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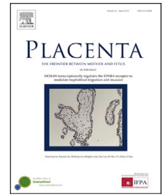

Increased NDRG1 expression attenuate trophoblast invasion through ERK/MMP-9 pathway in preeclampsia

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ABSTRACT

Objective: The aim of this study was to investigate the expression of *N*-myc downstream-regulated gene1(NDRG1)in the placentas of pregnancies complicated with early-onset and late-onset preeclampsia (PE) and its underlying mechanism on the pathophysiology of PE.
Methods: The expressions of NDRG-1 in placentas of pregnancies complicated with early-onset PE and late-onset PE were detected using immunohistochemistry, western blot assays and fluorescence quantitative PCR. The expressions of MMP-2, MMP-9 and ERK1/2 protein were detected by western blot analysis and cell invasion assay was performed using transwell chambers in NDRG1 silenced JEG-3 cells.
Results: Compared with the normal term pregnancies, the expression of both NDRG1 mRNA and protein were significantly high in placentas from PE, and the expression of NDRG1 in early-onset PE was higher than that in late-onset PE. In NDRG1-silenced JEG-3 cells, MMP-2, MMP-9 and phosphorylation of ERK1/2 protein increased obviously and the number of cells that penetrated the membrane increased.
Conclusion: Upregulation of NDRG1 is associated with impaired trophoblast invasion in PE by inhibition ERK/MMP-2 and MMP-9 Pathway.

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1. Introduction

Preeclampsia (PE) is a pregnancy-specific systemic complication, characterized by the new onset hypertension, edema and proteinuria after 20 weeks of gestation. According to the gestational age by which it develops, PE can be classified as early-onset (before 34 weeks of gestation) or late-onset (at or after 34 weeks of gestation) [1]. Even though unremitting efforts have been taken for many years, there is no consensus about the exact etiology and pathogenesis of PE. Therefore, PE remains high incidence and a major cause of maternal and neonatal mortality and morbidity worldwide [2–4]. The pathophysiology of PE is characterized by impaired trophoblast invasion of decidua spiral arteries, which subsequently leads to inadequate uteroplacental blood flow, injury to trophoblast tissue, and maternal multisystem dysfunction [5]. Trophoblast differentiation and apoptosis are regulated by several genes and *N*-myc downstream-regulated gene is involved [6].

N-myc downstream-regulated gene belongs to a family of proteins (NDRG1-4) implicate in many cellular processes, including differentiation, proliferation and invasion [7]. Diverse physiologic or pathologic conditions, such as hypoxia, cellular differentiation, exposure to heavy metal and neoplasia, modulate NDRG1 expression [8–10]. NDRG1 expression in tumor cells is directly involved in the regulation of invasion and downregulation of NDRG1 can be enhanced *in vitro* invasion [7]. NDRG1 has been identified to express in the placenta. In the second- and third-trimester placenta, NDRG1 mRNA is expressed predominantly in syncytiotrophoblast. Recent studies [11,12] have shown the expression of NDRG1 in trophoblasts is upregulated under hypoxic conditions, such as intrauterine growth restriction. Nevertheless, the expression of NDRG1 in placenta tissue of early-onset or late-onset PE and the associated regulatory mechanisms remain unknown. Therefore, the aim of the present study was to investigate the expression and mechanism of NDRG1 in the placentas of early-onset and late -onset PE.

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2. Materials and methods

2.1. Patient information and placenta collection

The ethics committee of Affiliated Hospital of Qingdao University approved the present protocol of the study. The experimental groups were shown as follows. Control group (n = 20) included normal singleton term pregnancies. Early gestation group (n = 20) included healthy women undergoing legal abortion for nonmedical reasons. Premature delivery group (n = 20) included 24–37 years old women with gestational age less than 37 weeks without other complications. PE group included 40 individuals with PE between the ages of 25–44 years who were recruited from September 2014 to March 2015. The diagnosis of the PE was based on clinical evidence. According to onset time, they were divided into two subgroups, early-onset PE (early PE, n = 20), and late-onset PE (late PE, n = 20). Women were excluded from the study who were smokers, drinkers, chronic hypertension, gestational diabetes mellitus, eclampsia, or women carrying multiple pregnancies. Each patient signed the informed consent.

Placentas were collected immediately after a vaginal or abdominal delivery under sterile conditions. After removing the decidual layer, three pieces of tissue (each about $1.0 \times 1.0 \times 1.0$ cm^3) were obtained from each placenta, and rinsed several times with ice-cold phosphate-buffered saline (PBS) to remove excess of blood cells. Two samples were immediately placed into cryotubes and stored at −80 °C refrigerator for later analysis, and the other piece of tissue was fixed in 4% paraformaldehyde for immunohistochemistry. Villous tissues were collected after artificial abortion, with the same treatment method as the placentas.

