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Expression of angiogenic factor with G patch and FHA domains 1 (AGGF1) in placenta from patients with preeclampsia

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Abstract

Introduction. Preeclampsia (PE) is a major contributor to maternal and foetal morbidity and mortality worldwide. It manifests as high blood pressure and proteinuria in women at more than 20 weeks of gestation. Abnormal levels of anti- and pro-angiogenesis factors are known to be associated with PE. In the present study, we aimed to determine the localisation of angiogenic factor with G patch and FHA domains 1 (AGGF1) in the placenta and to compare the expression levels of AGGF1 in the third-trimester placentas of preeclamptic and normotensive pregnancies.

Materials and methods. Placental tissue samples were collected from women with PE (n = 28) and without PE (n = 28). The normotensive controls without PE were matched for gestational age at delivery with the patients with PE. The expression levels of AGGF1 in the placental tissues were evaluated using immunohistochemistry, quantitative reverse transcription polymerase chain reaction and Western blot.

Results. The immunoexpression of AGGF1 was localised in the syncytiotrophoblast tissue. Notable, the mRNA and protein expression levels of AGGF1 were decreased in preeclamptic placentas as compared with the normotensive control group ($P < 0.05$).

Discussion. Our results suggest that the decreased AGGF1 in preeclamptic placentas may be related to the pathogenesis of preeclampsia. *(Folia Histochemica et Cytobiologica 2020, Vol. 58, No. 2, 83–89)*

Key words: angiogenesis; AGGF1; preeclampsia; placenta; syncytiotrophoblast; IHC; qPCR; WB

Introduction

Preeclampsia (PE), which affects 3–6% of all pregnancies, is a major contributor to maternal and foetal morbidity and mortality worldwide [1, 2]. It can be diagnosed by the combined presentation of high blood pressure and proteinuria after 20 weeks of gestation. The current definition of PE also includes maternal organ dysfunction [3, 4]. Despite advances in PE research, its aetiology and pathogenesis remain unclear [5].

Angiogenesis refers to the formation of new blood vessels from existing vasculature [6]. Placental angiogenesis can dramatically influence the outcome of pregnancies. During pregnancy, a vascular network is built between the mother and the foetus. Aberrant vascularization during this process may results in imbalance between pro-angiogenic and anti-angiogenic factors [7–10].

In pregnant women with PE, placental tissue overproduces two important anti-angiogenic proteins, which enter into maternal circulation: soluble endoglin (sEng) and soluble fms-like tyrosine kinase-1 (sFlt1). Furthermore, these patients have low circulat-

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Table 1. Clinical characteristics of patients with preeclamptic and normotensive pregnancies

Parameters	Preeclamptic (n = 28)	Normotensive (n = 28)
Maternal age (years)	30.32 ± 5.54	29.86 ± 4.34
BMI in pregnancy [kg/m^2]	27.89 ± 3.25	26.58 ± 2.44
Gestational age (weeks)	36.77 ± 3.24	38.02 ± 1.07
SBP at delivery [mm Hg]	**155.9 ± 15.5***	113.8 ± 7.8
DBP at delivery [mm Hg]	**100.2 ± 21.4***	75.9 ± 7.50
Proteinuria†	**100% (28/28)***	0% (0/28)
Newborn weight [g]	**2683 ± 860***	3416 ± 289
1-Min Apgar score	**14.3% (4/28)***	0% (0/28)
5-Min Apgar score	**7.14% (2/28)***	0% (0/28)

Data are presented as mean ± SD or percentage (number/total). *P < 0.05 compared to normotensive control, Student's t-test. †Quantity of 24-hour protein excretion ≥ 300 mg/24 h is proteinuria positive and ≤ 300 mg/24 h is proteinuria negative. Abbreviations: BMI — body mass index in pregnancy; SBP — systolic blood pressure; DBP — diastolic blood pressure.

ing blood levels of two pro-angiogenic factors: vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) [11, 12]. Zeisler *et al*. showed that the sFlt1/PlGF ratio is elevated in pregnant women before the clinical onset of PE. However, the predictive value of this ratio is not clear, although it can be used to predict the short-term absence of PE [13]. These angiogenesis factors are well-known biomarkers of PE and have been implicated in its pathophysiology [14, 15].

