Evaluation of expression pattern of Epidermal Growth Factor Receptor (EGFR) in Preeclampsia

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Abstract

EGFR is vital for normal placental formation and development. Over the last two decades, the understanding preeclampsia has increased greatly with the discovery of the anti-angiogenic molecules soluble Fms-like tyrosine kinase-1 (sFLT-1) and soluble endoglin (sEng). These factors are released at excessive levels from the preeclamptic placenta and cause the widespread maternal endothelial dysfunction that gives rise to the multi-organ injury that occurs in preeclampsia. However, upstream mechanisms like EGFR regulating the release of these molecules are still poorly described. In this study, we hypothesized that EGFR levels will differ in spectrum of PE. We aimed at analyzing EGFR levels in mild to moderate and severe PE by immunohistochemistry, western blotting and RT- PCR.

The study group consisted of placental bed biopsy tissues obtained from pregnancies with mild to moderate preeclamsia (PE) (n = 40) and without (n = 30) PE. The expression of EGFR in the placental villous tissue was evaluated quantitatively using immuno-histo-chemistry, western blot and real time-PCR. Immuno-histo-chemistry, western blot and RT PCR analysis illustrated the significant decrease in the expression of EGFR in PE group irrespective of its severity compared with the normotensive control group ($p<0.005$). This result suggests that altered expression EGFR proteins in placenta may be associated with the pathogenesis of PE by affecting angiogenesis.

Keywords: Preeclampsia, EGFR, Immuno-histo-chemistry.

Introduction

Preeclampsia is a pregnancy-specific disease, affecting 3-5% of all pregnancies worldwide. Its hallmark features are high blood pressure (hypertension) and endothelial dysfunction, leading to widespread end-organ injury[8]. There have been recent advances in our understanding of the pathophysiology of preeclampsia, many reported this very decade, but as yet there is no specific cure, delivery of the placenta remains the only definitive treatment[14]. Epidermal growth factor receptor (EGFR) signaling is one of the most avidly studied signaling networks in mammalian biology, with critical roles in cellular growth and survival[18]. Accumulating evidence suggests that human trophoblast survival and invasive capacity are linked to intercellular signaling by peptides related to epidermal growth factor (EGF). EGF can protect against apoptosis induced during *in vitro* culture of human term cytotrophoblast cells[22], indicative of the ability of EGF and related proteins to act as survival factors. The EGFR signaling pathway is one of the most versatile signalling units in mammalian biology where almost all cell types possess ErbB family members[20].

The placenta, however, has the highest expression of EGFR compared with all other human nonmalignant tissues[15]. The EGFR plays critical roles in placental development and survival[5,12]. EGFR nullizygous mice have placental defects that can be embryonically lethal[5,12].

Some growth factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), have been studied extensively in the context of PE, but the role that altered epidermal growth factor receptor (EGFR) might play in promoting PE remains comparatively unexamined. So far, reports on the EGF and EGFR expression have been limited to the first trimester placental tissue and only a few of them refer to placentas from PE pregnancies. The results of those studies were contrasting, some noted increased levels of EGFR whereas some reported lower or unchanged levels. Therefore, the present study aimed to see the expression of EGFR in different placental compartments in PE placenta compared to normal and also to see whether the expression of EGFR varies with severity of PE.

Material and Methods

This study was approved by Institutional Ethics Committee of our Institution. PE was diagnosed based on increased blood pressure (140/90 mmHg) in a pregnant woman after 20 weeks of amenorrhea, accompanied by proteinuria (0.3 g/24 h or 1+dipstick), as defined by the report of American College of Obstetricians and Gynecologists guidelines[16]. Cases of chronic hypertension or superimposed PE were excluded from the study.

Placental bed biopsies: Fresh placental bed biopsy tissues were obtained from 40 term pregnancies at the time of caesarean delivery after the patient's informed consent. The study groups consisted of pregnant women with PE (n = 40, PE group) and without PE (n = 30, normotensive control group). All pregnancies were also free of other complications such as gestational diabetes, chronic hypertension, or autoimmune disease. The expression of

EGFR was analysed by using immunohistochemistry, western blot and real time PCR.

