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ORIGINAL ARTICLE

Evaluation Of Epidermal Growth Factor-Like Domain (EGFL7) mRNA Expression and its Protein Level in Preeclampsia

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Abstract

Objectives To evaluate the mRNA expression of epidermal growth factor-like domain 7 (EGFL7) in maternal blood and its protein level in sera of pregnant women complicated with preeclampsia (PE).

Method Case–control study involving 25 pregnant women diagnosed with PE (cases) and 25 gestational age-matched normal pregnant women (controls). EGFL7 mRNA expression in normal and PE patients was quantified by (qRT-PCR), and EGFL7 protein level was estimated using ELISA.

Results The RQ values of EGFL7 in the PE group were significantly higher than in the NC group ($P < 0.001$). Pregnancies affected with PE showed higher serum EGFL7 protein compared with matched controls ($P < 0.001$). EGFL7 serum level cutoff value ≥ 38.25 µg/ml could be used in the diagnosis of PE with sensitivity = 92%, and specificity = 88%.

Conclusion EGFL7 mRNA is overexpressed in maternal blood of pregnancies complicated with preeclampsia. Serum EGFL7 protein is elevated in PE cases and can be used as a diagnostic marker for preeclampsia.

Keywords Preeclampsia · Epidermal growth factor · EGFL-7 · Gene expression

Introduction

Preeclampsia (PE) is a common complication of pregnancy that affects about 8% of pregnant women worldwide. It is the main cause of perinatal morbidity and mortality [1]. Prediction of preeclampsia earlier in pregnancy can help in preventing the disease or in preventing its complications [2]. PE was defined by The International Society for the Study of Hypertension in Pregnancy (ISSHP) as hypertension accompanying proteinuria (300 mg/d) which appears after 20 weeks of gestation or as hypertension presented in addition to one of the following: renal impairment, hepatic malfunction, hematological or neurological disturbances, intrauterine growth restriction (IUGR), or uteroplacental insufficiency [3].

Many factors are known to increase the incidence of PE including chronic illnesses of the mother, such as chronic hypertension, diabetes, cardiovascular and renal disease; family history of preeclampsia, previous preeclampsia, nulliparity, multifetal pregnancy, advanced maternal age, and obesity [4]. Delivery of the fetus and placenta is the only absolute treatment of PE, although intensive studies on novel therapies are going on. Management includes antenatal counseling, perinatal control of hypertension and early treatment of complications, the well-timed decision of termination of pregnancy, and postpartum follow-up [5].

Preeclampsia pathogenesis is still evidently unidentified; however abnormal placental development and maternal contributors as genetic and environmental factors cause placental malfunction [6].

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Proper vasculogenesis and angiogenesis in placental tissue is a necessity for normal placentation and proper fetal growth and development, and this requires balanced angiogenic antiangiogenic factors, so the imbalance in these factors will lead to placenta related diseases like PE, intrauterine growth restriction, and small for gestation age [7].

Angiogenic factors have been aroused as a promising diagnostic tool as they appeared imbalanced in preeclampsia. Factors currently studied include soluble FMS-like tyrosine kinase 1, placental growth factor, endogenous vascular endothelial growth factor inhibitor, tumor necrosis factor-alpha, and soluble endoglin [8]. One of the potent angiogenic factors is the Epidermal Growth Factor like domain 7 (EGFL7) which is expressed in endothelial cells and other highly vascular supplied tissues such as heart, lung, uterus, and ovaries. In addition, EGFL7 is expressed early in embryogenesis and trophoblast cells of the placenta and it regulates trophoblast migration and invasion, having a role in placental development [9, 10]. In this study, we aimed to evaluate the mRNA expression of EGFL7 in maternal blood of pregnancies complicated with PE and find out if its serum protein level could be a valuable diagnostic marker.

Subjects and Methods

Study Subjects

All study subjects were recruited from Obstetrics and Gynecology Department at Mansoura University Hospitals in duration from December 2018 to December 2020. This study was a case–control study involving 50 subjects divided into 2 groups: the first group included 25 Preeclampsia patients diagnosed according to ACOG guidelines: blood pressure >140mmHg systolic or 90mmHg diastolic and >0.3g/day of proteinuria, after 20 weeks gestation. The second group included 25 control subjects of matched gestation age normal pregnant women. Both early-onset preeclampsia (EOPE) (defined as preeclampsia that develops before 34 weeks of gestation) and late-onset preeclampsia (LOPE) (defined as preeclampsia that develops at or after 34 weeks of gestation) were included in the study. Patients with a history of systemic diseases such as immune disorders, inflammation, diabetes, renal disease, or chronic hypertension, patients receiving medical treatments or who had surgeries with a possible adverse effect on pregnancy outcomes, twin pregnancies, and pregnancies with obstetric, medical, or surgical complications were excluded.

