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Gestational Diabetes Mellitus (GDM), Hypothyroidism, and Gene Variants (Keap1 Rs11085735) in Patients with Preeclampsia

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Abstract

Background: Preeclampsia is a multifactorial hypertensive disorder of pregnancy with multisystem involvement. Recent studies have demonstrated that preeclampsia is associated with increased placental oxidative stress at the cellular level. The nuclear factor erythroid-2-like 2 (Nrf2) / Kelch-like ECH-associated protein 1 (Keap1) signaling is an antioxidant pathway that plays an important role in protecting cells against oxidative stress. Here, we aimed to determine the possible association between the Keap1 variants and genetic susceptibility to preeclampsia.

Methods: In a case-control study, 150 preeclampsia patients and 150 women with normal pregnancy from Northern Iran were selected to evaluate the genotypes of Keap1 (rs11085735) using the polymerase chain reaction (PCR)-restriction length polymorphism (RFLP) method.

Results: A significant association between genotypes of Keap1 rs11085735 polymorphism with the renal function biomarkers and the risk of preeclampsia was not found. However, the aspartate aminotransferase (AST) level was higher in the presence of the Keap1 AA genotype compared to AC and CC genotypes. We found a significantly higher prevalence of gestational diabetes mellitus (GDM) in mild- and severe- preeclampsia and also hypothyroidism in severe preeclampsia compared to controls.

Conclusions: We found an association between preeclampsia with GDM and hypothyroidism. Our findings suggest that the Keap1rs11085735 polymorphism may not be a risk factor for susceptibility to preeclampsia in our studied population; however, this polymorphism could affect the activity of AST.

Keywords: Gestational diabetes mellitus, Hypothyroidism, Keap1 variants, Oxidative stress, Preeclampsia.

Introduction

Preeclampsia, a serious hypertensive complication of pregnancy, occurs after 20 weeks of gestation in previously normotensive women (1). It affects 3-8% of pregnancies and increases the risk of maternal and fetal morbidity and prenatal mortality (2).

Gestational diabetes mellitus (GDM) is the presence of hyperglycemia first diagnosed during pregnancy. Gestational diabetes mellitus could be a risk factor for preeclampsia development (3). Thyroid hormones are involved in proliferation, differentiation, and trophoblast invasion of the decidual and decidual angiogenesis. Women with reduced thyroxine levels had been at a higher risk of hypertension disorders during pregnancy (4).

Although the etiology of preeclampsia is still unclear, it is believed that preeclampsia originates from abnormal placentation and

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impaired remodeling of the spiral uterine arteries, leading to decreased placental perfusion. Reduction in placental perfusion results in placental hypoxia/ischemia and the release of cytotoxic factors into the maternal circulation (2). These factors could be responsible for systemic vascular endothelial dysfunction, cardiovascular complications, and an exaggerated inflammatory response involved in developing preeclampsia symptoms (2). Moreover, in preeclampsia uteroplacental hypoxia/reoxygenation increases the release of free radicals from the poorly perfused fetoplacental unit that initiates oxidative stress in placental cells. When the circulating blood is passing through the ischemic placenta, oxidative stress is propagated to different maternal tissues.

Normal biological levels of reactive oxygen species (ROS) are necessary for cellular and molecular processes (5). However, excess ROS can produce serious deleterious effects on cellular components that may lead to cell death. Therefore, the cell possesses numerous antioxidant defenses to regulate ROS levels. Superoxide dismutase, catalase, and glutathione peroxidase are the major antioxidant enzymes that scavenge the molecules responsible for generating free radicals (6,7).

The Kelch-like-ECH-associated protein1 (Keap1)/Nuclear factor erythroid 2-related factor 2 (Nrf2)/ antioxidant response element (ARE) signaling pathway is a central defense mechanism that plays a key role in the protection of cells against oxidative and electrophilic stresses by inducing the expression of many cytoprotective genes.