Angiogenic factor with G patch and FHA domains 1 (AGGF1, also known as VG5Q), a newly described angiogenic factor, is encoded on chromosome 5q13.3. The full-length AGGF1 complementary DNA (cDNA) encodes a protein with 714 amino acids, which contains a forkhead-associated domain (FHA), a G-patch domain, and a coiled-coil motif, the functions of these domains are unknown [16, 17]. Recent studies have shown that the purified AGGF1 protein can promote angiogenesis as potently as VEGF in chick embryos, and that the knockdown of AGGF1 expression can inhibit endothelial vessel formation [18, 19]. However, whether AGGF1 plays a role in the development of preeclampsia remains to be addressed.

The roles of many angiogenic factors in the development of PE have aroused wide concern in the past few decades. However, the expression pattern of AGGF1 in the placenta has not been investigated yet. In this study, we aimed to investigate the localisation of AGGF1 expression in the placenta and to analyse the difference in the expression patterns of AGGF1 in third-trimester placentas from preeclamptic and normotensive pregnancies.

Materials and methods

Ethical approval. The protocols for this study were approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All patient-derived tissue samples were obtained after receiving informed written consent from the patients.

Study populations. All placentas were obtained after caesarean sections performed at the Union Hospital between January 2016 and December 2016. The study groups consisted of 28 preeclamptic pregnancies and 28 normotensive pregnancies. All participants were at more than 28 weeks of gestation. PE is traditionally diagnosed if the patient presents with hypertension (blood pressure ≥ 140/90 mmHg) and proteinuria (≥ 300 mg/24 h) after week 20 of gestation. In the absence of proteinuria, hypertension combined with maternal organ dysfunction, such as thrombocytopenia, liver involvement, renal insufficiency, pulmonary oedema, or new-onset cerebral or visual symptoms can also be diagnosed with PE [3, 4]. Exclusion criteria for patients in this study included multiple pregnancies, tobacco use, cardiovascular diseases, diabetes mellitus, rupture of membranes, foetal structural or genetic anomalies, blood diseases, severe pre-existing metabolic disorders, and infectious diseases. Controls were normotensive women without any pregnancy-related complications or chronic problems. The clinical characteristics of the patients are summarized in Table 1.

Sample collection and preparation. Immediately after caesarean section, placental tissue (~10 g each) was collected from the area around the umbilical cord attachment site by cutting a vertical plane through the full thickness of an

apparently normal area, including both the maternal and foetal surfaces. Tissues with calcification or clots were excluded. The collected tissues were washed with sterile phosphate-buffered saline (PBS) and placed in 10% formalin and embedded in paraffin for immunohistochemical (IHC) analysis. The remaining parts of the placental segments were aliquoted and stored at –80°C for RNA and protein extraction.

