast in pregnancies complicated with foetal growth restriction, a disease of placental origin similar to PE.[5] Alternatively, TP53 can activate apoptotic cascade by repression of antiapoptotic gene survivin.[6] Survivin is an oncoprotein located on the inner mitochondrial membrane. It regulates apoptosis by inhibiting activation of caspases, the proteolytic component of the apoptotic pathway. Increased apoptosis in PE is accompanied by reduced messenger

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ribonucleic acid (mRNA) expression of survivin and this decline in mRNA levels of survivin has been inversely correlated with the severity of the disease.

MicroRNAs (miRNAs), as epigenetic regulators, can modulate the process of apoptosis by targeting different genes implicated in intrinsic and extrinsic apoptotic pathways.[7] MicroRNA-16 (MiR-16) is widely expressed in placental tissue and a considerable upregulation of its level has been documented in PE.[8] MiR-16 has been proclaimed to trigger apoptosis by repression of B cell lymphoma 2 (BCL2), an antiapoptotic factor.[9] The current study was planned to measure and compare TP53 and survivin mRNA levels in peripheral blood mononuclear cells (PBMCs) and expression of miR-16 in the serum of preeclamptic and normal pregnancies, and to check the correlation of miR-16 with mRNA expression of TP53 and survivin. It was hypothesised that mRNA expression of TP53 and survivin along with the expression of miR-16 is deregulated in the blood in cases of pregnancy complicated with PE.

Materials and Methods

The observational cross-sectional comparative study was conducted at the Department of Physiology and Cell Biology, University of Health Sciences, Lahore, Pakistan, from 2016 to 2018. After approval from the institutional ethics review board, the sample size was calculated using the World Health Organisation (WHO) calculator[10] on the basis of formula:

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

The sample was raised using purposive sampling technique from among PE cases aged 18-40 years in the third trimester (28-40 weeks) of their respective pregnancies in group A. The PE criteria comprised new onset of systolic blood pressure (SBP) >140mmHg or diastolic blood pressure (DBP) ≥90mmHg at >20 weeks of gestation accompanied by 24hrs proteinuria ≥300mg (≥1+ on dipstick), in at least two random urine samples collected 4-6hrs apart.[1] Healthy pregnant women with normal blood pressure (BP) and comparable age in the final trimester (28-40 weeks) were recruited as controls in group B. PE cases were divided into subgroup A1 having early-onset preeclampsia (EOP) ranging from 28 to 32 weeks, and A2 having late-onset preeclampsia (LOP) ranging from 32.1 to 40 weeks. Patients with PE history in the previous pregnancy, multiple gestations and chronic HTN were excluded, and so were those with chronic diseases, like chronic arthritis, kidney disease, inflammatory disease of the bowel, high BP, cardiac pathology, like ischaemic disorder of heart, diabetes mellitus (DM), or any other persistent inflammatory diseases. Smokers were also excluded.

Data was collected after taking informed written consent from each participant.

Three ml of blood was placed in ethylenediaminetetraacetic acid (EDTA)-added vacutainer and 3ml in serum separator tubes. The sample was centrifuged at 4500 rpm for 5 min; the buffy coat was separated and kept at -20°C. RNA extraction was done with FavorPrep RNA Isolation Kit (Favorgen, Taiwan) as specified by the manufacturer. Nanodrop technology was used to quantify cell-free RNA and stored at -80°C in ribonuclease (RNase)-free water. Complementary DNA (cDNA) was synthesised with Thermo Scientific, First Strand revert aid cDNA Synthesis kit as specified by its protocol. The cDNA was amplified by conventional polymerase chain reaction (PCR) followed by product confirmation on the agarose gel. MiRNA was extracted from maternal serum with FavorPrep miRNA Isolation Kit (Favorgen, Taiwan). The concentration of miRNA was measured using nanodrop. Reverse transcription of miRNA was done with the miScript II RT kit (Qiagen).

Primers were designed for the specific gene sequence to estimate levels of expression of miRNA and mRNA. CFX 96, Real-time PCR machine was used with the SYBR Green master mix (Fermentas, USA). For mRNA, to 1µl of diluted (1:10 i.e. 2.5 ng/µl) cDNA sample, 8µl of Master Mix, 0.5 µl of reverse and forward primers were added. Finally, the reactions were completed in 10µl of volume by the addition of RNase-free water. All samples were estimated in duplicate. Data normalisation was done with three housekeeping genes. Real-time PCR protocol was denaturation for 4 minutes at 95°C, followed by 40 cycles of 95°C for 30s, annealing at 60°C for 30s, and extension phase at 72°C for 45s. For miRNA, initial denaturation was done at 94°C for 3min with 15 cycles of denaturation at 95°C for 10s, annealing at 58°C for 20s. Melt curve analysis was done with 65°C to 95°C with an increment of 0.2°C for 0.01s. Relative analysis of the gene expression was done by utilising the $2^{-\Delta\Delta ct}$ approach.[11] Primer sequence and product length for PCR was noted (Table-1).

