Preeclampsia and Keap1 (rs11085735) variants and lncRNA MEG3 methylation status: Association with lncRNA MEG3 hypermethylation

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Research Article

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Abstract

Background. Preeclampsia (PE) is one of the complications of pregnancy. Genetic and epigenetic mechanisms are involved in the preeclampsia pathophysiology. The aims of present study were to investigate the role of Keap1 (rs11085735) variants and the methylation status of long non-coding RNA-maternally expressed gene 3 (lncRNA MEG3) in the pathogenesis of PE in a population from Western Iran with Kurdish ethnic background.

Results. There was no significant difference in the frequency of Keap1 genotypes comparing 75 patients with PE and 75 women with normal pregnancy. The frequencies of hemi methylated and full methylated lnc-MEG3 were 94 and 6% ($P=0.04$), respectively in all patients (50 women), 86.4, and 13.6% ($P=0.04$), respectively in patients with severe preeclampsia and 98 and 0% in controls (50 women). The frequency of full methylated lnc-MEG3 was 14.3% in early-onset and 2.8% in late-onset preeclampsia ($P=0.12$). The frequency of full methylated lnc-MEG3 was higher in patients with BMI $>25 \text{ kg/m}^2$ compared to normal weight patients. The PE patients had significantly higher levels of liver biomarkers (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin) and significantly lower platelet count than healthy pregnant women.

Conclusions. Hypermethylation status of lnc-MEG3 increased in patients with PE compared to controls that could be contributed in the pathogenesis and development of preeclampsia and its severe form. However, Keap1 variants might not be involved in the pathogenesis of preeclampsia.

Background

Preeclampsia (PE) is the most challenging disorder of pregnancy hypertension [1]. The pathogenesis of PE is not well understood [2, 3]. Evidence suggested several theories in this regard as trophoblast cell invasion dysfunction into the myometrium that could play the leading role in PE incidence [4–6]. Endothelial cells dysfunction by trophoblast invasion and fluctuations in blood flow causes oxidative stress in trophoblast cell. Oxidative stress, a homeostatic imbalance dysregulation between oxidants and antioxidants, occurs in the early onset PE and makes it progress [4]. As Stepan et al. indicated, pregnant women with trophoblast pathologic invasion had a lower total antioxidant capacity [7]. One of the cytoprotective pathways against oxidative stress is the Nrf2-Keap1 pathway. Oxidative stress disrupts the KEAP1-NFE2L2 complex, consequences Nrf2 translocation to the nucleus, which acts as a regulatory factor and promotes the expression of antioxidant response element-dependent genes. Kelch-like ECH-associated protein 1 (Keap1) is a cysteine-rich-protein and critical negative regulator of Nrf2; therefore, it acts as a sensor for oxidative and electrophile stress. In normal conditions (not under oxidative stress), keap1 directs NRF2 through ubiquitination to proteasomal degradation to preserve cellular hemostasis [8].

Recently two studies investigated the effect of the Keap1-Nrf2 pathway on the biological function of trophoblast cells (HTR8/SVneo cells) in the oxidative stress model at the cellular level. Their results

demonstrated the expression level of Keap1 in the placenta of patients with PE was slightly lower than the normal placenta [3, 6]. Another important factor is maternally expressed gene 3 (MEG3), a long non-coding RNA (lncRNA) member, which contributes to apoptosis, proliferation, and growth. LncRNA MEG3 is typically produced by placenta cells. It has been suggested a reduction of MEG3 in placental samples of patients with preeclampsia accounts for increased apoptosis and decreased migration that might have a role in remodeling failure of uterine spiral and PE incidence [9]. Also, Yu et al. proposed declining MEG3 via downregulation of epithelial-mesenchymal transition (EMT) by the TGF- β pathway inhibitor, which can be involved in the occurrence of PE [5].

There are no available reports related to the role of Keap1 polymorphism and MEG3 methylation in the pathogenesis of preeclampsia. So, to find the effect of Keap1 and MEG3 in the risk of PE we aimed to investigate the frequency of Keap1 (rs11085735) variants and the Lnc-MEG3 methylation status in preeclamptic patients compared with healthy individuals in a population from Western Iran with Kurdish ethnic background.

Methods

In a case-control study, 150 participants recruited, after providing written consent under approval by the Ethics Committee of Kermanshah University of Medical Sciences. The study was under the Declaration of Helsinki II principles. All participants were selected from women referring to the obstetrics clinic of Imam Reza Hospital of Kermanshah University of Medical Sciences (Kermanshah, Iran) and consisted of 75 women with PE and 75 women with normal pregnancy as controls. A sample of 75 patients was used to evaluate the variants of Keap1, and a sample of 50 patients was used to examine the methylation status of lncMEG3. The number of healthy individuals in both analyses was equal to patient samples.