Sample size

The sample size was calculated by using G*Power software (version 3.1.9.7). Based on a previous study by [17], the authors hypothesized a large effect size for the EGFL7 mRNA expression in preeclampsia (PE) vs. control subjects (d=0.8). Group sample sizes of 25 patients with PE and 25 pregnant females without PE achieve 87.36% power to reject the null hypothesis of zero effect size when the population effect size is 0.80 and the significance level (α) is 0.050 using a one-sided two-sample equal-variance t-test**.**

Blood Sample Processing

5 ml of venous blood was collected from all subjects (patients and controls) and divided into two aliquots: one added on a falcon tube containing the RBCs lysis buffer for mRNA extraction and the other added to clot activating gel vaccutainers and let for 15 min at room temperature followed by centrifugation at 1000 g for 5 min [11]. Serum aliquots were frozen, and stored at -20° C until assessment by ELISA.

Total mRNA Extraction and Quantification

Total RNA was extracted based on the previously described method by using the miRNeasy mini kit (Qiagen, cat no. 217004, Germany) [12]. Total RNA was eluted with 30 mL of RNase-free water and stored at -80o C until further analysis. RNA was quantified using a NanoDrop 2000/2000c Spectrophotometer *(Thermo Scientific, USA)* and was converted to cDNA using the COSMO cDNA synthesis Kit (WF-1020500X) provided by Willofort, UK. Cat. No. WF10205002. Based on the previously described method [13]. The real-time PCR assays were performed using the Applied Biosystem 7500 device, a real-time PCR detection system with 96-well plates (Life technology, USA). It was done by using HERA SYBR® Green qPCR Lo-ROX Kit provided by Willofort, UK. *(Cat. No. WF10304002)*, according to the previously described method [14]. The following cycling conditions were used: 95 °C for 2 min, and 40 cycles of 95 °C for 10 s, 60 °C for 30 s. Fold changes in EGFL7 expression were determined by the ΔΔCt method normalized against the mean expression of a housekeeping reference gene (B-actin). EGFL-7 gene and B-actin specific primers were designed by the Primer blast program.

(NCBI/Primer BLAST[https://www.ncbi.nlm.nih.gov/tools/primerblast/].

Primer sequences are listed below:

- EGFL7 (forward): (5`- CTGTCTCCGAGTCGTTCGTG-3’)
- EGFL7 (reverse): (5`-TAGATGGTTCGGTAGGTG CTG–3`)

- B-actin (forward): (5`- GTGGCCGAGGACTTTGATTG –3`)
- B-actin (reverse): (5`-GTGGGGTGGCTTTTAGGA TG–3`)

Enzyme-linked Immunosorbent Assay

The level of EGFL-7 protein in serum was estimated by enzyme-linked immunosorbent assay technique (ELISA) using Inova human EGFL7 ELISA kit (*Inova biotechnology, Beijing, China*) according to the manufacturer's instructions. The optical density (OD) of each well was determined at 450 nm with the microtiter plate reader (*Chromate 4300 Microplate Reader, serial number EQOLE1408, Austria, Europe*).

Statistical Analysis

Quantitative data were initially tested for normality using Shapiro Wilk's test with data being normally distributed if $P > 0.050$. Continuous data were presented as median and interquartile range IQR when not normally distributed. A nonparametric analysis (Mann–Whitney U test) was used to compare not normally distributed data. The Spearman's rank-order correlation which is a measure of the strength and direction of the association/relationship was done between two continuous or ordinal variables. The diagnostic performance of a test or the accuracy of a test to discriminate diseased cases from non-diseased cases was evaluated using ROC curve analysis. IBM SPSS Statistics, Version 22.0 was used for data entry and analysis.