Immunohistochemistry: By using scalpel, 4–5 biopsies of villous parenchyma (1 cm3 each) from the central and marginal regions of part of the placental disc were collected. Expression of EGFR was analysed in 40 placental villous tissues. 3µm thick sections were obtained from formalin fixed and paraffin embedded placental tissues. The levels of EGFR proteins in the 40 placental villous tissues have been detected using immunohistochemical staining according to the method described previously[17]. In brief, the sections were heated in 0.01 M citrate buffer solution (pH 6.0) in the water bath at 98 ° C for 20 min following de-paraffinization and endogenous peroxidase blockage.

Now incubate with the mouse monoclonal antibody against EGFR (rabbit monoclonal, BD Biosciences, CA-9061) and GAPDH (Santa Cruz Biotechnology, CA-166574) at 1:100 dilution overnight at 4^0C and visualize with 3,3′-diaminobenzidine (DAB) detection kit (Vector Labs). During IHC optimization, the 1:100 dilutions were determined to be the best. Anti-rabbit and anti-mouse IgG whole molecule (Sigma-Aldrich) was used at a dilution of 1:1000 for negative control. Two pathologists analyzed IHC-stained samples and all samples were blinded.

The staining intensity of proteins was graded at a scale of 0 (no staining) to 3 + (strong stain). The positivity of EGFR was scored based on the percentage of positive cells: 0 = 0 percent of stained positive cells; 1, weakly stained tissue or 1–25 percent of positive cells; score 2 = moderate stained tissue, or 26–50 percent of positive stained cells and score 3, strongly stained tissue or more than 50% of stained cells[17].

Western blot analysis: For the protein expression analysis in placental tissue, placental samples of human were homogenized at 4°C in 500µL RIPA lysis buffer containing protease and phosphatise inhibitors. The lysates were centrifuged at 14000 rpm at 4°C for 15 min to remove the debris of cells. Bicinchoninic acid assay (BCA assay) was used to measure the protein concentrations. Tissue lysate (40µg) were subjected to SDS-PAGE by using Tris–HCl buffer. The proteins were transferred to nitrocellulose membranes (Himedia). The membrane was subsequently incubated with primary antibodies.

Total protein was extracted and quantitated for the expression analysis in placental tissue as previously described[10]. Using Tris – HCl buffer, the total protein (40µg) was separated from 10 per cent Bis-Tris PAGE gel. The antibodies were used against EGFR (rabbit monoclonal, BD Biosciences, CA-9061) and GAPDH (Santa Cruz Biotechnology, CA-166574). Appropriate secondary antibodies conjugated to horse radish peroxidase (Bio-Rad) were incubated with respective membranes for 2 hours at room temperature. The membranes were developed using ECL plus (Bio-Rad) and the image was captured using enhanced chemi-luminescence system, G: BOX Chemi XX6/XX9. Immunoblot for GAPDH was considered as internal control for loading. The protein bands were quantified and normalized relatively as the control band with Image J, version 1.35d (National Institutes of Health Image Software).

RNA preparation, RT-PCR and real-time PCR: Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized from 2µg of total RNA by using Takara cDNA synthesis kit using a random hexamer 42°C for 1 h. Template cDNA was subjected to PCR amplification using gene-specific sense and antisense primers (Table 1). RT-PCR conditions were at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, annealing at 60°C for 30s and extension at 72°C for 30 s in a thermal cycle (Quant Studio 5 by Applied Bio systems). The quantitative amount of each gene was standardized against the house-keeping gene β-actin. The RNA levels were expressed as a ratio, using the 'delta-delta' method for comparing the relative expression results between normotensive control and patients with PE[10].

Statistical analysis: Statistical analysis was carried out by using Graph Pad Prism version 7.04. Statistical analysis of expression of EGFR in mild to moderate and severe PE for IHC was carried out with the Mann-Whitney U-test. Results for normally distributed data were analysed using student t-test. Chi-square test was used to see the neonatal gender difference. $P< 0.05$ was considered to be statistically significant.

Results

The demographic characteristics of the normotensive women and preeclamptic patients were studied. There were no statistical differences between the PE and normotensive control groups with respect to their age, BMI, neonatal gender. Almost all the deliveries in the control group were at full term. Compared to normal control group, the mean gestational age is shorter in PE group. In patients with PE, when compared with the normotensive control group, birth weight of the baby is reduced and the systolic and diastolic blood pressures were significantly higher ($P < 0.05$).