Immunohistochemistry. The expression patterns of AGGF1 in the placental tissues were evaluated. The placental tissues embedded in paraffin were sectioned into slices 4 μm thick and incubated with rabbit polyclonal antibodies against AGGF1 (5.0 μg/ml; Abcam, Cambridge, MA, USA). The sections were then deparaffinised and rehydrated in graded ethanol before antigen retrieval was performed in citrate buffer (pH 6.0) for 3 min at full pressure using a pressure cooker. To inhibit the activity of endogenous peroxidase, the sections were then treated with 3% hydrogen peroxide for 5 min. Next, blocking was performed by incubating the sections in a solution of 0.01 mol/L Tris, 0.3% Triton X-100, and 10% normal goat serum for 30 min, after which they were incubated overnight at 4 °C with the primary antibody. After rinsing in phosphate-buffered saline (PBS), the sections were incubated with peroxidase-labelled anti-rabbit immunoglobulin G (IgG) for 30 min. Finally, all slides were incubated with DAB (Beyotime, Shanghai, China) before being rinsed with distilled water, counterstained with haematoxylin, dehydrated, and mounted. A Motic microscope (Motic, Xiamen, China) was used to visualise and photograph the slides. Semiquantitative evaluation of immunohistochemical (IHC) reaction was performed by two observers who were blinded to the identity of the slides. Remmele and Schicketanz immunoreactive score (IRS) and IHC score (IHS) were selected to analyse the data [20]. The IHSs were determined based on the staining intensity (SI) and the percentage of immunoreactive cells (PR). The SI scale was divided into four categories (0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining), and the PR scale was divided into five categories (0 = no staining, 1 = 1–10% staining, 2 = 11–50% staining, 3 = 51–80% staining, and 4 = 81–100% staining). A final semiquantitative score ranging from 0 to 12 points was calculated for each sample. Samples with IHS above 4 were considered positive, and those with IHS below 4 were considered negative.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was isolated from the placental tissues using RNAiso Plus (TaKaRa, Tokyo, Japan), according to the manufacturer's protocol. The RNA quality and purity were determined spectrophotometrically (NanoDrop; Thermo Fisher Scientific, CA, USA). Samples with optical density$_{260/280}$ ratios between 1.8 and 2.0 were chosen for the subsequent steps. One microgram of total RNA from each sample was reverse transcribed into cDNA in a 20-μl using PrimeScript™ RT Master Mix (TaKaRa), which was stored at –20°C for further analysis. Quantitative PCR was performed using the cDNA as the template. SYBR Premix Ex Taq II (TaKaRa) was used for the PCR. Each reaction mixture contained 5 μl 2× SYBR Taq II, 0.2 μM forward PCR primer, 0.2 μM reverse PCR primer, 0.2 μl 50 × ROX reference dye, 1 μl cDNA, and 3.4 μl sterile distilled water for a final volume of 10 μl. The qRT-PCR was performed using the pre-set PCR program in StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) to quantify mRNA expression levels, with GAPDH as internal control. Primers for the RT-PCRs were synthesized based on the GenBank database. The primer sequences were: GAPDH, forward primer: 5'-ACCACAGTCCATGCCATCAC-3', reverse primer: 5'-TCCACCACCCTGTTGCTGTA-3'; AGGF1, forward primer: 5'-AAACGTAGGAGCAGGTTGG-3', reverse primer: 5'-TGAGGATGGTTTGCCTGTCC-3'. The qRT-PCR consisted of a denaturation step at 95°C for 30 s, 40 cycles of an annealing step (95°C for 5 s, 65°C for 30 s, and 60°C for 45 s), and an extension step at 72°C for 60 s. The expression of target genes was established using the comparative cycle threshold (Ct; $2^{-\Delta\Delta CT}$) method.

Western blot. The placental tissues were homogenized on ice for 30 min in pre-cooled RIPA buffer. The homogenates were centrifuged at 12000 *g* for 15 min at 4°C and the supernatants were collected. After isolating the protein from the tissues, the concentration of lysates was determined in the samples using a bicinchoninic acid protein assay kit (Beyotime). After boiling the samples for 10 min, equal amounts of the proteins (30 μg) were separated using 10% SDS-PAGE and transferred to 0.45-μm polyvinyl difluoride (PVDF) membranes in 20% methanol and 0.1% SDS. These mixtures were then blocked for 1 h at room temperature with 0.1% TBST (20 mM Tris-HCL, 500 mM NaCl, and 0.05% Tween 20 at pH 7.5) with 5% fat-free dried milk power. The membranes were then separately incubated overnight at 4°C with rabbit polyclonal AGGF1 antibody (1.0 μg/ml; Abcam), and rabbit monoclonal GAPDH antibody (0.33 μg/ml; Affinity, Cincinnati, OH, USA). After washing and incubation with the secondary anti-rabbit antibody (0.2 μg/ml; Affinity) for 1 h at room temperature, the membranes were washed again and visualised using ECL-PLUS (Amersham Biosciences, Uppsala, Sweden). Finally, the intensity of the bands obtained was quantified using Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

Statistical analysis. All the data obtained were normally distributed, and all statistical analyses were performed