Results

Maternal and perinatal findings are described in Table 1. There were no statistically significant differences between normal control and PE groups as regards maternal age, gestational age, and BMI. Blood pressure, mean arterial pressure, and protein/creatinine ratio were found higher in pregnancies affected with PE ($P < 0.001$ by Mann–Whitney U test). Neonatal birth weight was found statistically significantly lower in the PE group vs. control group ($P < 0.001$ by Mann–Whitney U test). There were no statistically significant differences between EOPE and LOPE subgroups as regards maternal age, BMI, blood pressure, MAP, and protein-creatinine ratio. Neonatal birth weight was found statistically significantly lower in the EOPE subgroup vs. LOPE subgroup ($P < 0.001$).

Table 1 Maternal and perinatal findings

parameter	Group		P-value	Group		P-value
	NC N = (25)	PEN = (25)		EOPEN = (7)	LOPE N = (18)	
Maternal age	27.6 ± 3.07	29.1 ± 3.8	0.105	31 ± 4.5	28.4 ± 3.3	0.131
Gestational age in weeks	36 (10.5)	35(8)	0.406	26.8 ± 1.3	36.1 ± 1.2	**< 0.001**
BMI	29 (24–40)	30 (21.50–44.93)	0.748	32.4 ± 7.1	32.1 ± 8.3	0.932
Birth weight	3610 (295)	3400(650)	**0.001**	2800(100)	3500(300)	**< 0.001**
SBP	110(100–130)	150 (130–170)	**< 0.001**	150(140–170)	150(130–170)	0.642
DBP	80(70–90)	100 (80–110)	**< 0.001**	97(90–110)	100(80–110)	0.521
MAP	86.6 ± 10	113.3 ± 13.3	**< 0.001**	115 ± 8	115 ± 10	0.956
Protein/creatinine ratio	0.29 (0.15–2.1)	2.9 (1.7–3.5)	**< 0.001**	2.9(1.9–3.5)	2.9(1.7–3.5)	0.879

Data were expressed as mean ± SD, median (min–max), and median (IQR)

NC: Normal control, *SD*: Standard deviation, *BMI*: Body mass index, *SBP*: Systolic blood pressure, *DBP*: Diastolic blood pressure, *MAP*: Mean arterial pressure

Bold values indicates the statistical significant *P*-value

Table 2 RQ values of EGFL7 mRNA expression and its protein serum concentrations in µg/ml

RQ of EGFL7 gene	Group				*P*-value
	NC N = (25)	PE N = (25)	EOPEN = (7)	LOPEN = (18)	
	0.23 (0.64)	0.98 (1.73)	2.8 (5.7)	0.92 (0.55)	**< 0.001**
EGFL7 protein	31 (14.3)	45.8 (25.4)	70.7 (7.3)	40.9 (9.52)	**< 0.001**

Data are median (IQR), *IQR* = Interquartile range, *P*-value by Mann–Whitney U test

Bold values indicates the statistical significant *P*-value

The Relative Quantitation (RQ) Value of EGFL7 mRNA Expression and Serum Levels of EGFL7 Protein in µg/ml Assessed by ELISA

Both were statistically significantly higher in the PE group than the normal control group, ($P < 0.001$), and in EOPE cases than in LOPE cases ($P < 0.001$) as shown in Table 2.

The expression of the EGFL7 gene was detected in the maternal blood of both groups studied (PE vs. normal group), and it was found that EGFL7 was overexpressed in the blood of PE cases compared to normal control groups (P-value < 0.001). And it was found that the EGFL7 gene was overexpressed in EOPE vs. LOPE (P-value < 0.001). The same was done for serum EGFL7 level estimation using ELISA.

For defining the accuracy of relative quantitation (RQ) of EGFL-7 mRNA expression by real-time PCR, and estimation of serum EGFL7 protein level in discriminating PE cases from normal controls, ROC curve analysis was performed, cutoff values were defined for (RQ) of

Table 3 Diagnostic accuracy of serum EGFL7 levels and RQ of EGFL7 expression in discriminating case vs. control

Marker	Discriminating case vs. control							
	SN	SP	PPV	NPV	ACC	F1 score	MCC	AUC
Serum EGFL7	92%	88%	88.4%	91.6%	90%	90.2%	80%	0.959
mRNA expression	84%	76%	77%	82%	80%	80%	60%	0.847

SN: Sensitivity, *SP*: Specificity, *PPV*: Positive predictive value, *NPP*: Negative predictive value, *ACC*: Accuracy, *MCC*: Matthews correlation coefficient, *AUC*: Area under the curve