2.2. Cell line

The behavior of trophoblast invasion is similar to tumor cell, so JEG-3 was used in the study. JEG-3 was obtained from the cell bank at the Chinese Academy of Sciences (Shanghai, China) and cultured in minimum essential medium (MEM, Gibco, USA), containing 10% (v/v) fetal bovine serum (FBS, HyClone, USA), and 1% penicillin/streptomycin (Gibco, USA), maintained at 37 °C in a humidified atmosphere of 5% CO_2.

JEG-3 cells were seeded in 6-well plates and allowed to attach overnight to reach 30%–50% confluence at the time of transfection. To suppress native NDRG1 expression, transfections were performed with 50 nM NDRG1 siRNA for 72 h according to the manufacturer's instructions (Ribobio Life Technologies, Guangzhou, China), same volumes sterile PBS were added as the control cells.

2.3. Immunohistochemistry (IHC) analysis

Immunohistochemical staining for all paraffin-embedded placental tissues were performed by the streptavidin ebiotin-peroxidase complex method. Briefly, 5-μm thick sections were prepared and incubated with primary antibody (NDRG1,1:150, Rabbit anti human monoclonal antibody, Sigma) at 4 °C overnight, then incubated with the secondary antibody (Gene Tech, Shanghai, China) for 30 min at 37 °C. After rinsing in PBS, the sections were incubated with 3,3'-diaminobenzidine liquid (DAB, ZSGB-BIO, Beijing, China), counterstained with Mayer's hematoxylin, then dehydrated. Immunoreactivity was evaluated independently by two investigators who were blinded to experimental protocol according to the intensity and extent of staining. The Image-Pro Plus software (version 6.0; Media Cybernetics) was used to for densitometry analysis.

2.4. Fluorescence quantitative PCR analysis

The total RNA was extracted from the tissue of the placenta using Trizol regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) from 2 μg of total RNA. Fluorescence quantitative PCR was performed using the TaKaRa SYBR *Premix Ex Taq* II kit by ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR primers were as follows: NDRG1, 5′-GAGGCTAGAGGCATTTGGAA-3′ (Forward),5′-CTTTTGCTGCACATTAAGAGGA-3' (Reverse), β-actin,5′-ATAGTTGCG TTACACCCTTTCTTG-3' (Forward), 5′–TCACCTTCACCGTTCCAGTTT-3(Reverse). A total of 2 μl of cDNA were mixed with 1.6 μl primers, 10 μl SYBR Premix Ex *Taq* II and 0.4 μl ROX Reference Dye II. Initial denaturation was performed at 95 °C for10min, followed by 40 cycles of denaturation at 95 °C for 30s, then annealing at 60 °C for 30s and extension at 68 °C for 30s. A melting curve for primer validation and a template standard curve were performed to show template independent amplification results. Relative mRNA levels were calculated using the $2^{-\triangle\triangle Ct}$ method and normalized against β-actin.

2.5. Western blot analysis

Frozen placental tissues were homogenized in lysis buffer containing: 20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1% Triton-X100, 20 mM leupeptin, 10% glycerol, 0.1% SDS, 0.5% deoxycholate and 5 mg/ml aprotinin. To prepare whole-cell extracts, JEG-3 cells were washed in PBS before incubation with RIPA lysis buffer. The insoluble material was removed by centrifugation at 15 000 g at 4 °C for 15 min and the protein concentration of the supernatant was measured using the Bradford method of protein quantitation by spectrophotometry at 595 nm (Beckman DU530). Equivalent amounts of protein from each lysate sample were mixed with protein loading buffer, heated at 95 °C for 5 min. The protein samples (20 μg) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and then electrotransferred to a nitrocellulose membrane. After being blocked with 5% nonfat milk for 1 h at room temperature, the membranes were incubated with primary antibodies (1: 2000, Abcam) against NDRG1, MMP2, MMP9, ERK1/2 and PERK, GAPDH (1:2500 Abcam) at 4 °C overnight. The membranes were washed and incubated with monoclonal HRP-conjugated antibody for 1 h at room temperature. The immunoreactive bands were detected by using enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL, USA).

2.6. Cell invasion assay

Cell invasion assay was performed using transwell chambers precoated with Matrigel (BD, USA). MEM with 10% FBS was added into the lower chamber. First JEG-3 cells were pre-cultured with or without NDRG1 siRNA (50 nM) for 72 h. Then the cells were washed with PBS and suspended in MEM. 200 μl cell suspension (1×10^5 cells/ml) was added into the upper chamber. After culturing at 37 °C for 24 h, the upper cells that did not invade through the membrane were wiped out by a cotton tipped swab. The filters were fixed in methanol and stained with DAPI (4′,6 -diamidino-2-phenylindole). The number of invasive cells whose nuclei were stained in blue were counted under fluorescence microscopic. The experiment was performed three times.

2.7. Statistical analysis

The data were analyzed by statistical package SPSS22.0. Data are presented as mean ± SD. Statistical analysis was performed using

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