Preeclampsia was defined as systolic and diastolic blood pressure equal to or higher 140 and 90 mmHg, the presence of proteinuria confirmed by excreting more than 300 mg protein in a 24-hours urine test or detecting 30 mg/dl or more in a randomized urine sample (1 + reaction on dipstick), the urine protein to creatinine ratio bigger than 0.3. Severe preeclampsia was defined as having one or more of the following criteria, blood pressure equal or higher than 160/110 mmHg after twice measurement and 6 hours apart while the patient was resting on the bed. Blood pressure equal or higher than 160/110 mmHg after twice measurement, which at least 6 hours apart, and the patient was resting on the bed. Proteinuria > + 3 on 2 random urine samples collected at least 4 h apart, elevated serum level of creatinine and transaminase, thrombocytopenia, visual disturbances, upper abdominal pain, headache, and fetal growth restriction [10]. Early-onset preeclampsia was defined as preeclampsia before 34 weeks of gestation that was detected in 20 patients. Patients with diabetes, gestational diabetes, infection, premature rupture of the bladder, oligohydramnios, polyhydramnios, valvular heart disease, chronic hypertension, and chronic kidney disease were excluded from the study.

Six ml blood samples were obtained from each participant. Two ml of each sample were transferred to a falcon tube containing a 0.5 mM EDTA used for DNA extraction and further genetic analysis. The

remaining blood sample without anticoagulant was centrifuged at 300 g for 10 minutes. Serum separated and restored in cryotubes at -20 C. The serum concentration of blood urea, creatinine, total and direct bilirubin, and liver enzymes (including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)) were measured by an enzymatic colorimetric assay kit (Pars Azmon kit, Tehran, Iran) according to the manufacturer's instructions and performed by automated RA-1000 (Technicon, CA).

Genomic DNA was extracted from peripheral blood leukocytes using the phenol-chloroform method [11]. The Keap1 polymorphism (rs11085735) was detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Using designed forward primer of 5'-CTCAGCCTCCCAAAGTCCCT-3' and the reverse primer of 5'-CTCCCACGGCTGCATCCAC-3' a fragment with 300-bp was amplified. The obtained 300-bp PCR product was digested with HinfI restriction enzyme.

Methylation Specific PCR (MSP). For bisulfite treatment of extracted DNA, 30 µL of melted 2% low melt agarose was added to about 200 ng DNA and was incubated at 65°C for a few minutes. To denature DNA strands, it was incubated with NaOH at 80°C for 15 minutes and re-solidifies the agarose bead by chilling on ice. By adding 10 mM hydroquinone and 40.5% sodium bisulfite at 50°C for about 4 hours treatment was done. To stop bisulfite reaction and desulfonation, the beads were washed with Tris-EDTA buffer, 0.2 M NaOH, 1M hydrochloric acid, and pure water, respectively. The washed beads were diluted in 30 microliter deionized water, heated at 80°C and aliquoted for PCR reaction. The MSP-PCR method was used to determine the methylation status of the lncRNA MEG3 gene promoter. After bisulfite treatment, methylation status was detected using two pairs of specific primers, un-methylated primers (forward GAGGATGGTTAGTTATTGGGGT and reverse CCACCATAACCAACACCCTATAATCACA) and methylated primers (forward GTTAGTAATCGGGTTTGTCTGGC and reverse AATCATAACTCCGAACACCCGCG). In MSP-PCR one pair specific is used to detect methylated status and the other is used for identifying the unmethylated template [12].

Statistical analysis. The frequencies of Keap1 variants and the lncRNA MEG3 methylation status in preeclamptic patients and controls were investigated with the χ^2 test. The Student t-test was used to compare the quantitative data. Quantitative data presented by mean $\pm$ SD and qualitative data presented by frequency and percentage. The SPSS version 16.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. Statistical significance level was considered at the $P < 0.05$.