Data was analysed using SPSS 22. The mRNA expression was normalised against the mean of three housekeeping genes, i.e., β-actin, 18srRNA, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). For normalisation of miRNA, the U6 was utilised as an internal control. Distribution of data was tested using Shapiro-Wilk test.

Table-1: Primers sequence and polymerase chain reaction (PCR) product length.

Gene	Sequence	PCR product length (bp)
MiRNA-16	5′ –GGGCCAGTATTAACTGTGC- 3′	
Uni-R	5`GTGCAGGGTCCGAGGT`3	
U6-F	5′ –CTCGCTTCGGCAGCACA- 3′	
U6-R	5′ –AACGCTTCACGAATTTGCGT- 3′	
TP53-AIPI -FP	5′-CTCCTCAGCATCTTATCCGAGTG- 3′	124-bp
TP53-AIPI –RP	5′-GTGGTACAGTCAGAGCCAACC- 3′	
Survivin-FP	5′-TTCTCAAGGACCACCGCATC- 3′	127-bp
Survivin-RP	5′-GCCAAGTCTGGCTCGTTCTC- 3′	
GAPDH-FP	5′ -ACG GAT TTG GTC GTA TTG GG- 3′	214bp
GAPDH-RP	5′ -CGC TCC TGG AAG ATG GTG AT -3′	
β-actin-FP	5′ -TCC ACC TTC CAG CAG ATG TG -3′	75bp
β-actin-RP	5′ -GCA TTT GCG GTG GAC GAT -3′	
18s rRNA-FP	5′-AGA AAC GGC TAC CAC ATC CAA -3′	91bp
18s rRNA-RP	5′ -CCT GTA TTG TTA TTT GTC ACT ACC T -3′	

MiRNA-16: Micro ribonucleic acid-16, TP53AIP1: Tumour protein p53-regulated apoptosis-inducing protein 1, FP: forward primer, RP: Reverse primer, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, rRNA: Ribosomal ribonucleic acid.

The comparison of mRNA levels was done for fold change values among the groups using Mann Whitney U test. Gene expression levels were expressed as the median of fold change. Spearman correlation test was applied to measure the correlation of miR-16 with mRNA levels of survivin and TP53. P<0.05 was considered significant. Part of data and its findings have been shared earlier.[12]

Results

Of the 54 patients, 27(50%) were in each of the two groups. Within group A, 14(52%) patients were in group A1 and 13(48%) in group A2. Basic attributes of the sample were not significantly different between the groups (p>0.05) (Table-2).

A significant upregulation of miR-16 and mRNA TP53 was observed in group A compared to group B. In contrast, mRNA expression of survivin showed a significant downregulation in group A. There was significant difference in terms of mRNA expression of TP53 and survivin in subgroups A1 and A2, but the expression of miR-16 was not significantly different between the subgroups (Table-3).

MiR-16 was inversely correlated with survivin (Figure-1) and it had positive correlation with TP53 (Figure-2).

Table-2: Clinical characteristics of the participants.

	Preeclampsia n = 27	Normotensive Group n = 27	p value
Maternal age (years)	26(23-30)	25(22-28)	0.49
BMI (kg/m^2)	28.9(27.6-30.3)	26.1(23.8-28.6)	*< 0.01
Gestational Age at sampling	32(29.2-36)	30.5(28-33.5)	0.17
Systolic BP	150(140-160)	100(100-110)	*< 0.001
Diastolic BP	100(90-100)	70(60-70)	*< 0.001
Parity	2(2-3)	1(1-2)	0.64

Values presented as Median with Inter Quartile Range (IQR).
*Significant difference between preeclampsia and normotensive group, calculated by Wilcoxon Rank Sum test.
BMI: Body Mass Index. BP: Blood Pressure.