Expression of EGFR: There was moderate staining intensity of EGFR in syncitiotrohoblast membrane and weak staining intensity in chorionic villous stromal cells, villous vascular endothelial cells and vascular smooth muscle cells in normal as well as in PE placenta. In normal placenta staining was strong compared to PE placenta ($P<0.05$). By western blot method, expression of EGFR decreased in PE placenta by 1.67-fold compared to normal placenta. Relative mRNA expression decreased by 4.8-fold in normal placenta compared to preeclampsia ($P < 0.005$).

Discussion

This study showed decreased expressions of EGFR in the placental villous tissue in third trimester placental bed from

pregnancies with PE compared with the normotensive control group. Although the mechanisms which are responsible for the etiopathogenesis of preeclampsia, have not been completely understood, there is an agreement that it is associated with reduced invasion and failed remodelling of maternal endometrial spiral arteries in the placenta[19].

Table 1
Sequence of primers used for reverse transcriptase -polymerase chain reaction (RT-PCR)

Gene	Sequence
EGFR	5'-GAGAGGAGAACTGCCAGAA-3' 5'-GTAGCATTTATGGAGAGTG-3'
β-actin	5'-GGGAAATCGTGCGTGACATTAAG-3' 5'-TGTGTTGGCGTACAGGTCTTTG-3'

Table 2
Demographic characters in preeclamsia (PE) and normotensives

Variables	Normal N =30	Mild PE N=20	Severe PE N=20	F value	P	P1	P2	P3
Age	25.53±3.9	25.75±2.9	25.55±3.3	0.025	0.975	0.836	0.987	0.843
Height	1.53±0.049	1.51±0.03	1.52±0.02	1.36	0.262	0.153	0.337	0.495
Weight	60.63±4.0	60.05±4.3	63.05±5.7	2.375	0.101	0.630	0.08	0.07
BMI	25.83±2.0	26.05±1.6	25.45±2.1	2.88	0.06	0.602	0.020	0.05
GA	37.67±0.9	36.85±1.4	34.60±1.5	35.09	0.000*	0.02	0.000	0.000
SBP	106.07±9.2	145.10±6.4	163.50±3.3	417.6	0.000*	0.000	0.000	0.000
DBP	70.10±6.8	91.00±5.7	102.30±4.3	191.8	0.000*	0.000	0.000	0.000
weight (Kg)	2.9±0.29	2.72±0.22	2.2±0.40	33.22	0.000	0.001	0.000	0.000
Placental weight	503±18.4	478±77.6	495±47.7	1.517	0.227	0.095	0.435	0.395
Placental weight	2.77±0.4	2.55±0.5	2.35±0.4	4.775	0.012*	0.07	0.007	0.460
PT	12.0±0.0	12.7±0.8	13.1±0.7	21.615	0.000*	0.0001	0.000	0.102
APGAR at 5min	7.8±0.68	7.1±0.6	6.8±0.61	18.01	0.000	0.0002	0.0001	0.139
APGAR at 10min	8.8±0.68	8.3±0.65	7.9±0.307	16.67	0.0001	0.005	0.000	0.018

BMI: Body mass index; GA: Gestational age; PT: Prothrombin time; SBP: Systolic blood pressure; DBP: Diastolic blood pressure. Statistical significance between the groups was analysed by one way ANOVA followed by Tukey's post hoc multi comparisons (total comparison between groups). Statistical analysis is carried out by Student's unpaired t test between two groups (*p <0.05). (P1- Normotensives with mild PE, P2 –Normal to Severe PE, P3- Mild to severe PE). Above data is expressed as Mean ± SD

Table 3
Statistical analysis of expression status of EGFR in normal and PE placental tissue.