Results

Characteristic of patients with PE and healthy controls are represented in Table 1. Compared to the healthy controls, all preeclamptic patients had significantly higher levels of liver function biomarkers (ALT, AST, ALP, and total bilirubin). Preeclamptic patients had a significantly lower level of platelet (PLT) count compared to controls. In all preeclamptic patients and also in severe PE group BMI values before (26.3 ± 6.2 , $P = 0.008$, and 25.9 ± 7.5 kg/m², $P = 0.1$, respectively) and after pregnancy (30.2 ± 7.2 , $P = 0.004$, and 29.9 ± 8.4 kg/m², $P = 0.1$, respectively) were higher than controls (22.8 ± 9.2 and 25.4 ± 11.9 kg/m²,

respectively). Distribution of Keap1 variants and lncRNA MEG3 methylation status in both patient and control groups are demonstrated in Table 2. As indicated in Table 2, there was no significant difference in the frequency of Keap1 genotypes comparing patients with PE and control women. Although, the frequency of Keap1 CC genotype was higher in all preeclamptic patients (93.3%) and patients with severe preeclampsia (96.4%) compared to controls (84%) it did not reach to a statistical significant. The unmethylated lncRNA MEG3 was not detected among patients. However, the lnc-MEG3 full methylated was not found in controls. The frequencies of hemi methylated and full methylated lncRNA MEG3 were 94 and 6% ($P = 0.04$), respectively in all patients, 86.4, and 13.6% ($P = 0.04$), respectively in patients with severe preeclampsia and 98 and 0% in controls (Table 2). In Table 3 the frequencies of Keap1 genotypes and lnc-MEG3 methylation status have been compared between patients with early-onset and late-onset preeclampsia. The frequency of Keap1 genotypes was not significantly different comparing patients with early-onset with late-onset preeclampsia. The frequency of full methylated lnc-MEG3 was 14.3% in early-onset and 2.8% in late-onset preeclampsia ($p = 0.12$). Among early-onset preeclampsia 12 out of 14 patients (85.7%) had BMI before pregnancy $> 25 \text{ kg/m}^2$ compared to 21 out of 36 (58.3%) in late onset preeclampsia ($\chi^2 = 3.36$, $P = 0.066$). Also, the methylation status of lnc-MEG3 was detected according to BMI. The full methylated lnc-MEG3 was not found among patients with BMI before pregnancy $\leq 25 \text{ kg/m}^2$ and its frequency was 9.1% among patients with BMI before pregnancy $> 25 \text{ kg/m}^2$ ($\chi^2 = 1.64$, $P = 0.2$).

Table 1
Characteristics of patients and controls

Variables	All Patients n = 75	Severe Preeclampsia n = 28	Controls n = 75
Age, year	31.5 ± 6.6; p = 0.018	29.6 ± 6.9;p = 0.8	29 ± 6.1
Gestational age, week	35.3 ± 2.2; p < 0.001	35.2 ± 2.1; p < 0.001	37.5 ± 2.1
Before pregnancy weight, kg	71.2 ± 10.7; p = 0.09	70.9 ± 13.4;p = 0.5	68 ± 12
After pregnancy weight, kg	81.3 ± 12.7;p = 0.4	80.3 ± 14.4;p = 0.9	79.7 ± 12.7
Before pregnancy BMI, kg/m ²	26.3 ± 6.2;p = 0.008	25.9 ± 7.5;p = 0.1	22.8 ± 9.2
After pregnancy BMI, kg/m ²	30.2 ± 7.2;p = 0.004	29.9 ± 8.4;p = 0.1	25.4 ± 11.9
Systolic blood pressure, mm Hg	152 ± 16.3; p < 0.001	165 ± 17.5; p < 0.001	113.3 ± 7.5
Diastolic blood pressure, mm Hg	93.3 ± 15.2; p < 0.001	102.6 ± 11.9; p < 0.001	73.5 ± 5.4
Platelet count	152.7 ± 36.3; p = 0.026	152 ± 33.6; p = 0.02	187.2 ± 47.7
Urea, mg/dL	26.2 ± 7.3; p < 0.001	26.8 ± 7.4; p < 0.001	19.6 ± 6.5
Creatinine, mg/dL	0.7 ± 0.2;p = 0.3	0.8 ± 0.2;p = 0.5	1.4 ± 3.8
AST, U/L	39.2 ± 45.5;p = 0.001	38.3 ± 47.3 ;p = 0.1	19.9 ± 7.1
ALT, U/L	28.5 ± 27.5; p < 0.001	28.7 ± 25;p = 0.05	15.3 ± 8
ALP, U/L	350.5 ± 131;p = 0.006	352.6 ± 132.8;p = 0.05	280.1 ± 10.8.1
Direct bilirubin, mg/dL	0.31 ± 0.13;p = 0.2	0.2 ± 0.08;p = 0.9	0.2 ± 0.1
Total bilirubin, mg/dL	0.76 ± 0.29;p = 0.001	0.6 ± 0.2;p = 0.03	0.57 ± 0.26

Table 2

Distribution of *keap1* and *Lnc RNA MEG3* genotypes in patients with preeclampsia, severe preeclamptic patients and controls.

