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Original Paper

Down-Regulation of Neuropathy Target Esterase in Preeclampsia Placenta Inhibits Human Trophoblast Cell Invasion via Modulating MMP-9 Levels

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Key Words
Neuropathy target esterase • Preeclampsia • Placenta • Trophoblast • Invasion

Abstract:
Background/Aims: Neuropathy target esterase (NTE, also known as neurotoxic esterase) is proven to deacylate phosphatidylcholine (PC) to glycerophosphocholine as a phospholipase B. Recently; studies showed that artificial phosphatidylserine/PC microvesicles can induce preeclampsia (PE)-like changes in pregnant mice. However, it is unclear whether NTE plays a key role in the pathology of PE, a pregnancy-related disease, which was characterized by deficient trophoblast invasion and reduced trophoblast-mediated remodeling of spiral arteries. The aim of this study was to investigate the expression pattern of NTE in the placenta from women with PE and normal pregnancy, and the molecular mechanism of NTE involved in the development of PE. ***Methods:*** NTE expression levels in placentas from 20 pregnant women with PE and 20 healthy pregnant women were detected using quantitative PCR and immunohistochemistry staining. The effect of NTE on trophoblast migration and invasion and the underlying mechanisms were examined in HTR-8/SVneo cell lines by transfection method. ***Results:*** NTE mRNA and protein expression levels were significantly decreased in preeclamptic placentas than normal control. Over-expression of NTE in HTR-8/SVneo cells significantly promoted trophoblast cells migration and invasion and was associated with increased MMP-9 levels. Conversely, shRNA-mediated down-regulation of NTE markedly inhibited the cell migration and invasion. In addition, silencing NTE reduced the MMP-9 activity and phosphorylated Erk1/2 and AKT levels. ***Conclusions*:** Our results suggest that the decreased NTE may contribute to the development of PE through impairing trophoblast invasion by down-regulating MMP-9 via the Erk1/2 and AKT signaling pathway.

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Introduction

Preeclampsia (PE) is a pregnancy-related disease defined as on-set of hypertension and proteinuria after the 20th week of pregnancy, which affects approximately 6-8 % all pregnancies worldwide and is responsible for a large proportion of maternal and perinatal morbidity and mortality [1, 2]. Although the exact etiology of PE remains uncertain, abnormal placentation is thought to be the key component for the pathogenesis of PE because symptoms disappear after the placenta is removed from the maternal body [1, 3]. Generally accepted hypotheses propose that poor trophoblast migration and invasion impair remodeling of the spiral arteries, which results in maternal endothelial cell dysfunction and finally leads to hypoperfusion and clinical manifestations of PE [1, 4-6]. Consequently, investigations on the molecular mechanisms related to insufficient trophoblast invasion will be helpful to uncover the pathophysiological process of PE.

Neuropathy target esterase (NTE), also named neurotoxic target esterase, was originally discovered 40 years ago as the primary target of organophosphorus (OP) compounds that cause delayed neuropathy [7]. This syndrome is characterized by delayed paralysis of the lower limbs due to degeneration of nerve axons in the spinal cord and peripheral nerves [7, 8]. However, the human NTE gene was cloned only 10 years ago [9, 10]. NTE is a serine esterase protein which is highly conserved across species including bacteria, nematodes, yeast, insects and vertebrates [9]. In the adult, NTE is observed throughout the brain, particularly in the hippocampus and cortex [11, 12]. Additionally, NTE is also expressed in non-neural tissues, such as spleen, liver and placenta [7, 13, 14]. Gurba et al. showed that human placental NTE is similar to forms found in avian and human brain [14]. In mice, at E7.5, NTE is strong expressed in the ectoplacental cone [15], which will develop the whole placenta. Moreover, NTE knockout resulted in death of embryo due to failed placenta development and impaired vasculogenesis at mice mid-gestation [12, 14, 15]. Histological analysis indicated that NTE is essential for the formation of labyrinth layer and survival and differentiation of secondary giant cells [14, 15]. In addition, NTE has been demonstrated to have potential lysophospholipase activity and deacylate phosphatidylcholine (PC) to glycerophosphocholine (GPC) as a phospholipase B in Drosophila and mammalian cells [7, 16-18]. Furthermore, Omatsu et al. found that phosphatidylserine (PS) /PC microvesicles can induce preeclampsia-like changes in pregnant mice [19]. Huang et al. study indicated that the levels of PC as well as triglyceride were significantly increased in preeclamptic placenta compared to control [20]. These studies suggested that NTE was possibly involved in the placental pathology of PE by influencing phospholipid metabolism.