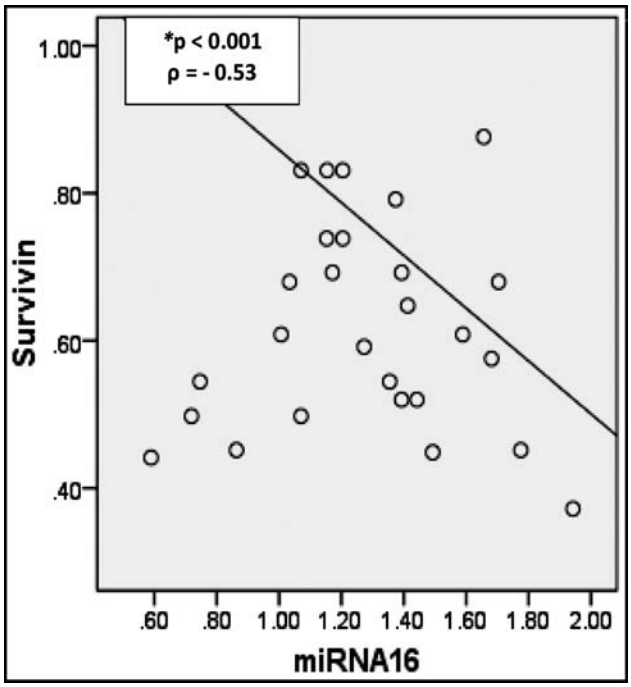

Figure-1: Inverse correlation between expression of micro ribonucleic acid-16 (miR-16) and messenger ribonucleic acid (mRNA) expression of survivin. *p value computed by Spearman correlation test, Spearman's rho (ρ).

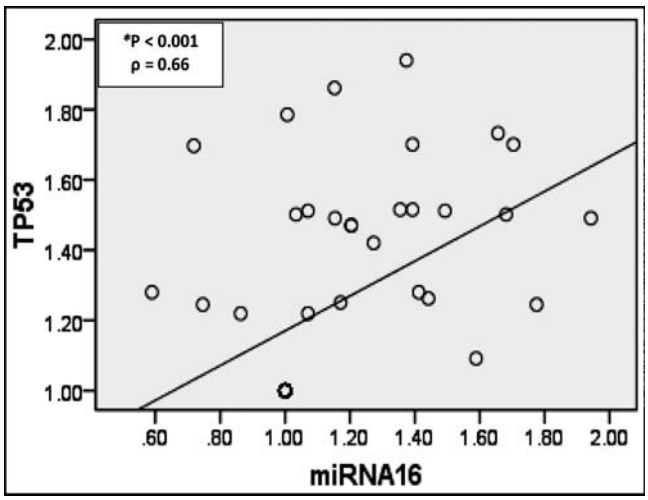

Figure-2: Correlation of micro ribonucleic acid-16 (miR-16) with messenger ribonucleic acid (mRNA) expression of tumour protein p53 (TP53). *p value computed by Spearman correlation test, Spearman's rho (ρ).

Table-3: Expression of micro ribonucleic acid-16 (miRNA) and messenger ribonucleic acid (mRNA).

	Normotensive controls (a) n=27	Preeclamptic cases (b) n=27	EOP (c) n=14	LOP (d) n=13	p value
miRNA-16	1	1.27(1.07-1.49)	1.27(1.16-1.57)	1.25(0.83-1.47)	a-b < 0.001 c-d 0.409 a-c < 0.001 a-d < 0.01
Survivin	1	0.60(0.49-0.73)	0.69(0.63-0.81)	0.51(0.45-0.60)	a-b 0.000 c-d < 0.01 a-c < 0.001 a-d < 0.001
TP53-AIPI	1	1.49(1.26-1.69)	1.51(1.47-1.71)	1.28(1.23-1.51)	a-b < 0.001 c-d 0.029 a-c < 0.001 a-d < 0.001

Fold Change expression presented as Median with IQR (Inter Quartile Range), n: sample size
*p value computed by Mann Whitney U test. p value <0.05 considered significant. EOP: Early-onset preeclampsia, LOP: Late-onset preeclampsia, TP53-AIPI: Tumour protein p53-regulated apoptosis-inducing protein 1.