Parameters	All patients	Severe PE patients	Controls
Keap 1 genotypes	N = 75	N = 28	N = 75
AA	5 (6.7%)	1 (3.6%)	7 (9.3%)
AC	0 (0.0%) $\chi^2 = 2.9$; P = 0.08	0 (0.0%) $\chi^2 = 0.67$; P = 0.4	5 (6.7%)
CC	70 (93.3%) $\chi^2 = 0.5$; P = 0.4	27 (96.4%) $\chi^2 = 1.1$; P = 0.2	63 (84.0%)
Lnc RNA MEG3 genotypes	N = 50	N = 22	N = 50
UU	0 (0.0%)	0 (0.0%)	1 (2.0%)
UM	47 (94.0%) $\chi^2 = 0.95$; P = 0.3	19 (86.4%) $\chi^2 = 0.38$; P = 0.5	49 (98.0%)
MM	3 (6.0%) $\chi^2 = 4.0$; P = 0.04	3 (13.6%) $\chi^2 = 4.0$; P = 0.04	0 (0.0%)
*Overall χ^2 comparing three Keap1 genotypes between all preeclamptic patients and controls is 5.7; P = 0.05			
** Overall χ^2 comparing three <i>keap1</i> genotypes between severe preeclamptic patients and controls is 0.3; P = 0.2			
# Overall χ^2 comparing three <i>LncRNA MEG3</i> genotypes between all preeclamptic patients and controls is 4.0; P = 0.1			
## Overall χ^2 comparing three <i>LncRNA MEG3</i> genotypes between severe preeclamptic patients and controls is 7.4; P = 0.02			

Table 3

Distribution of Keap1 and lncRNA MEG3 genotypes in early-onset and late-onset preeclampsia.

Parameters	Early-onset preeclampsia	Late-onset preeclampsia
Keap1 genotypes		
AA	1 (6.3%)	4 (6.8%)
AC	0	0
CC	15 (93.8%)	55 (93.2%)
lncRNA MEG3 genotypes		
UU	0	0
UM	12 (85.7%)	35 (97.2%)
	$\chi^2 = 0.1$; P = 0.6	
MM	2 (14.3%)	1 (2.8%)
*Overall χ^2 comparing three keap1 genotypes between early-onset preeclamptic patients and late-onset preeclamptic patients is 0.006; P = 0.94		
**Overall χ^2 comparing three lncRNA MEG3 genotypes between early-onset preeclamptic patients and late-onset preeclamptic patients is 2.4; P = 0.12		

Discussion

The present study investigated the biochemical and hematological parameters and also the frequency of Keap1 variants and lncRNA MEG3 methylation status in preeclamptic patients compared with women with normal pregnancy in a population from Western Iran. Compared with healthy pregnant women, a significant difference in liver function tests (ALT, AST, ALP, and total bilirubin) was detected in patients with PE. Patients with PE had higher serum levels of liver function tests except for direct bilirubin. During pregnancy, hormonal changes cause the dysfunction of hepatocytes, and a slight increase in ALP levels occurred in pregnancy. On the other hand, the serum concentration of ALT and AST usually remains normal. Evaluation of women with PE indicated dramatically increased serum levels of ALT, AST, ALP, and total bilirubin in these patients than normal pregnant women and these elevations might contribute to PE development. However, the direct bilirubin level did not significantly increase in patients compared to controls [13–15]. Also, elevated liver enzymes eventuate with emerging of HELLP syndrome. HELLP syndrome is a pregnancy disorder with clinical emersion hemolysis, elevated liver enzymes, and low PLT count and generally considered a type of severe PE [16], although studies demonstrated HELLP can be overlapped with early-onset preeclampsia [17]. Consistent with reports, our results showed a significantly lower PLT count in all and severe PE subjects than the healthy group. Evaluation of renal function can effectively diagnose gestational hypertension, as elevated urea and creatinine levels might be diagnostic and predictive biomarkers of PE [18, 19]. We found a higher level of blood urea in patients than in healthy

subjects. Previous studies have represented obesity as a risk factor for PE [20]. Our study demonstrated that mothers in the PE group before pregnancy were overweight and had BMI above 25 kg/m².