Based on the evidence noted above, we hypothesized that alterations in placental NTE expression might be involved in the development of PE. To test this hypothesis, here we investigated the expression pattern of NTE in the placenta from women with PE and normal pregnancy. We also observed the effect of NTE on trophoblast cell behavior by gene over-expression or knockout in human placental trophoblast cell line, HTR8/Svneo cells. The findings in the present study revealed that the decreased NTE in placenta may contribute to the development of PE through impairing trophoblast invasion by down-regulating MMP-9.

Materials and Methods

Samples collection

Ethical approval was granted by Ethical Committee of JiangXi Province People's Hospital, and informed consent was obtained from each patient. Placentas were collected from pregnancies with: (1) normal pregnancy (no medical complications and proteinuria, maternal blood pressure < 140/90 mm Hg), (2) Severe PE (new-onset hypertension, defined as systolic blood pressure of > 160 mmHg and/or diastolic blood pressure of > 110 mmHg, two measurements with 6 h interval, and proteinuria >5 g/24h or 3+or greater protein in two random samples collected at >4h interval after 20 weeks of gestation). Tissues were collected and transported to the laboratory within 30 min after delivery. To minimize blood contamination,

each piece of tissue was intensively washed with ice-cold phosphate buffered saline (PBS), and then fixed in Bouin's solution, or snap-frozen and stored at -80°C for subsequent RNA and protein analyses.

Cell culture and transfection

The immortalized first-trimester trophoblast cell line, HTR-8/SVneo, derived from the short-lived primary cytotrophoblast cell line [21], were cultured in the DMEM/F12 media (Invitrogen, CA) supplemented with 10% FCS and 100μg/ml penicillin/streptomycin in 95% air and 5% CO_2. The cells were maintained in the logarithmic phase of growth and sub-cultured at 3-4 days intervals. Before the experiments, cells were cultured with serum-free medium for 12 h. Transient transfection of pEGFP-N3 (Control), pNTE-EGFP (NTE-EGFP), pGenesil-1(empty vector), and scrambled shRNA and NTE shRNA into the HTR-8/SVneo cells was performed using FuGENE HD (Promega) according to the manufacturer's protocol. Transfected cells were further assayed by qPCR and Western blotting at 24h and 72 h post-transfection for the expression of NTE, respectively.

Scratch-wound assay

Cells were adjusted to 2×10^5 cells/ml in serum-free DMEM/F12 medium, and cultured in the 24-well plastic dishes. Scratch wounds were created by scraping the monolayer cell with a sterile 200μl pipette tip at 24h post-transfection, and then washed away suspended cells. Cell migration into the wound space was taken photographs at 0 and 24 h using a DS-Fi1 digital camera (Nikon, Japan) and estimated after wounding with the NIH ImageJ software. Migration areas of groups were normalized to the area of control group.

Invasion assay

Invasion assays were performed in 24-well transwell plates (Corning, USA) according to manufacturer's instructions. Firstly, the transwell inserts were firstly coated with 35 μl of 1 mg/ml Matrigel matrix (BD, USA) at 37°C, 4 h for gelling according to the manufacturer's recommendations. HTR-8/SVneo cells were transfected, trypsinized and seeded at a density of 2×10^5 cells in 200μl experimental medium with 1 % FCS on the upper chamber; the lower chamber was filled with 600 μl medium with 10% FCS. Then cells were incubated in 95% air and 5% CO_2 at 37°C for 24 h. The inserts were removed, washed with PBS and non-migrating cells in the upper chamber were removed with a cotton bud. Finally, the inserts were fixed in methanol and acetone (1:1) for 10 min at room temperature and stained with hematoxylin. For each experiment, the numbers of invasive cells were quantified in seven randomly chosen fields of each filter.

