QUANTITATIVE EPIGENETIC ANALYSIS OF MIRNA 200 IN PREECLAMPSIA

Shali Paremmal[1], Nidhi Sharma[2], Keerti Gopi[3*], Asha Latha Pogula[4]

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Abstract

Preeclampsia is a clinical condition whose aetiology is described to be multifactorial and the cellular mechanism of which has not been understood completely. Recent studies hugely concentrate on microRNAs due to their regulatory effect on the target gene at the epigenetic level. Since Epithelial-Mesenchymal Transition (EMT) is a common mechanism observed both in cancer cell lines and embryological development and miR200 is a regulator of EMT, a large body of studies has been carried out in this regard. Previous studies have shown the regulatory effect of miR200 on ZEB1/ZEB2 mRNA in EMT. Since the trophoblast invasion is found to be a deficit in Preeclampsia, the expression level of miR200 requires detailed studies. The present study shows that miR210 expression is significantly upregulated in preeclamptic placental tissue in comparison with age-matched normotensive controls in the rt-PCR study. Further studies are needed to explore whether the therapeutic regulation of miR200 reverses the EMT suppression imposed by miRNA200.

Keywords- Preeclampsia, Micro RNA 200, Epithelial-Mesenchymal Transition

[1]Department of Anatomy[1], Govt. Medical College Mahabubnagar , Telangana, India, 509001.
[2]Department of Obstetrics and Gynaecology, Saveetha Medical College Tamilnadu. India, 602105.
[3*]Department of Biochemistry, Govt. Medical College Mahabubnagar, Telangana, India,509001.
[4]Department of Physiology, Govt. Medical College Mahabubnagar, Telangana, India,509001

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Corresponding Author
Keerthi Gopi[3*]
[3*]Department of Biochemistry, Govt. Medical College, Mahabubnagar, Telangana.
Email id- [3*]keerthi.gopi77@gmail.com
Orcid Id-0000-0002-2632-1289

1. Introduction

10% of pregnancies worldwide are estimated to be affected by Hypertensive disorders, including gestational hypertension, eclampsia and preeclampsia. The prevalence rate of preeclampsia alone is estimated to be 2-8% [1], the range of which changes from geographical to ethnical distribution. Preeclampsia refers to the new onset of hypertension and proteinuria or the new onset of hypertension and significant end-organ dysfunction with or without proteinuria after 20 weeks of gestation or postpartum, in a previously normotensive woman. The expulsion of the placenta relieves the symptoms indicating the significant role of placentation in the clinical manifestation of preeclampsia (PE). Although its aetiology and pathogenesis remain to be elusive, the current concept of PE pathogenesis favours a two-stage model hypothesis that applies to a majority of PE cases [2]. The first stage is poor placentation, as evidenced by colour Doppler ultrasound, which shows a tight link between reduced spiral arterial blood flow before the 20th week of gestation and an increased risk of developing PE in the later period of pregnancy. Utero-placental blood flow is found to be considerably reduced in women, who are destined to develop preeclampsia in the later stage of pregnancy [3]. This reduced placental blood flow causes placental ischemia/hypoxia, resulting in trophoblast debris and induces placental secretion of harmful factors, such as soluble FMS-like tyrosine kinase 1 (sFlt1) inflammatory cytokines, and antibodies of angiotensin-I receptors, to be circulated into the maternal blood[4]. The second stage of the disease is the maternal response to abnormal placentation, which is featured by systemic endothelial dysfunction.

During the normal course of placentation, a switchover mechanism happens where a set of cytotrophoblast cells in the developing embryo invade the spiral arterioles of the endometrium and give up their epithelial nature to assume an endothelial phenotype. This causes the conversion of high-resistance, small-diameter vessels into high-capacitance, low-resistance vessels to accommodate a large volume of blood delivered into a newly formed uteroplacental unit [5]. The subjects who are destined to develop PE in the later stage of pregnancy, this highly orchestrated cascade of events goes awry. The degree of cytotrophoblast invasion is seen to be inadequate in them. The end result is the failure of arterial remodelling where the vessels remain highly resistant and low diameter vessels, supplying an inadequate amount of blood to the uteroplacental unit making it prone to hypoxic [6]. A tremendous amount of studies are underway to elucidate the exact mechanism underneath this aberration and molecular factors responsible for the highly orderly executed cascades of events observed in the normal course of action. They range from various transcription factors to trans-membrane proteins [7].