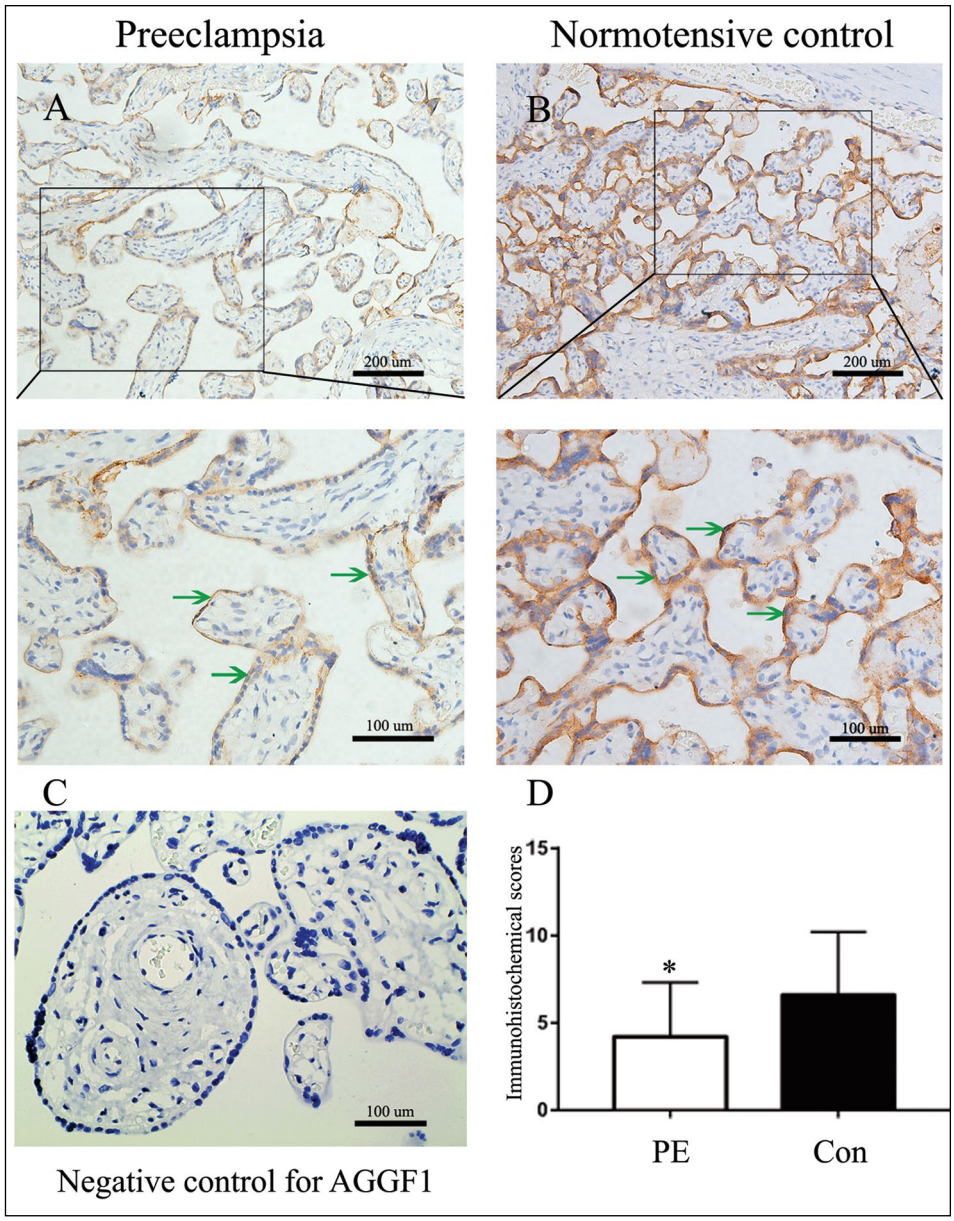

Figure 1. AGGF1 immunoreactivity in human placenta. Immunolocalisation of AGGF1 in placentas of patients with preeclampsia (**A**) and normotensive patients (**B**). (**C**). No AGGF1 immunoreactivity in tissue section of a patient with preeclampsia. (**D**). Immunohistochemical scores of AGGF1 in placentas of patients with preeclampsia (PE) and normotensive patients (Con) (4.21 ± 3.11 *vs*. 6.61 ± 3.59, respectively). Green arrows indicate syncytiotrophoblasts. The AGGF1 localises in the syncytiotrophoblastic layer of placental villi. *P = 0.0101, PE *vs.* Con. AGGF1 — Angiogenic factor with G patch and FHA domains 1.

using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The results were presented as mean ± standard deviation (SD). Comparisons between groups were performed using Student's t-test. The chi-square test was used to analyse categorical variables. Statistical significance was defined as $P < 0.05$.