Nrf2 is a transcription factor that regulates the expression of genes modulating antioxidant and phase II detoxifying enzymes and related proteins. Under physiological conditions, Nrf2 exists in the cytoplasm as a complex with Keap1. Keap1, a negative regulator of Nrf2, acts as an adaptor molecule for the CUL-E3 ligase and mediates Nrf2 ubiquitination and subsequent proteasomal degradation. Keap1 is a cysteine-rich protein that acts as a critical sensor for oxidative and electrophilic stress. Electrophilic/oxidative stress modifies the cysteine residues of Keap1, especially cysteine 151, leading to the stabilization of Nrf2 (8). In response to oxidative stress, Nrf2 is released from the Keap1–Nrf2 complexes and translocates into the nucleus where it binds to the ARE in the target gene promoters and induces the expression of antioxidant genes (9). Once the redox balance of the cell is returned, Keap1 moves to the nucleus and binds to the Nrf2 to take it back to the cytoplasm for proteasomal degradation (10).

The single nucleotide polymorphism of rs11085735 is located in Keap1 intron 3 which interferes with the exon sequence that encodes the Kelch-1 domain, so this polymorphism may influence the interaction between Keap1 and Nrf2.

The role and association of Keap1 gene polymorphism with the risk of preeclampsia have not been studied. The present study aimed to find the presence and the frequency of GDM and hypothyroidism and also the frequency of Keap1 rs11085735 gene variants in patients with preeclampsia compared to healthy pregnant women.

Materials and Methods

The present case-control study was conducted on 150 women with preeclampsia (95 women with mild preeclampsia and 55 women with severe preeclampsia) and 150 women with normal pregnancies. The participants were referred to obstetric clinics in the northern part of Iran, from September 2018 to October 2019. The criteria for defining preeclampsia were new-onset hypertension (systolic blood pressure equal to or higher than 140 mmHg and diastolic blood pressure equal to or higher than 90 mm Hg on 2 occasions at least 6 h apart) accompanied by proteinuria (≥ 300 mg/L in urine/24 h), and/or evidence of maternal renal failure (creatinine ≥ 1.1 mg/dl), liver dysfunction (serum transaminases elevation), hemolysis or thrombocytopenia (platelets <100,000/mμl), pulmonary edema, headache, visual disturbances, and/or uteroplacental dysfunction with fetal growth restriction (1). Patients with blood pressure

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more than 160/110 mmHg accompanied by proteinuria >3+, and/or evidence of maternal organ dysfunction (liver, kidney, neurological dysfunction), thrombocytopenia, and/or fetal growth restriction were considered as severe preeclampsia. However, the definitive diagnosis of preeclampsia was given by a perinatologist based on the medical histories, physical examination, clinical findings, and special tests like doppler sonography.

Exclusion criteria were multiple pregnancies or any evidence of previous medical disorders such as known hypertension, diabetes mellitus, autoimmune, kidney, liver, and heart diseases. Among patients, there were 40 women with early-onset preeclampsia (preeclampsia before 34 weeks) and 110 women with late-onset preeclampsia (preeclampsia after 34 weeks). Data related to renal and liver function including urea, creatinine, blood urea nitrogen (BUN), uric acid, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels, and also information related to the GDM, and hypothyroidism were collected from the file of individuals in the hospital. All individuals with hypothyroidism were on therapy with levothyroxine.

Biochemical analysis

Blood samples were obtained from each individual, A portion of whole blood was put into plain tubes and centrifuged. Then the serum was stored at -80 ℃ until analysis. The remaining portion of the blood samples was treated with EDTA and used for DNA extraction.