Type of tissue		Overall Score	EGFR		
			STM	CVSC	VVEC
Normal N =30		0	1	4	6
		1	8	23	24
		2	17	3	0
		3	4	0	0
Preeclampsia N =40	Mild PE N = 20	0	2	10	9
		1	11	9	11
		2	4	1	0
		3	3	0	0
	Severe PE N = 20	0	3	0	8
		1	10	7	12
		2	7	13	0
		3	0	0	0
P			0.019	0.017	0.136
P1			0.056	0.009	0.061
P2			0.007	0.033	0.127
P3			0.614	0.465	0.752

STM: Syncytiotrophoblast membrane; CVSC: Chorionic villous stromal cells; VVEC: Villous vascular endothelial cells. Immunohistochemical staining score: 0, no staining; 1+, 1–10%; 2+, 11–50%; 3+, 51–70%; 4+, 71–100%. P-Value is the level of significance calculated by Kruskal–Wallis rank-sum test. Mann-Whitney U test were used for comparison between two groups

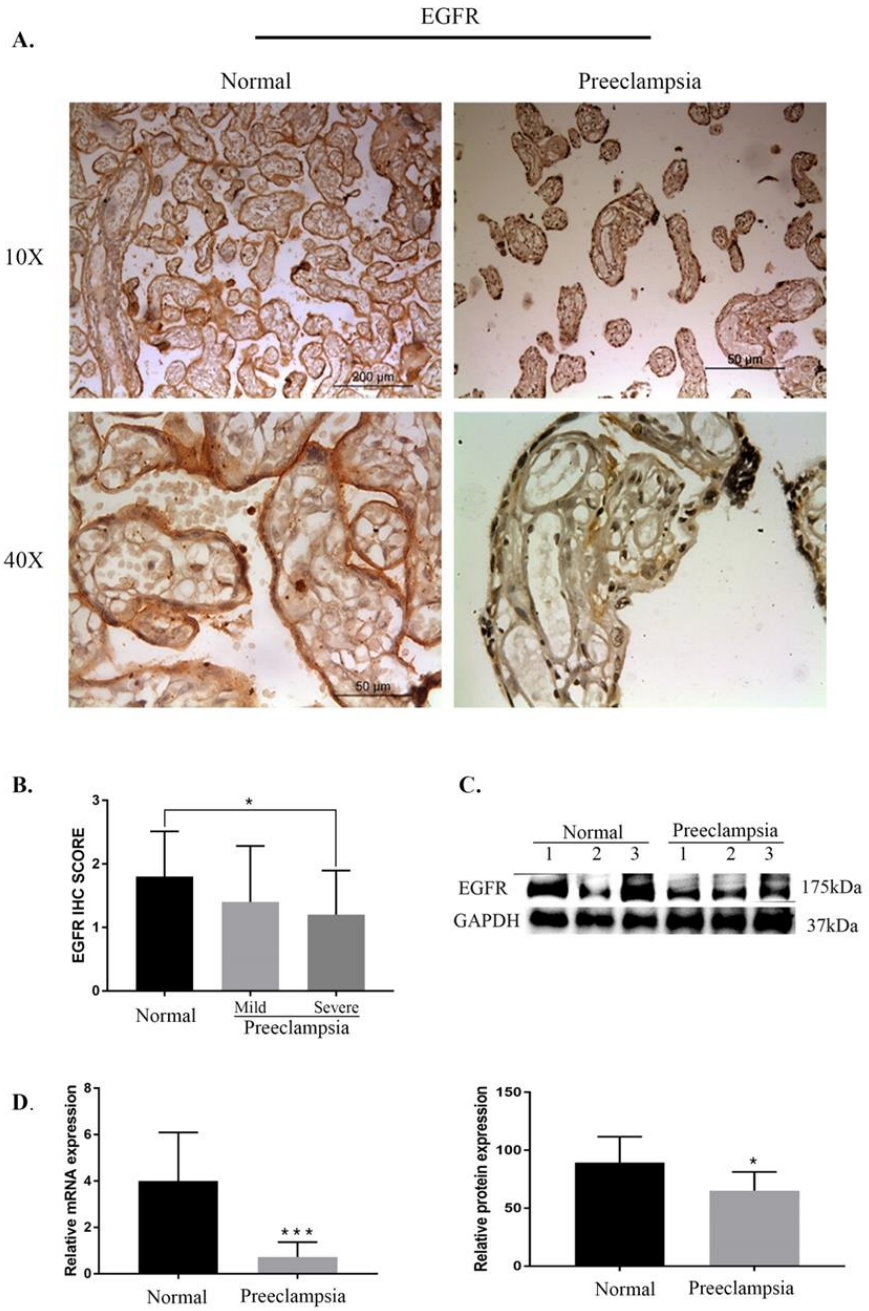

Figure 1: The expression of EGFR in normal and preeclamptic A. Immunohistochemical expression of EGFR antibody shows strong immunostaining in the membrane of syncytiotrophoblast in normal placenta compared to mild expression in preeclamptic placenta. B. The EGFR staining intensity scores indicate reduced expression of in preeclamptic placenta compared to normal. C&D: Western blot analysis and RT-PCR showed that EGFR in PE placentas were significantly lower on protein and mRNA levels.

The Epidermal Growth Factor Receptor (EGFR) is one of the most avidly studied molecules with important roles in both physiological and pathological states[7,13]. After fertilization and repeated mitotic activity, the blastocyst is formed. The blastocyst outer layer is trophoblast (TB), a specialized epithelial cell layer with the ability to proliferate and differentiate toward the placenta. In the post implantation period, TB is proliferating at an accelerated rate giving rise to two specific cell lines, mononucleated cytotrophoblast (CTB) and multinucleated syncytiotrophoblast (STB).

Later on, TB is divided into villous trophoblast (VTB) lining the placental villi and extra villous trophoblast (EVTB) with the ability to invade maternal spiral arteries in the process of so-called endovascular remodeling, thus allowing for the newly formed placental tissue and embryo constant and fluent nutrition supply[2]. Numerous growth factors and their

receptors, hormones and cytokines regulate TB differentiation and the EVTB remodeling process. One of the key growth factors during placental development is the epidermal growth factor (EGF) and its receptor (EGFR). EGF is a polypeptide composed of 53 amino acids with the ability to provoke the mitogenic effect on epidermal and mesothelial cells[6].