Immunohistochemistry

Tissues were fixed in Bouin's solution, dehydrated, and embedded in paraffin. The sections were deparaffinized in xylene, and rehydrated in graded alcohols. Immunohistochemical staining was performed by the streptavidin–biotin -peroxidase complex method and detected with diaminobenzidine solution. Monoclonal mouse anti-human NTE (1:150; Sigma) and cytokeratin (1:300; Santa Cruz) were used as the primary antibody. In some sections, the primary antibodies were replaced with mouse preimmune IgG as a negative control.

Quantitative real-time PCR

Total RNA was extracted from placental tissues using RNAiso Plus solution (TaKaRa, China) according to the manufacturer's protocol. RNA samples were reverse-transcribed into cDNA in a 25 μl reaction mixture (TaKaRa, China). Then, cDNA was applied as a template for real-time PCR analysis on an ABI Prism 7500 System (Applied Biosystems, USA). Real-time PCR was performed in a 20 μl reaction volume containing 10 μl of SYBR Green Master Mix (TaKaRa, China), 2 μl of template cDNA, 0.5 μM primers, and water. Melting curve analysis and agarose gel electrophoresis were conducted following real-time PCR assays to monitor PCR product purity. 18 S served as constitutive control. The following primers were used: NTE: sense, 5'- CCAAGAGTTCCGGCTGTCA -3', antisense, 5'- CACAATGAGGATGCAGTCGG -3'; 18S: sense, 5'-GCTGAGAAGACGGTCGAACT--3', antisense, 5'-TTAATGATCCTTCCGCAGGT -3'.

Gelatin zymography

Matrix metalloproteinase-2 (MMP-2) and MMP-9 levels were evaluated by substrate gel zymography. The conditioned media of cells were collected at 24 h and 48 h. Protein content of conditioned media was

measured according to the method of Bradford (Applygen Technologies Inc.,China), and media containing equal amounts of protein were subjected to electrophoresis in a 10% polyacrylamine gel containing 0.5 mg/ml gelatin (Sigma, USA). The relative densities of bands were counted by comparing their densitometry values with that of corresponding control group.

Western blot analysis

Cells were lysed in the RIPA buffer supplemented with a phosphatase inhibitor cocktail and phenylmethylsulfonyl fluoride (Applygen Technologies Inc.,China). Equal amounts of protein extracts were subjected on the 12% SDS-polyacrylamide gel for electrophoresis, and then transferred onto the polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked with 5% BSA for 1 hour at room temperature and incubated with the following primary antibodies at 4°C overnight: beta-actin antibody (SANTA CRUZ, USA), NTE antibody (Sigma), Erk1/2, phospho-Erk1/2, AKT, phosphor-AKT (Cell signaling Technology, USA). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies and visualized via enhanced chemiluminescence (Pierce, USA). The relative band intensity was acquired by using the Quantity One software.

Statistical analysis

Data were presented as means ± SD. Statistical analysis was performed using one-way ANOVA followed by LSD's post-hoc test for multiple groups. Non-parametric Mann-Whitney U-tests were used to compare levels of placental NTE mRNA in normal controls and pregnancies with PE, and a value of $P<0.05$ was considered to be statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL) 13.0.

Results

Clinical data analysis

Our study sample included 20 pregnancies with PE and 20 normotensive controls. The clinical characteristics of patients are presented in Table 1. Higher systolic and diastolic blood pressures were observed for women with PE. Moreover, in the PE group, gestational age was 2.4 weeks shorter at delivery ($P=0.012$) and birth weight was at least 0.5 Kg lower ($P<0.001$). However, there was no significant difference in fetal gender and maternal age between normal pregnancy and PE.