Genotyping

Genomic DNA was extracted from the leukocyte of the EDTA- treated whole blood according to the phenol-chloroform protocol. Then, the purity and quantity of extracted DNA were determined by using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

The genotypes of Keap1 rs110857735 were detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR primers were designed using the Allele ID 7.0 (Primer BioSoft, USA) and Gene Runner (Version 3.02, Hastings Software Inc.) (Table 1). PCR reactions were performed in a 25-µl final reaction volume, containing 17 µl H2O, 1 µl of 2.5 mM dNTPs, 1 µl of 50 mM MgCl2, 1 µl of each primer (10 pmol/µl), 20–50 ng of genomic DNA, 2.5 µl of 10× PCR buffer, 0.5 µl of 5 U/µl Taq DNA polymerase. The PCR procedure included the initial denaturation at 94°C for 4 min followed by 35 cycles; each cycle comprised a denaturation step at 94 °C for 20 s, annealing temperature for 30 s (Table 1), then extension at 72 °C for 40 s, and a final extension step of 72 °C for 5 min.

To detect the Keap1 polymorphism, 10 µl of the PCR products of the Keap1 gene were digested with the HinfI (Thermo Fisher Scientific) restriction enzyme in a 15 µl final volume according to the manufacturer's instructions and then separated on 3% agarose gels. The details of PCR primers, the restriction enzyme used to identify Keap1 polymorphism, and the PCR-RFLP products' sizes are summarized in Table 1.

Table 1. Primer sequences used for Keap1 gene amplification and obtained products after digestion with HinfI restriction enzyme.

Polymorphism	**rs110857735**
Primer sequence (5′ → 3′)	Forward: GGTCACTGACTAGAACTCTCCAAGG
	Reverse: GCTGCATCCACCACAACAGTGT
Allele	C/A
Region	Intron 3
Annealing temperature (°C)	57.5
Amplicon size (bp)	192
Restriction enzyme	HinfI
Product size (bp)	CC (104, 88)
	AC (192, 104, 88)
	AA (192)

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Statistical analysis

The data were analyzed using SPSS version 16.0. The clinical and biochemical characteristics of different groups were compared by Student's unpaired *t*-test, and Mann-Whitney *U* test, as appropriate. The Kolmogorov-Smirnov test was used to determine the normality of the data.

Allele and genotype frequencies of the studied polymorphism in patients and healthy controls were compared by Pearson's Chi-square (χ^2) statistical test. The odds ratios (ORs) were calculated by regression analyses to examine the potential association between Keap1 variants and the risk of preeclampsia by computing the ORs with their 95% confidence intervals (CIs). The clinical and laboratory data were compared between Keap1 genotypes by the analysis of variance. The P-values < 0.05 were considered as statistically significant.

Results

Clinical characteristics of studied subjects

Table 2 demonstrates the statistical analysis of patients' and controls' demographic and clinical characteristics. The mean maternal age in preeclampsia patients and healthy pregnant women were 31.31 ± 6.16 years, and 27.19±5.99 years, respectively. As indicated in Table 2 the levels of body mass index (BMI), systolic, and diastolic blood pressure in patients were higher than in controls (P <0.001). Gestational age was significantly lower (P<0.001) in severe- and mild- preeclampsia women compared to healthy pregnant women.

AST and ALT levels were higher in all preeclampsia patients compared with the healthy pregnant group (P=0.14 and P=0.145, respectively). In severe preeclampsia, the AST and ALT levels were higher compared to mild preeclampsia (P=0.05 and P=0.01, respectively).

Higher levels of urea, creatinine and uric acid were found in preeclampsia patients compared to the healthy pregnant group (P<0.001, P=0.001, and P<0.001, respectively). Higher levels of urea, creatinine, BUN, and uric acid were found in severe preeclampsia patients compared to the healthy pregnant group (P<0.001, P<0.001, P=0.03, and P=0.003, respectively). Also, we observed higher levels of urea, creatinine, and BUN in severe preeclampsia patients in comparison with mild preeclampsia (P= 0.005, P=0.03, and P=0.003, respectively).