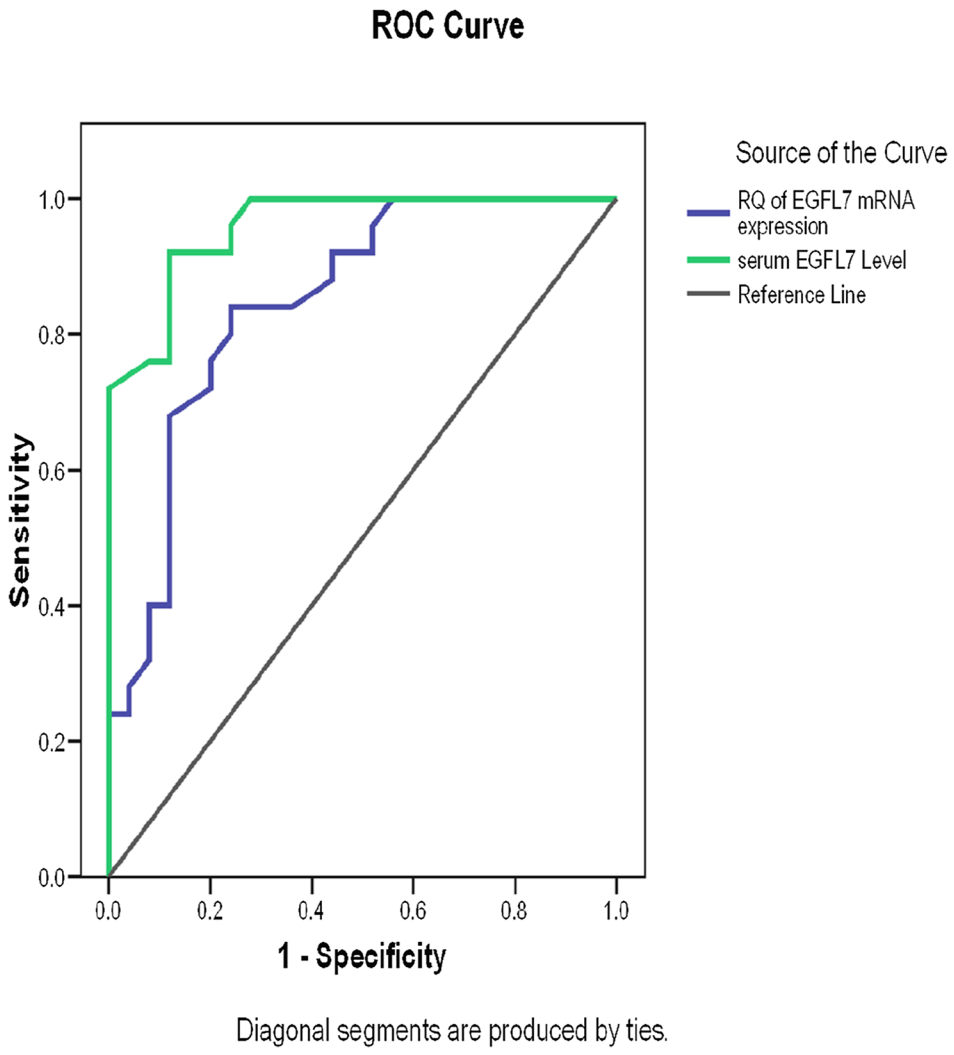

Fig. 1 ROC curve for determination of the accuracy of RQ of EGFL7 mRNA expression and serum EGFL-7 in discriminating PE cases from normal controls

EGFL-7 mRNA expression and serum EGFL7 protein level ≥ 0.70, ≥ 38.25 µg/ml respectively and results are shown in Table 3 and Fig. 1.

Table 3 shows the sensitivity, specificity, and accuracy of both tests in discriminating PE cases vs. the normal control group.

A strong positive correlation was found between both studied biomarkers with r_s (0.452) and *P*-value (0.001) by using Spearman's rank-order correlation.

Discussion

Preeclampsia (PE) is a pregnancy-induced hypertensive disorder affecting many pregnancies [15]. The pathogenesis of preeclampsia is still unknown, but placental and maternal factors are thought to be major contributors [16]. Proper vasculogenesis and placental function require a balance of angiogenic and antiangiogenic factors, and an imbalance in these factors will impact placental development [7, 17].