NTE expression in human placentas

qPCR results indicated that expression level of NTE mRNA in preeclamptic placenta was significantly lower than that of the normal sample ($P<0.01$, Fig. 1). We then analyzed the spatiotemporal expression of NTE protein in the placental tissues by immunohistochemistry method (Fig. 2). Immunostaining results from normal pregnant samples showed that NTE protein was strongly expressed in cytotrophoblasts and syncytiotrophoblasts of villi (Fig. 2D), and moderately expressed in endothelial cells of blood vessels (Fig. 2D). Trophoblast identity was confirmed by staining for cytokeratin on a serial section (Fig. 2A, B). Compared to normal control group, the staining intensity of NTE-positive area was significantly lower in the

Table 1. Clinical characteristics of the women with and without pre-eclampsia

	Normal pregnancy (n=20)	Severe Preeclampsia (n = 20)	P-value
Maternal age (years±SD)	26.4±3.1	30.3±4.9	0.36
Gestational age (weeks±SD)	37.2±2.5	34.8±2.2	0.012
Birth weight (g±SD)	3342.2±315.3	2813.4±672.1	<0.001
systolic blood pressure (mmHg±SD)	108.1±10.8	167.6±10.8	<0.001
diastolic blood pressure (mmHg±SD)	68.5±9.1	106.7±11.2	<0.001
Fetal gender (male/female)	11/9	10/10	1.000

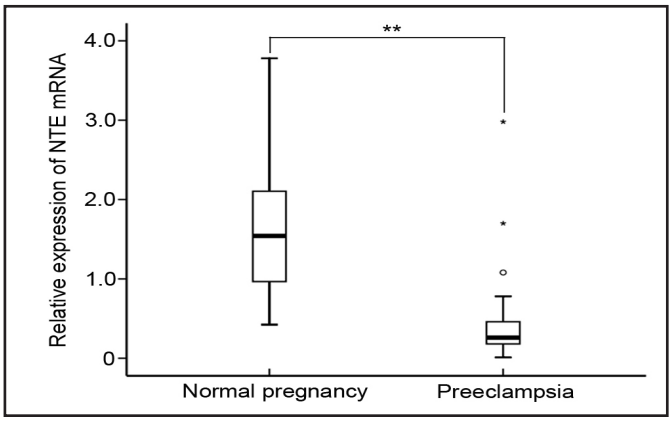

Fig. 1. Expression levels of NTE mRNA in human placentas derived from normal pregnant women and severe preeclamptic patients. mRNAs levels were quantified using quantitative PCR and normalized to 18S and presented as box and whisker plots. The median value is represented by a horizontal line in each box. The 75th (upper margin) and 25th (lower margin) percentiles of the values are shown. **P<0.01 compared with control (Mann-Whitney U-test, n=20)

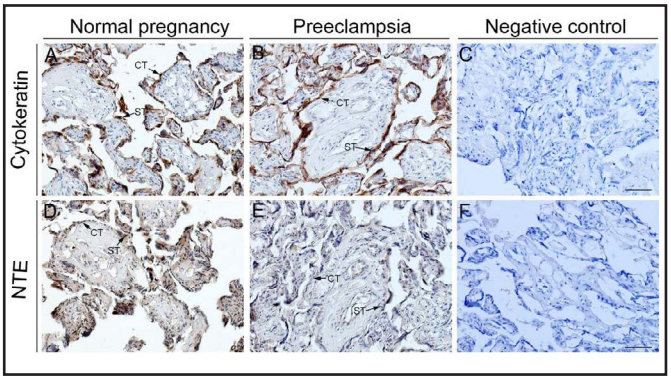

Fig. 2. Immunohistochemical analysis of NTE protein in human placentas derived from normal pregnant women and severe preeclamptic patients. Serial sections of human placental tissues from normal controls (A, D) and preeclampsia (B, E) were stained with cytokeratin (A, B), NTE (D, E) antibodies or without primary antibodies (C, F). Trophoblast cells of placenta were identificated by cytokeratin staining (A, B). CT, cytotrophoblast; ST, syncytiotrophoblast. Scale bar, 50 μm.

placental villi of PE (Fig. 2E), in accordance with qPCR results. Negative control omitting primary antibodies showed no specific staining (Fig. 2C, F).