In the present study, we investigated the frequency of Keap1 variants. The Nrf2-keap1 pathway is one of the leading mechanisms against oxidative stress. Keap1 acts as an adaptor protein for Cul3-based E3 ubiquitin ligase; therefore, via Nrf2 proteasomal degradation regulates its function negatively. The expression of Nrf2 downstream target genes are regulated by genetic variations in the Keap1 or NFE2L2 gene [8]. There is no available study related to the frequency of Keap1 genotypes in preeclampsia and their influence on the risk of preeclampsia. Some studies established intronic rs11085735 SNPs polymorphism of Keap1 correlated with several diseases. Siedlinski et al., in a prospective study, expressed that rs11085735 in Keap1 was associated with a higher Forced Expiratory Volume in 1 second and might be associated with the risk of Chronic Obstructive Pulmonary Disease [21]. Variant rs11085735 minor allele (A) was associated with lower Keap1 and higher nuclear Nrf2 expression and reduced overall survival in breast cancer patients treated with radiotherapy and tamoxifen [22]. Testa et al. demonstrated that the rs11085735 polymorphism of the Keap1 gene strongly predicts incident cardiovascular events in chronic kidney disease patients [23]. Studies on trophoblast cell line (HTR8 / SVneo) obtained from PE patients showed decreased Keap1 and increased Nrf2 expression in HTR8 / SVneo cells [3, 6]. However, we could not find an association between the rs11085735 Keap1 variant and PE, even between early-onset and late-onset PE subjects. Lnc RNA is a transcript with more than 200 nucleotides that plays role in biological regulations such as DNA methylation, cell-cycle regulation, apoptosis, and angiogenesis with the incidence and the progression of numerous diseases. Recent observations illustrated the role of lnc RNA in trophoblast motility and migration. The lnc-MEG3 is one of the long non-coding RNAs that located in chromosome 14q. The lnc-MEG3 downregulation is correlated with PE through suppression of migration, invasion, and epithelial-mesenchymal transition of placental trophoblast cells [24]. Zhang et al. indicated down-regulation of MEG3 triggered apoptosis and repressed cell migration in trophoblast cells in vitro; consequently, it correlated with uterine spiral artery remodeling failure and participated in PE pathogenesis. The lnc-MEG3 provoked apoptosis of trophoblast cells via NF- κ B and Bax/Bcl2 signaling pathways [9]; also, Yu et al. declared that the lnc-MEG3 downregulation is involved in PE through suppression of migration, invasion, and EMT of placental trophoblast cells by TGF- β pathway inhibitors [5]. Epigenetic changes such as DNA methylation are influenced by environmental factors including life style [25].

In the current study, we found a significant difference between PE patients and also severe PE with the healthy group regarding methylation status of lnc-MEG3. Patients with PE had a higher frequency of lnc-MEG3 methylation than the healthy group. Also, among patients with preeclampsia, women with early-onset PE had a higher rate of lnc-MEG3 methylation than late-onset preeclampsia. Since, hypermethylation of this gene decreases its expression and the lnc-MEG3 downregulation is involved in PE pathogenesis [5] it seems hypermethylation of this gene in preeclamptic patients especially in severe preeclampsia be associated with the risk of preeclampsia and its severe form. Also, the frequency of lnc-MEG3 full methylated was higher among overweight patients compared to normal weight ones. Further,

overweight patients had higher frequency among patients with early-onset preeclampsia compared to late-onset preeclampsia. So, there might be a role for obesity in the pathogenesis of severe form of preeclampsia through lnc-MEG3 methylation that needs to be elucidated.

Conclusions

We found biochemical and hematological parameters such as ALT, AST, ALP, total bilirubin, urea, and PLT count levels were significantly different comparing PE patients with the healthy group that is considered in PE diagnosis. The present study detected increased hypermethylation status of lnc-MEG3 in patients with PE compared to controls that could be associated with the risk of preeclampsia and its severe form. Also, the role of obesity in the pathogenesis of preeclampsia through gene methylation should be elucidated. However, we found no difference in the frequency of Keap1 genotypes between the preeclamptic patients and controls.

Abbreviations

ALT

Alanine aminotransferase

ALP

Alkaline phosphatase

AST

Aspartate aminotransferase

BMI

Body mass index

HELLP

Hemolysis, elevated liver enzymes, and low PLT count

Keap1

Kelch-like ECH-associated protein 1

LncRNA

Long non coding RNA

MEG3

Maternally expressed gene 3

MSP-PCR

methylation-specific PCR

PCR-RFLP

Polymerase chain reaction-restriction fragment length polymorphism

PE

Preeclampsia

TGF

transforming growth factor

Declarations

Ethics approval and consent to participate. Written consent was provided from participants. The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (the research project code 990654) and was under the Declaration of Helsinki II principles.

Consent for publication. Not Applicable.

Availability of data and material. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests. The authors declare that they have no conflict of interest.

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Authors' contributions. MZ diagnosed patients and provided samples. SH and ZS diagnosed patients and involved in doing experiments. MK wrote the first draft of the manuscript. ZR designed the study and critically revised the manuscript. All authors read and approved the final manuscript.

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