The frequency of severe preeclampsia was significantly different between patients with early-onset and late-onset preeclampsia (χ2=7.89, P=0.005). The frequency of severe preeclampsia was 55% in early-onset preeclampsia compared to 30% in late-onset preeclampsia (OR=2.85; 95%CI= 1.35-6).

Systolic blood pressure was significantly higher in patients with early-onset preeclampsia (153.72±16.1 mm Hg, P=0.012) in comparison with late-onset preeclampsia (146.80±11.56 mm Hg), while diastolic blood pressure showed no significant difference in patients with early-onset preeclampsia (95.75±10.89 mm Hg) compared with late-onset preeclampsia (92.77±7.43 mm Hg, P=0.059). Serum urea level was significantly higher in patients with early-onset preeclampsia compared with late-onset (33.38±22.56 versus 22.32±5.96 mg/dl), P=0.014).

Table 3 demonstrates the prevalence of hypothyroidism and GDM in patients and controls. The frequency of GDM was significantly higher in all patients (%36), severe- (%32.1), and mild- (%37.9) preeclampsia women compared to healthy pregnant women (0 %, P < 0.001). No significant difference was observed in the frequency of hypothyroidism between all patients and healthy pregnant women. However, a significantly higher frequency of hypothyroidism was found in severe preeclampsia compared with the healthy pregnant group (24.5% versus 12%, P=0.029).

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Table 2. Clinical, demographic, and biochemical characteristics of studied subjects. The Mean ± SD and P values are shown.

Character	All patients n=150	Severe preeclampsia n=55	Mild preeclampsia n=95	Controls n=150
Maternal age (Years)	31.31 ± 6.16 (<0.001)	32.40±5.96 (<0.001)	30.62±6.23(<0.001)	27.19±5.99
Gestational age (Weeks)	35.12 ± 3.77(<0.001)	34.09±3.82 (<0.001)	35.75±3.64 (<0.001)	38.97±1.14
Body mass index (Kg/m^2)	34.43 ± 5.85(<0.001)	33.17± 5.68 (<0.001)	35.07 ± 5.90 (<0.001)	29.93 ± 5.16
Systolic blood pressure (mm Hg)	148.64± 13.25(<0.001)	157.52± 15.93 (<0.001)	143.24 ± 7.37 (<0.001)	108.93±13.33
Diastolic blood pressure (mm Hg)	93.56 ± 8.55(<0.001)	98.20 ± 10.51 (<0.001)	90.84 ± 5.86 (0.006)	75.74 ± 5.76
Alanine amino transferase (U/L)	21.58 ± 37.02 (0.15)	31.56±57.4 (0.07)	16.28±14.25(0.99)	16.5±13.53
Aspartate amino transferase (U/L)	27.24 ± 22.58 (0.14)	32.26±28.84 (0.11)	24.36±17.58 (0.99)	23.95±7.84
Blood urea nitrogen (mg/dl)	10.29 ± 3.42 (0.11)	11.77± 4.10 (0.03)	9.46 ± 2.68 (0.92)	9.08 ± 2.15
Creatinine (mg/dl)	0.83± 0.19 (0.001)	0.88± 0.2 (<0.001)	0.80 ± 0.18 (0.14)	0.74±0.14
Urea (mg/dl)	25.59 ± 13.89 (<0.001)	31.43 ± 19.87 (<0.001)	21.54 ± 4.65 (0.13)	16.72 ± 5.58
Uric acid (mg/dl)	6.14 ± 1.44 (<0.001)	6.15 ± 1.47 (0.003)	6.14 ± 1.47 (<0.001)	4.69 ± 0.99

P-value was compared to controls.

Table 3. Comparing the presence of hypothyroidism and gestational diabetes between patients and controls.