Over-expression and knockdown of NTE in HTR-8/SVneo Cells

At 24 h and 72 h post-transfection, we examined NTE mRNA and protein expression by qPCR and Western blotting, respectively. Transfection with NTE-EGFP plasmid significantly increased NTE mRNA (P<0.001, Fig. 3A) and protein levels (P<0.01, Fig. 3B), whereas, transfection with NTE-shRNA significantly reduced NTE mRNA (P<0.05, Fig. 3A) and protein levels (P<0.01, Fig. 3B) compared with non-transfected HTR-8/SVneo cells.

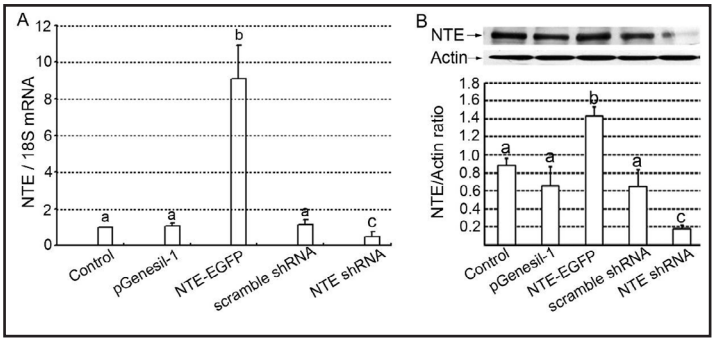

Fig. 3. NTE expression in the HTR-8/SVneo cells. (A) Quantitative PCR analysis of NTE mRNA expression in HTR-8/SVneo cells. mRNAs levels were quantified using quantitative PCR and normalized to 18S. (B) Western blot analysis of NTE protein expressed in HTR-8/SVneo cells after transfection. (Top) Representative immunoblotting results of NTE protein; (Bottom) Densitometric values from western blot analyses of NTE protein. The data were normalized to β-actin expression and shown as the means± SD of three independent experiments. Groups with different superscript letters are significantly different (ANOVA followed by LSD multiple range test).

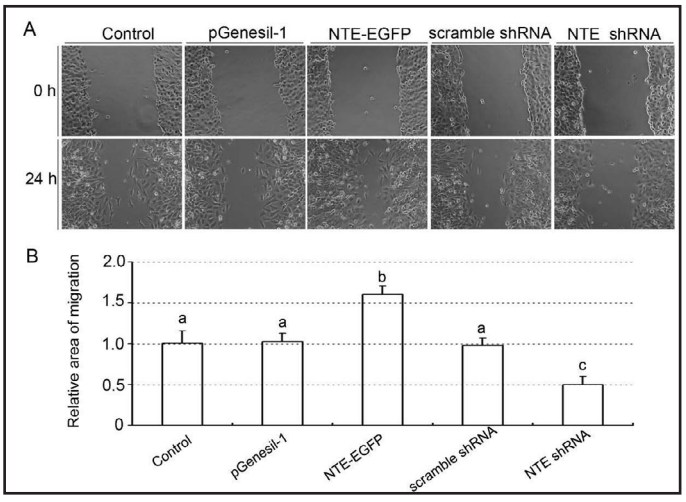

Fig. 4. The effect of NTE on HTR-8/SVneo cells migration. (A) Scratch wound assays of HTR-8/SVneo cells after transfection. Serial photographs of HTR-8/SVneo cells were taken under the light microscope at 0 h and 24 h after the wounds were made. Original magnification: ×200. (B) Relative areas of HTR-8/SVneo cells migration. Results are shown as mean±SD of five independent experiments. Groups with different superscript letters are significantly different (ANOVA followed by LSD multiple range test).