Angiogenic factors were found to be imbalanced in preeclampsia, so they can be used as biomarkers for the diagnosis of PE [18]. Epidermal Growth Factor like domain 7 (EGFL7) is one of the potent angiogenic factors that have an important role in placental development [10]. In our study, EGFL7 was overexpressed in the blood of PE cases vs. normal control groups with a *P*-value < 0.001 and showed overexpression in EOPE vs. LOPE with a P-value < 0.001. ROC curve analysis was performed to determine the accuracy of relative quantitation (RQ) of EGFL7 mRNA expression in discriminating PE cases from normal pregnancies, it was found that for a cutoff value ≥ 0.70, sensitivity was 84%, and specificity was 76%. To the best of knowledge, the only study that evaluated EGFL7 expression in maternal blood in PE cases was the study conducted by [17], that found statistically significant overexpression of the EGFL7 gene in maternal blood in cases of EOPE with a P-value < 0.01 when compared to the gestational age-matched control group[17]. While there was no significant difference in EGFL7 gene expression in the placentas of EOPE and LOPE when compared to normal pregnant women. RNA in the maternal circulation might be produced by either maternal, fetal, or placental origin, with most of the circulating fetal RNA being found packed and released from the placenta. However, because EGFL7 is overexpressed in the endothelium, this could explain the elevated levels of EGFL7 mRNA seen in preeclampsia and may result from endothelial dysfunction of maternal blood vessels commonly seen in preeclampsia [19, 20].

EGFL7 gene expression and circulating levels vary throughout pregnancy stages [21]. *G*estation age at sampling may explain the EGFL7 overexpression in EOPE cases when compared to LOPE.

It was found that EGFL7 mRNA expression in the maternal blood of pregnant women complicated with fetal growth restriction was higher when compared with matched controls [22]. We consider this study supportive to our results as it was found that fetal growth restriction is a pregnancy complication that shares common pathophysiological events with PE as regards defective placentation, placental hypoperfusion, releasing angiogenic factors in maternal circulation, and oxidative stress of placental tissue [23].

As the EGFL7 gene encodes a secreted protein, we performed a sandwich ELISA test to evaluate the role of EGFL7 as a biomarker for the diagnosis of PE. EGFL7 serum levels were statistically significantly higher in the PE group than the normal control group median (IQR) 45.8 (25.4) µg/ml in PE cases vs. 31 (14.3) µg/ml in the normal control group, P-value < 0.001, and also was statistically significantly higher in EOPE cases than LOPE cases P-value < 0.001.

For defining the accuracy of the test, ROC curve analysis was performed and showed that serum EGFL-7 had high accuracy with Area under the curve (AUC) = 0.959 for cutoff value ≥ 38.25 µg/ml, sensitivity = 92%, and specificity = 88% in discriminating PE cases from normal control.

In support of our finding, it was found that serum and plasma levels of EGFL7 protein were significantly higher in the PE group at term when compared to matched gestational age normal pregnant group P-value 0.003 [24]. In addition, *a study* found that levels of EGFL7 protein in plasma samples of EOPE cases were significantly higher than those measured in controls [21]. Placental villous culture studies performed by [24] suggested that villous trophoblast cells may be the main origin of circulating EGFL7 protein. However, stressed maternal vessels in pregnancy and increased oxidative stress in PE could be additional sources [25].

Other studies on the placental expression of the EGFL7 gene found statistically significant downregulation in PE, particularly EOPE, when compared to matched control groups [26–30]. Downregulation of expression of EGFL7 in PE placentas and its less integration into the villous cytotrophoblast and syncytiotrophoblast layers may cause instability of villous trophoblast cells, resulting in widespread detaching and release of extracellular matrix-bound EGFL7 into the maternal bloodstream, which could explain the high levels of circulating EGFL7 protein despite low expression of its gene in placental villi [31].

Conclusion

We came to the conclusion that the EGFL7 protein can be measured in serum and elevated in cases of PE with a high diagnostic accuracy, suggesting that it may be utilized as a cost effective diagnostic tool. EGFL7 mRNA expression may be detected in the blood, and it is overexpressed in PE pregnancies.

Recommendations

To confirm the possibility of using EGFL7 as a diagnostic marker, we urge more multicentric investigations with a larger sample size. Prospective studies combining evaluation of EGFL7 in the early stages of pregnancy with other clinically used PE biomarkers could prove EGFL7's potential as an early PE predictor.

Declarations

Conflict of interest This material is the authors' own original work, which has not been previously published elsewhere. The research was ethically approved by Institutional Review Board, Mansoura university, faculty of medicine. There is no conflict of interest.

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