Notch signalling pathway, the transcription factor stork head box 1 (STOX1), various components of the renin-angiotensin-aldosterone system and the intracellular serpin proteinase inhibitor-9 are a few among them.

The activation of Notch signalling in cell-to-cell contacts of adjacent cells has a vital role in cytotrophoblast proliferation, deduction, and remodelling of endometrial arterioles in the human placenta [8]. The main mechanism involved is 4 transmembrane receptors (Notch1, 2, 3 &4) and five ligands (DL L1, 3& 4 and JAG1&2) binding to them [4]. On ligand binding, the transmembrane receptor undergoes proteolytic cleavage, and an intracellular domain gets translocated to the nucleus and binds to the transcription factor, triggering changes in cell-to-cell adhesion, culminating in angiogenesis. The studies show that the absence of Notch 2 leads to reduced spiral arterial wall diameter and placental profusion in mice· [9].Studies also showed that peripheral and endovascular cytotrophoblast fail to express Notch ligand JAG 1 in preeclamptic subjects [9]. *In vitro* and *in vivo* studies evidenced that mutation of the Winged transcription factor family STOX1 profoundly affects the expression of genes associated with preeclampsia [4]. The variability of genes coding for major histocompatibility complexes and receptors for natural killer cells receptor molecules is known to correlate with the probable outcome of preeclampsia. Multiple studies reveal the correlation of a specific combination of fetal histocompatibility molecules and maternal natural killer receptors with the risk of preeclampsia, recurrent miscarriage, and fetal growth restriction.
Epigenetic mechanisms play a crucial role in the regulation of gene expression in the differentiated cell and even in embryological development [10]. These include DNA methylation, actions of non-coding RNA (nc RNA) at the transcriptional level and histone modification and biogenesis at the translational level. Discovered in 1993 as a small noncoding RNA, the micro RNA became a hot topic for current research because of its regulatory effect on the expression of its host gene. Hundreds of mi RNA have been discovered through cloning and size-fractionated RNA techniques. Their regulatory role is diverse from cell proliferation, cell differentiation, cell death, fat metabolism, neuronal patterning and immunity [11-14]. Recent studies focusing on the role of mi RNAs in early embryogenesis emphasise the regulatory role played by mi R200 in Epithelial-Mesenchymal Transition [15]. In order to establish and retain the epithelial cell identity, the approach taken by mi RNA 200 family

differs in different cellular contexts. For example , during neural crest cell migration, mi R 200c and miR145 target the Sox 1 and Sox9 genes, thereby upregulating E-Cadherin expression to ensure EMT suppression [16]. miRNA 200 targets the ZEB1 gene in hepatocyte differentiation from embryonic stem cells and ZEB2 in postnatal forebrain neurogenesis respectively. By directly targeting the transcription factors involved, the miR200 family plays a crucial role in EMT/MET and in metastasis, cell cycle regulation apoptosis, cancer stemness, deregulating chemoresistance and recently intra-tumoral immune cell function[17]. In general, the upregulation of the miR 200 family in primary carcinoma suppresses the epithelial to mesenchymal transition by down-regulating the EMT-inducing factors like ZEB and ZEB 2 (The EMT-inducing transcriptional factors ZEB1 and ZEB2 repress E-cadherin expression and promote cancer cell migration and invasion), and thereby impede cancer cell dissociation, migration and invasion. Thus highlighting the tumour-suppressing effect of the miR200 family. So it is worth seeing the expression profile of miRNA 200 in preeclamptic placenta where the trophoblast invasion is seen to be affected, which may helpful for the further development of therapeutic strategies.
Materials and methods:

Subject selection
The study is conducted at Govt. Medical College Mahabubnagar, Telangana , India. The work was carried out after obtaining an ethical committee clearance certificate from the Institutional Ethical Committee (Protocol no. IEC/GMCMBNR/AP/07/2021) and informed consent from the patients and healthy controls. Placental tissues are collected from age-matched 45 pre-eclamptic cases and 15 normotensive pregnant women after delivery. The inclusion criteria set for the sample selection are as follows. De Novo appearance of hypertension (Systolic Blood Pressure ≥140 mmHg or Diastolic Blood Pressure ≥90 mmHg). Proteinuria (≥0.3 g/24 h of urinary protein or ≥2 + reading on a dipstick) after the 20^{th} week of gestation in normotensive women matched for maternal age. Women were tested for normal liver function tests and are euthyroid with BMI <25 and with no evidence of any other endocrinopathy. Subjects with diabetes mellitus, ischemic heart disease, stroke, peripheral vascular disease, cardiac, renal, hepatic dysfunction, chronic hypertension, pre-existing seizure disorder, eclampsia, pre-gestational diabetes, placental abruption, gestational diabetes, thyroid disease, dyslipidaemia are excluded from the study.

Sample Collection
Small pieces of tissue from the foetal surface of the middle portion of the placenta are collected, after the removal of the amniotic membrane, washed in phosphate-buffered saline($P^{H=}$ 7.4) [PBS] to remove blood contamination and transferred to 1x phosphate buffer saline, stored at -80^0C till the experiment is carried out. The phenol/alcohol method was adopted for total mRNA isolation, and isolated messenger RNA (mRNA) was stored at -20^0 C degrees for further procedure. cDNA synthesis of micro RNA is done using Universal Stem Loop Primer(USLP) and Universal 6 Reverse Primer(U6RP) [18].

Quantification of miRNA210using quantitative real-time polymerase chain reaction (q rt- PCR)
Quantification of miRNA210 was performed based on SYBR –Green assay using qRT -PCR. (Quant studio 5, Applied biosystem) Primers of miR-200 were designed as follows.

mi RNA 200-FP-5'-
AGTGGGGCTCACTCTCCAC-3'
mi RNA 200 RP-3'-
AGGAGGAGGAGGAGGAGAA5'
U6-forward-5-CTCGCTTCGGCAGCACA-3,
U6reverse-3'-ACGCTTCACGAATTTGCGT5'

Human microRNA-specific forward and reverse primers were used to quantify the samples. The following conditions were set for conducting qRT-PCR. Denaturation at 94°C for 2 min and 40 cycles of 94°C for the 30s, 55-60°C for 30s and 72°C for 30s and melting curve of 10 min. The expression level of mi RNA 210 is calculated using relative fold change in both controls and preeclampsia subjects in relation to the amount of U6Sn RNA present in the same sample. Each sample is performed in triplicate and the mean value is calculated.