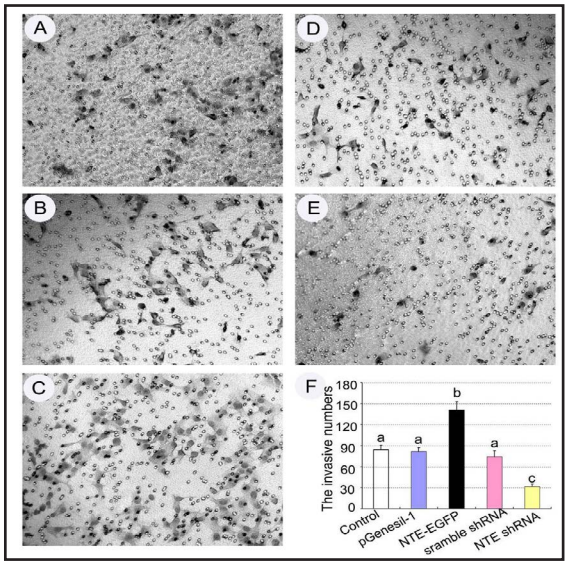

Fig. 5. The effect of NTE on HTR-8/SVneo cells invasion. (A-E) The representative microscope images of invasive HTR-8/SVneo cells 24 h post-transfection with Control (A), pGenesil-1(B), NTE-EGFP(C), and scrambled shRNA (D) and NTE shRNA (E). Original magnification: ×200. (F) The invasive numbers of HTR-8/SVneo cells. Results are shown as mean±SD of five independent experiments. Groups with different superscript letters are significantly different (ANOVA followed by LSD multiple range test).

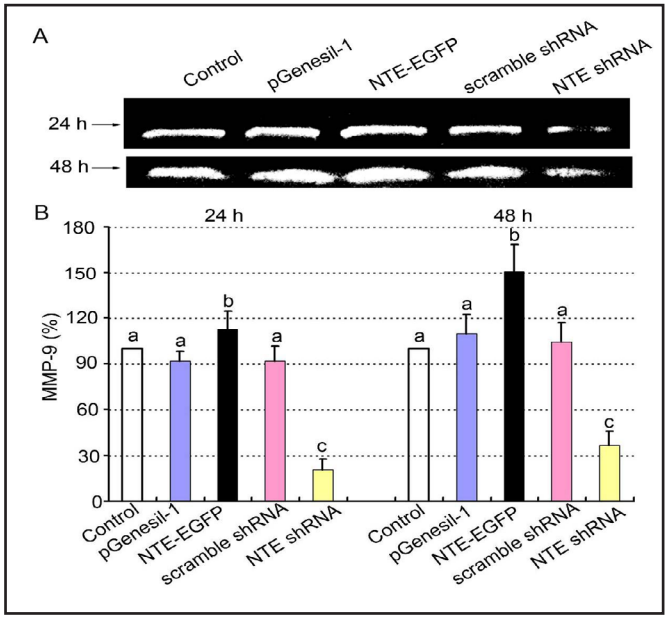

Fig. 6. The effect of NTE on secreted MMP-9 levels. (A)Representative gelatin zymography of supernatants pooled from HTR-8/SVneo cells after transfection 24h and 48 h. Proteolytic activity was noted for the 92-kDa gelatinases corresponding to MMP-9. (B) Relative densitometric analysis of MMP-9 in the HTR-8/SVneo cells. Results are shown as the mean ± SD fold change compared to control group of three independent experiments. Groups with different superscript letters are significantly different (ANOVA followed by LSD multiple range test).

Effect of NTE on migration and invasion of HTR-8/SVneo Cells

HTR-8/SVneo Cells were chosen as model to investigate cell migration. In scratch wound migration (Fig. 4) and Matrigel invasion assays (Fig. 5), we found that over-expression of NTE significantly increased the migration and invasion of HTR-8/SVneo cells (all $P< 0.01$). In contrast, knockdown of NTE markedly decreased the migration and invasion of HTR-8/SVneo cells (all $P< 0.01$). We observed no significant differences among control, empty vector, and scrambled shRNA cells.