	All preeclamptic patients n=150	Severe preeclampsia n=53	Mild preeclampsia n=95	Controls n=150
	N (%)			
Hypothyroidism Presence				
Presence	24 (16)	13 (24.5)	11 (11.6)	18 (12)
Absence	126 (84)	40 (75.5)	84 (88.4)	132 (88)
P value	=0.31	=0.029	=0.92	
Gestational diabetes mellitus				
Presence	54 (36)	17 (32.1)	36 (37.9)	0 (0)
Absence	96 (64)	36 (67.9)	59 (62.1)	100 (100)
P value	<0.001	<0.001	<0.001	

P-value was compared to controls.

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In 54 preeclamptic patients with GDM, the mean level of before-pregnancy body mass index (BMI) was 31.1±5.3 Kg/m2 compared to 28.7±5.4 Kg/m2 in those patients without GDM (P=0.01). Also, among preeclamptic patients with GDM, the mean level of after-pregnancy BMI was 35.7±5.9 Kg/m2 compared to 33.7±5.7 Kg/m2 in those patients without GDM (P=0.048).

Genotyping

Table 4 demonstrates the genotype and allele frequencies of Keap1 rs11085735 in patients and controls. The genotype and allele frequencies of Keap1 rs11085735 were not significantly different comparing patients with controls.

Table 4. Genotype and allele frequencies of Keap1rs11085735 in studied groups.

Polymorphism of Keap1 A> C n (%)								
Genotype		**All patients**		**Severe preeclampsia**		**Mild preeclampsia**		**Controls**
AA	6 (4)			2 (3.6)	-	4 (4.2)	-	3 (2)
AC	25 (16.7)	**OR**	0.32 *	8 (14.5)	0.3 *	17 (17.9)	0.32[a]	39 (26)
		(95%CI)	(0.073-1.4)		(0.04-2.2)		(0.066-1.62)	
		P	0.11		0.21		0.15	
CC	119 (79.3)	**OR**	0.55*	45 (81.8)	0.62[b]	74 (77.9)	0.51[b]	108 (72)
		(95%CI)	(0.134-2.25)-		(0.1-3.9)		(0.112-2.4)	
		P	0.4		0.57		0.38	
A	37 (12.3)		-	12 (10.9)	-	25 (13.2)	-	45 (15)
C	263 (87.7)	**OR**	1.25	98 (89.1)	1.44	165 (86.8)	1.16	255 (85)
		(95%CI)	(0.78-2)		(0.73-2.8)		(0.68-1.97)	
		P	0.34		0.28		0.57	

Comparisons were made with controls. *Compared with AA genotype.

Considering all individuals, the AST level was significantly higher in the presence of the Keap1 AA genotype (46±22.1 U/L) compared to AC (26.3±21.2 U/L, p=0.05) and CC genotypes (25.7±19.6 U/L, p=0.025). Also, the presence of the Keap1 AA genotype in mild preeclamptic patients increased the AST level (56±23.7 U/L) compared to AC (22±8.9 U/L, P=0.001) and CC genotypes (23.2±17.3 U/L, P=0.001).

Discussion

Preeclampsia is a hypertensive disorder of pregnancy with multisystem involvement. Among the various genetic, immunologic, metabolic, and inflammatory factors that contribute to the pathophysiology of preeclampsia, persistent oxidative stress is the key contributor to its pathogenesis. Given the well-known role of oxidative stress in preeclampsia, this study investigated the frequency of Keap1 variants and their possible association with kidney and liver function in patients with preeclampsia.

Oxidative stress is defined as an imbalance in the production of ROS and cellular antioxidant capacity. Pregnancy is well-known to increase oxidative stress (11). In normal pregnancy, increased ROS production is coupled with the elevated antioxidant capacity

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to prevent oxidative damage (12). However, in preeclampsia the excessive production of ROS is thought to overwhelm the antioxidant capacity, leading to a state of oxidative stress (13). Oxidative stress causes apoptosis and necrosis of syncytiotrophoblasts, leading to the release of inflammatory cytokines, eicosanoids, peroxides, ROS, and anti-angiogenic factors into the maternal circulation from damaged cells (14). These factors cause systemic maternal endothelial cell dysfunction and microangiopathy that may be involved in maternal placenta, liver, kidney, and brain injury.