2. Result

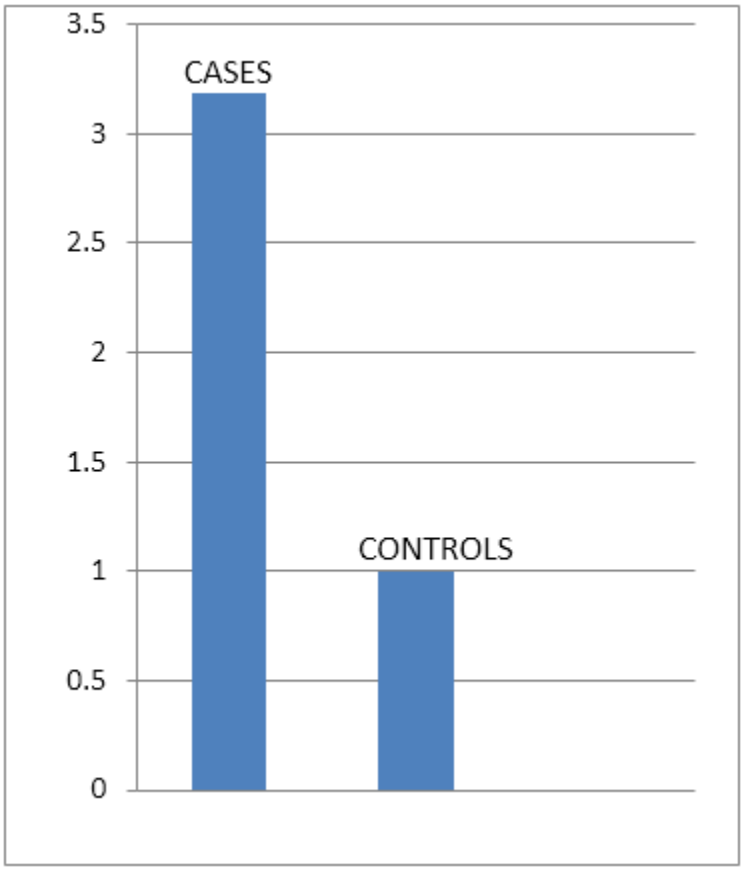

Figure 1: The analysis of the relative expression profile of the mi RNA200 showed a significantly increased expression level in pre-eclamptic placental tissue compared to the healthy controls. PE group showed a fold change of 3.18 compared to the control group.

The change in expression levels of each miRNA was analysed using the relative quantification method (RQ = 2−ΔΔCT) as described by the Livak method [19]. The graphs of all mi RNA levels have been represented as Ln < delta<Ct. One sample t-test was applied for column statistics to find significance between control and patient groups.

Statistical Analysis

The Mann-Whitney U test for a 2-group comparison determined the significance of gene amplifications. $P < 0.05$ was considered statistically significant. The change in expression levels of miRNA was analysed using the relative quantification method (RQ=2−ΔΔCT).

3. Discussion

The present rt-PCR analysis study showed significant (>0.05) upregulation in the expression level of miR200 in preeclamptic placental tissue compared with the age-matched normal placenta. Study in ovarian cancer line cells demonstrated that the target for mir200 family is m RNA of E-Cadherin repressor ZEB1 (TCF8/_EF1) and ZEB2 (SMAD-interacting protein 1 [SIP1]/ZFXH1B), the transcription factors involved in EMT [20]. Ectopic expression of miRNA200 upregulated the expression of E-Cadherin and conversely prevented the epithelial-mesenchymal transition and motility of cancer cells. Su et al (2021) reported that EMT markers in the placenta of preeclamptic patients showed higher E-cadherin and lower ZEB1 and TGF-β1 protein expression and aspirin-mediated suppression of miRNA200 resulting in the reversal of trophoblast invasion regulation through miRNA200/ZEB1/ TGF-β1signalling network [21].In kidney biopsy, the reactive oxygen species H2O2 downregulated the KLOTHO, a transmembrane protein in proximal and distal convoluted tubules, (Kenichi Morii et al 2019) [22]. Further, Magenta et al showed that H2O2 upregulated miR200c in umbilical vein endothelium [23] . MiR200c was found to down-regulate the KLOTHO expression without changing the KLOTHO m RNA level by binding at the untranslated 3' UTR of KLOTHO. KLOTHO protein confers the ability to prevent Epithelial-Mesenchymal Transition (EMT) by various mechanisms such as PI3K/Akt.GSKβ3/Snail signalling, Wnt/β-catenin signalling, and TGF-β1 signalling [24-26].

A study by Wang Y, et al showed that the endothelial cells from the umbilical vein of preeclamptic women showed reduced expression of

the junctional proteins cadherin and occludin and increased monolayer permeability [27]. Gene ontology (GO) analysis study by Zhirui Chen et al revealed the fact that cell junction regulation is one of the major mechanisms acting on endothelial cells, as fibronectin 1 and occludin, components of the cell junction by being the target of circulatory miRNA 200c [28] .

4. Conclusion

The present study shows that the expression of mi RNA 200 is upregulated in preeclampsia patients than in normal healthy controls. Regulatory effect by mi RNA200 on cellular activity seen to be tissue specific. The mechanism by which the elevated level of miRNA200 in preeclampsia acts up on the trophoblast EMT and migration needed further investigation, thereby regulating the miRNA200 can prove beneficial for patient care therapeutically.

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