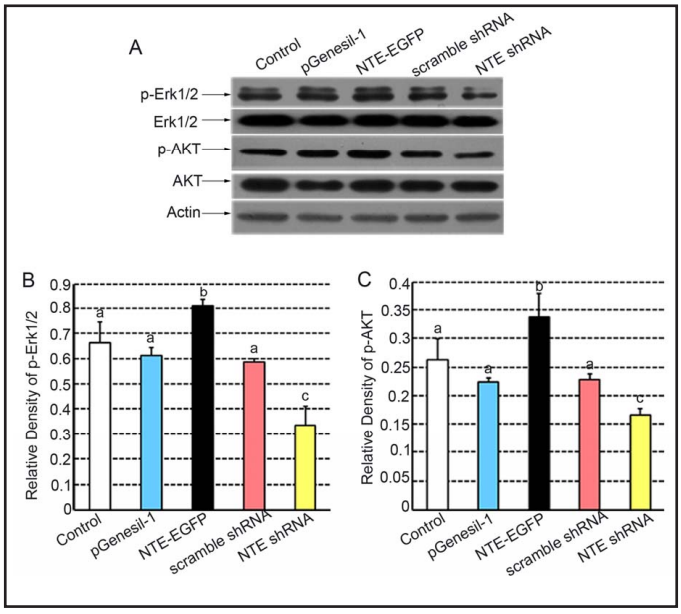

Fig. 7. The effect of NTE on the phospho-Erk 1/2 and AKT levels. (A)Representative immunoblotting results of phospho-Erk1/2 and phospho-AKT protein. (B) Densitometric analyses of phospho-Erk1/2 / total Erk1/2. (C) Densitometric analyses of phospho-AKT / total AKT. The results were shown as the means± SD of three independent experiments. Groups with different superscript letters are significantly different (ANOVA followed by LSD multiple range test).

The effect of NTE on secreted MMP-2, 9 levels of HTR-8/SVneo Cells

Gelatin zymography was performed to detect the gelatinase MMP-2 and MMP-9 activity (Fig. 6). The results revealed that over-expression of NTE significantly increased activity of MMP-9. Conversely, knockdown of NTE markedly inhibited the production of MMP-9 at 24 h and 48 h (Fig. 6). MMP-2 levels did not evidently change in supernatants of over-expression or knockdown HTR-8/SVneo Cells compared to control at 24 h and 48 h (data not shown).

The effect of NTE on phosphorylation of Erk1/2 and AKT

NTE over-expression of HTR-8/SVneo cells drastically increased phospho-Erk1/2 and phospho-AKT levels (Fig. 7). In contrast, knockdown of NTE significantly decreased levels of phospho-Erk1/2 and phospho-AKT compared to control (Fig. 7).

Discussion

The current study is, to the best of our knowledge, the first to examine the expression and localization of NTE in the placenta from women with PE and normal pregnancy. qPCR results showed that expression level of NTE mRNA in preeclamptic placenta was significantly lower than that of the normal sample. Immunostaining assay from normal pregnant samples showed that NTE protein was strongly expressed in cytotrophoblasts and syncytiotrophoblasts of placental villi, and moderately expressed in endothelial cells of placental blood vessels. NTE was firstly detected in homogenates of placental tissue by colorimetric assay of esterase activity with phenyl valerate (a non-physiological substrate) [13]. Subsequently, Gurba et al.'s study showed that human placental NTE is similar to forms found in avian and human brain, and placenta may be an ideal source of material for further purification and characterization of NTE [14]. In addition, LacZ staining also revealed that NTE was strongly expressed in the mice extraembryonic ectoderm of chorion and the ectoplacental cone at E7.5. From E8.5 onward, NTE expression was observed in many tissues

throughout the embryo [12, 15]. The distribution of NTE in tissue suggests that NTE protein may have, in addition to potential vital roles in the nervous system, more-general functions.