Results

Twenty-eight paired tissue samples were analysed by immunohistochemistry for AGGF1 expression. AGGF1 expression was localised in syncytiotrophoblasts. Positive expression of AGGF1 was found in both normotensive (18/28) and preeclamptic (9/28) samples. The immunohistochemical expression of AGGF1 in 32% of preeclamptic placentas (Fig. 1A) were dramatically decreased ($P < 0.05$) than those in normotensive ones (Fig. 1B). However, the majority (68%) of patients with preeclampsia did present AGGF1 immunoreactivity (Fig. 1C). Immunohistochemical scores of AGGF1 in two groups were shown in Figure 1D.

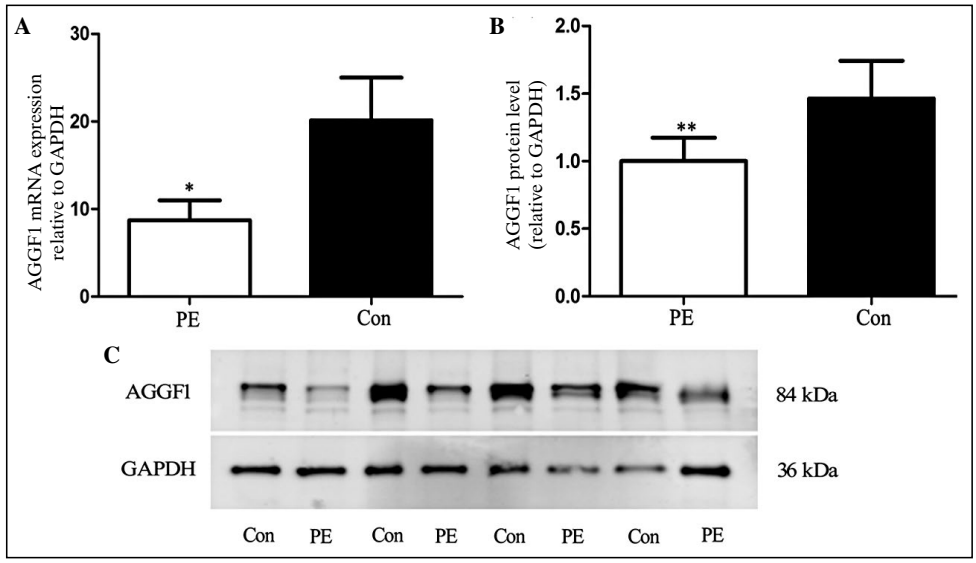

Figure 2. mRNA and protein expression levels of AGGF1 in placental tissues of patients with preeclampsia (PE) and normotensive patients (Con) assessed by qRT-PCR and Western blot. **A**. AGGF1 mRNA levels were lower in preeclamptic than control placentas (8.70 ± 2.28 *vs*. 20.13 ± 4.88 respectively, P = 0.0385). **B**. AGGF1 protein levels, as assessed by Western blot, were lower in preeclamptic than control placentas (1.00 ± 0.08 *vs.* 1.46 ± 0.14, respectively, P = 0.0054). **C**. Representative Western blots show the expression levels of AGGF1 protein in the studied groups. Bands were observed at 84 and 36 kDa, corresponding to the sizes of AGGF1 and GAPDH, respectively. *P < 0.05; **P < 0.01, preeclamptic placenta tissue (PE) *vs.* normotensive placenta tissue (Con).