Binding of EGF and EGFR stimulates intracellular tyrosine kinase activity which induces phosphorylation of the receptor resulting in cellular growth, proliferation, differentiation, migration and sometimes apoptosis[15]. During the 4th and 5th weeks of gestation, EGF and EGFR are primarily expressed on the CTB while later on the expression is shifted to STB, demonstrating a dual role of the EGF–EGFR complex: TB proliferation and later on TB differentiation[3,11]. The main idea behind the study was to study the expression pattern of EGFR in PE placenta indifferent placental compartment with respect to their severity and to compare them with a control group.

Our results show that EGFR was expressed on the membrane of syncytio-trophoblast, chorionic villous stromal cells and villous vascular endothelial cells of the placenta and the expression of EGFR is reduced in all the cellular compartments in PE placenta compared with normal with more reduction in severe PE compared to moderate PE.

So far, reports on the EGF and EGFR expression have been limited to the first trimester placental tissue and only a few of them refer to placentas from PE pregnancies. The results of those studies were dubious; some demonstrated higher levels of studied factors in PE placentas whereas others report on lower or unchanged levels. Proliferation is crucial in early placental development both in TB and DC; furthermore, it is highly connected with the EGF–EGFR complex since they act synchronously[8].

Findings reported by Ferrandina et al[5] suggest that hypertensive disorders in pregnancy are associated with elevated placental EGFR concentrations detected by the radioreceptor technique. Milchev et al[12] reported on lower EGFR expression in VTB of PE placentas, while Dong et al[4] reported on lower EGFR expression in VTB of placentas with pregnancy induced hypertension[4,12].

Decreased expression of EGFR show altered plasminolytic, inflamamatory and angiogenic pathway. Altered expression of these proteins could be of clinical relevance in designing the new standard markers in PE. The expression of proteins in the syncytiotrophoblast membrane, chorionic stromal cells and and fetal endothelial cells of the placental villi may presumably play essential role in cell signalling events in fibrin homeostasis, angiogenesis and inflammatory activity. The expression analysis of EGFR in normal and PE placenta could be of interest in PE research in delineating the molecular networks that could bridge the gap between the involvements of these molecules in the development of PE.

Conclusion

This result suggests that altered expression EGFR proteins in placenta may be associated with the pathogenesis of PE by affecting angiogenesis.

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