The invasion of placental trophoblast cells into the maternal decidua is a key process in the establishment of successful pregnancy [22, 23]. Following trophoblast invasion, the placenta is anchored in the uterine wall, and extravillous trophoblasts of placenta further remodel the uterine spiral arteries to form low-resistance vessels, which is vital to ensure an adequate blood supply for optimal fetal growth [22, 24]. Excess invasion of trophoblast may result in chorionic epithelial carcinoma, whereas insufficient invasion of trophoblast may result in PE, early abortion, and intrauterine growth retardation [1, 15]. Our results showed that over-expression of NTE significantly increased the migration and invasion of HTR-8/SVneo cells. Conversely, knockdown of NTE markedly decreased the migration and invasion of HTR-8/SVneo cells. Moreover, Moser et al. reported that NTE was required for placenta formation, and trophoblast giant cells of ectoplacental cone showed a significantly delayed and reduced invasion in NTE-null mice [15]. This is consistent with our results. Taken together, these evidence suggested that down-regulation of NTE in preeclamptic placenta will result in disorder of trophoblast differentiation and inhibit invasion of human trophoblast cell.

Some studies have indicated that NTE has potent lysophospholipase B activity and deacylates PC to GPC, and regulates the homeostasis of membrane PC in mammalian cells [7, 25]. Our study suggested that down-regulation of NTE in preeclamptic women would result in up-regulation of membrane PC levels and down-regulation of choline in human placenta, which could be related to the toxicity trophoblast cells. Furthermore, epidemic studies showed that the levels of placental PC, PS and triglyceride were significantly increased in preeclamptic pregnancy compared to normal pregnancy [20, 26]. Kobayashi et al. also reported that eclamptic seizures may be induced when patients with severe PE were administrated by an anticholinergic (scopolamine butylbromide), blocking the function of choline [27]. Omatsu et al. demonstrated that PS/PC microvesicles can induce preeclampsia-like changes in pregnant mice. Additionally, mice injected with PS/PC showed a significant elevation in SBP, an increase in proteinuria, and a significant reduction in fetal weight and placental weight compared with controls [19]. However, the limitations of our study did not examine the levels of placental PC and analyze the correlation of NTE and PC in the placenta. Accordingly, the confirmation of our postulated mechanism requires further investigation.

During placentation and early pregnancy, trophoblast invasion are closely related to the expression of matrix metalloproteinase (MMP) [28-30], which are capable of degrading extracellular matrix. Among members of MMP family, MMP-2 and MMP-9 were suggested to play an important role in the regulation of trophoblast invasion [31, 32]. Our results showed that reduction of NTE markedly inhibited the production of MMP-9 of HTR-8/SVneo Cells, which was in correspondence with the change of trophoblast cells migration and invasion. However, the underlining mechanisms of how NTE regulates the secretion and activity of MMP-9 require further study. Recent studies have reported that CDX1/2 restricts the invasion of HTR-8/SVneo trophoblast cells by inhibiting MMP-9 expression independently of the PI3K/AKT pathway [33, 34]. Our study showed that knockdown of NTE significantly decreased levels of phospho-Erk1/2 and phospho-AKT compared to control in HTR8/SVneo cells. Prast et al. also demonstrated that hCG promotes trophoblast migration and invasion of SGHPL-5 cells and extravillous trophoblasts through activation of ERK and AKT signaling involving their downstream effector MMP-2 [35]. These results suggested that growth factors at maternal-fetal interface were involved in the invasion of trophoblast cells via different matrix metalloproteinase and cell signaling pathway.

Collectively, our results demonstrated that NTE mRNA and protein expression was down-regulated in preeclamptic placentas. Furthermore, decreased NTE may contribute to the development of PE through impairing trophoblast invasion by down-regulating MMP-9 via the Erk1/2 and AKT signaling pathway. The research provides a possible pathological mechanism of NTE in the inducing PE. Thus, NTE is a potential target for PE therapy and prevention.

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Disclosure Statement

The authors declared that there are no conflicts of interests of this work.

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