The mRNA levels of total AGGF1 were also significantly decreased in the PE group than in the normotensive controls (Fig. 2A). The presence of the AGGF1 protein in placental tissues was determined by Western blot (Fig. 2B). The AGGF1 antibody detected one major band at approximately 84 kDa, which is similar to the reported molecular weight of AGGF1 (Fig. 2C) [19]. Protein levels of AGGF1 were lower (P < 0.05) in preeclamptic placentas than in normotensive ones.

Discussion

The gene encoding AGGF1 has been discovered in 2004 [19], and its physiological relevance needs to be further studied, especially in relation to PE. In the present study, we demonstrated that AGGF1 expression was localised by immunohistochemistry in the syncytiotrophoblasts of 64.3% of normotensive term placenta and 32.1% of preeclamptic placenta. Moreover, the mRNA and protein expression levels of AGGF1 were significantly lower in placental tissues from pregnancies complicated with PE than in those from normotensive pregnancies in the whole studied groups of patients.

Placental development involves the cooperation and proper functioning of various cell types [21]. Trophoblast tissue, the most critical component of placenta, acts as the interface between foetal and maternal tissues. Both cytotrophoblast's cells and syncytiotrophoblast of the placental villi release numerous angiogenic factors and present also their receptors [22].

Decreased AGGF1 expression in preeclamptic syncytiotrophoblast tissue of some preeclampsia patients may not be a specific characteristic of PE, but it is possibly associated with abnormal placental ischaemia and hypoxia in PE patients. Hypoxic regions are known to exist in and be important for the pathology of many diseases, including preeclampsia [23–25]. Placental ischaemia is considered a major pathogenic mechanism underlying PE and is known to result in hypoxia [26]. The ability of cells to adapt to hypoxia is important for survival in both physiological and pathophysiological states [27, 28].

Xu *et al*. reported that AGGF1 was down-regulated in human high-grade urothelial cancer cells after exposure to hypoxia, and this down-regulation may partly reduce hypoxia-induced apoptosis of the cancer cells [29]. However, the mRNA levels of AGGF1 were not different in hypoxic and normoxic conditions in cell cultures [29]. Conversely, Lu *et al*. reported that AGGF1 expression increased in response to hypoxia in endothelial cells that isolated from mouse hearts, which suggested that ischaemia induced AGGF1 expression in myocardial infarction mouse model [30]. Wang *et al*. reported that AGGF1 expression was up-regulated in hepatocellular carcinomas (HCCs),

compared to adjacent non-cancerous tissues and that a significant correlation (r = 0.548, 70 cases) was found between tumoural expression of VEGF and AGGF1 [31]. It has to be noted that these observations may be related to different cell types in various diseases or experimental models.

Our immunohistochemical analysis showed that the expression of AGGF1 was confined to the syncytiotrophoblast and was absent in the endothelial cells of the placenta. Previous studies have detected the AGGF1 protein in human umbilical vein endothelial cells and human microvascular endothelial cells [19]. AGGF1 is also known to be expressed in ovarian cancer cells, vascular smooth muscle cells, osteoblasts, HCCs, and human heart fibroblasts [19, 31]. In this study, we found no expression of AGGF1 in the endothelial cells of the placenta. This may be due to a differential expression in various organs or to the sensitivity of the technique.

Besides angiogenesis, AGGF1 has been suggested to be involved in multiple other functions. It has been suggested that AGGF1 can fully reverse the effects of hyperglycaemia on endothelial progenitor cells [32]. AGGF1 may also inhibit TNF-α-mediated monocyte adhesion to endothelial cells and affect the expression of adhesion molecules and chemokines [17]. In addition, AGGF1 has been shown to have anti-inflammatory effects, both *in vitro* and *in vivo*. As an anti-inflammatory agent, AGGF1 has been associated with TNF-alpha-induced endothelial activation and the regulation of liver fibrosis *via* TGF-beta signalling modulation [17, 33].

In summary, we preliminarily demonstrated that the AGGF1 expression was decreased in third-trimester preeclamptic placenta compared with normotensive term placenta. The exact role of decreased AGGF1 expression in PE remains unclear, and the molecular mechanisms remain to be defined.

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The authors declare that they have no conflicts of interest related to this study.

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