Discussion

The implication of miRNA in PE pathogenesis is an upcoming discipline of interest. Expression of miR-16 has been studied in PE pregnancies in serum[13] as well as placental tissue.[8] To the best of our knowledge miR-16 has not been quantified in PBMCs in PE, but increased expression of miR-16 has been observed in PBMCs in cases with recurrent miscarriage.[14] The current study observed an increase in the expression of miR-16 in preeclamptic cases. The results are supported by previous studies.[8] However, the difference in the expression was not significant among EOP and LOP cases in the current study. MiR-16 has been reported to increase apoptosis in different cells by targeting and repressing BCL2, an inhibitor of apoptosis. It causes increased apoptosis by directly inhibiting BCL2.[9] Likewise, a decline in the expression of miR-16 has been documented in adenomas of the pituitary gland characterised by unbalanced cell growth. The results suggested the probable implication of miR-16 in the process of cell growth, as its expression showed an inverse correlation with the size of the tumour.[15] Moreover, miR-16 was reported to target Nodal type II receptor Acvr2a, and hinder the process of development in animal studies. Nodal belongs to the family of transforming growth factor β, fundamental for early development and growth.[16] Role of miR-16 in constraining the growth as well as the progression of the cell is further reinforced by the observation that it directly modulates Cyclin D1 (CCND1) expression with resultant G1 cell arrest.[9] All these observations enlighten the contribution of miR-16 in the arrest of the cell cycle as well as apoptosis in different types of tissues. Increased apoptosis has been observed and documented in the placental tissue in PE pregnancies.[17,18] In the light of these observations, the current study measured miR-16 and observed an upregulation in its expression levels in PE pregnancies.

The present study also observed an increase in the mRNA expression of TP53, a regulatory component of apoptosis, in PBMCs in PE. Detailed analysis revealed significant divergence in the mRNA level of TP53 in the two PE subgroups, with a more marked increase in EOP. Increased expression of TP53 has previously been reported in villous trophoblast in PE, with upregulation of p53 in the placenta-induced apoptosis in trophoblast cells.[4] Apoptosis of the cells due to p53 usually follows the intrinsic mitochondrial pathway, but it can involve the extrinsic pathway by modulating the death receptors. Overexpression of p53 promotes the trafficking of Fas from the Golgi complex enhancing its levels at the cell surface. The increase in the cell surface Fas levels results in sensitisation of these cells to Fas-induced apoptosis.[3]

In contrast to p53, several inhibitory factors, known as members of the inhibitor of apoptosis (IAP) group, are involved in the regulation of apoptosis. Survivin is an important member of the IAP group. Survivin suppresses programmed cell death by inhibiting caspase activation.[19] Trophoblastic cells express survivin and it has been observed to play a crucial part in the proliferation as well as the viability of these cells.[20] The fact is supported by the inverse correlation of apoptosis with survivin in the trophoblastic cells.[21] The current study observed a significant downregulation in the mRNA expression level of survivin in PBMCs in preeclampsia compared to healthy pregnancies. The findings are supported by previous

studies.[10,21] The difference in the expression of survivin was significant in the two PE subgroups with a more marked decrease in the LOP group. Previous observation of decreased placental expression of survivin with advancing gestational age supports our findings.[10] In addition to the deregulated expression of these genes and miRNA-16, the present study has reported a significant correlation of miRNA-16 with both survivin and TP53 ($p<0.001$). Upregulated miRNA-16 correlates positively with the mRNA expression of TP53. Recently, miR-16 has been observed to be involved in the regulation of TP53 in myoblasts, highlighting its role in the apoptotic pathway.[22] Regarding survivin an inverse correlation was observed between mRNA level of survivin in PBMCs and miRNA-16 transcript level. Recently miR-16 has been reported to be inversely correlated with the mRNA level of survivin in PBMCs in cases of systemic sclerosis.[23] Overexpression of miR-16 might be responsible for the increase in TP53 and decrease in survivin mRNA expression in PBMCs in PE. Excessive TP53 mRNA expression in the blood of PE pregnancies could provide an extra-placental source for higher levels of TP53 leading to enhanced apoptosis in the placenta. Moreover, T regulatory lymphocytes, involved in immune tolerance and increased in number in a healthy pregnancy, have been reported to be decreased in PE.[24] TP53 is known to increase the Fas expression on the surface of the cells promoting apoptosis. The increase in TP53 along with a decrease in survivin might be responsible for increased apoptosis and a decrease in the T regulatory lymphocyte in PE.

The current study observed that increased miRNA-16 expression was differentially correlated with overexpressed TP53 and underexpressed survivin in the blood of PE pregnancies, but further studies are needed to illuminate the exact role of miRNA-16 in the apoptotic pathway in PE. We recommend future cell culture studies with miRNA mimics and inhibitors to elucidate the implication of miR-16 in apoptosis, a disturbed cellular pathway in PE.

Conclusion

Deregulated miRNA-16 along with differentially expressed apoptotic genes, survivin and TP53, might result in altered apoptosis involved in PE pathogenesis.

Limitations of the study

The study has measured the expression of miRNA and mRNAs only in the blood samples from the preeclamptic and normotensive pregnancies. The expression was not studied in the placental tissue due to financial restraints. Concomitant expression of miR-16 and mRNA survivin and TP53 in the blood as well as placental tissue could have unfolded their role in the pathogenesis of the disease.

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