Our study showed renal function parameters, urea, creatinine, and uric acid significantly increased in all preeclamptic patients and severe preeclampsia women compared with healthy pregnant women. Although the relation between hyperuricemia and preeclampsia has not been completely understood, it is believed that placental hypoxia/ischemia (15) and reduction of glomerular filtration rate may be involved in the elevated level of serum uric acid in preeclampsia (16). Increased serum creatinine in preeclampsia is due to the reduction of glomerular filtration rate in women with preeclampsia (17).

We found AST and ALT levels were higher in preeclampsia patients compared with the healthy pregnant group. Abnormal liver function is due to reduced blood flow to the liver, leading to ischemia and periportal hemorrhage (18).

Our findings indicated that GDM prevalence was significantly higher in all preeclamptic patients, and in severe-, and mild- preeclampsia compared to healthy pregnant women who all were without GDM. Also, significantly higher levels of before and after-pregnancy BMI were detected in preeclamptic patients with GDM compared to patients without GDM. GDM is considered a risk factor for preeclampsia development. Excess gestational weight gain might enhance the risk of preeclampsia in GDM women and preexisting obesity results in a greater risk (3).

Our study demonstrated a significantly higher frequency of hypothyroidism in severe preeclampsia compared with healthy pregnant women. A positive relationship between the serum level of thyroid stimulating hormone and systolic and diastolic blood pressure has been detected. Decreased thyroxine levels increased the risk of hypertension disorders during pregnancy and hypothyroxinemia women had an increased risk of preeclampsia/eclampsia (4).

Keap1 is a highly sensitive redox sensor that regulates redox homeostasis by mediating the proteasomal degradation of Nrf2. Genetic polymorphism in the Keap1 gene could be associated with a variety of human diseases, especially oxidative stress-mediated disorders. The A allele of the Keap1 rs11085735 reduces the Keap1 protein expression (19). Testa et al. showed that the Keap1 rs11085735 polymorphism may serve as a strong predictor of cardiovascular events in chronic kidney disease patients. It has been reported that the A allele of the rs11085735 was associated with an increased risk of cardiovascular disease by 85% (20). Also, the minor A allele of Keap1 rs11085735 was associated with worse overall survival in breast cancer patients treated with radiotherapy and tamoxifen (19).

Our study demonstrated the absence of an association between the Keap1rs11085735 variants and the risk of preeclampsia. It seems genetic polymorphism of Keap1rs11085735 may not be involved in the pathogenesis of preeclampsia. However, the presence of this polymorphism affected the activity of AST and the level of AST was significantly higher in the presence of the Keap1 AA genotype compared to AC and CC genotypes.

Our findings indicated higher levels of urea, creatinine, and uric acid in preeclampsia patients compared with healthy pregnant women. Also, increased levels of urea, creatinine, and BUN were associated with the severity of preeclampsia. Serum urea level was significantly higher in patients with early-onset compared with late-onset preeclampsia. Further, the present study detected a

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significantly higher frequency of GDM in all preeclamptic patients, in severe-, and in mild-preeclampsia women compared to controls. A significantly higher frequency of hypothyroidism was found in severe preeclampsia compared with healthy pregnant women. Our findings suggest that the Keap1rs11085735 polymorphism may not be a risk factor for susceptibility to preeclampsia in our studied population; however, this polymorphism could affect the activity of AST.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of Kermanshah University of Medical Sciences approved this study. The institutional ethical committee at Kermanshah University of Medical Sciences approved all study protocols (IR.KUMS.REC. 1397.380). Accordingly, written informed consent taken from all participants before any intervention. Ethical issues (including plagiarism, data fabrication, and double publication) have been completely observed by the authors.

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