MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 5294-5301
DOI: 10.12659/MSM.903602

Received: 2017.02.03
Accepted: 2017.05.17
Published: 2017.11.07

Promoter Methylation Status of *WNT2* in Placenta from Patients with Preeclampsia

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Funds Collection G

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Source of support: The Natural Science Foundation of Shandong Province (No. 15 ZR2015HM006 2015)

Background: Preeclampsia is a serious multisystem disorder of human gestation, affecting up to 10% of pregnant women worldwide, and results in maternal morbidity and mortality. The aim of this study was to determine the gene expression pattern and methylation status of the promoter of the *WNT2* gene in placentas from patients with preeclampsia and to evaluate the potential role of the *WNT2* pathway in the pathogenesis of preeclampsia.

Material/Methods: Real-time quantitative polymerase chain reaction (PT-PCR) was used to determine the *WNT2* gene expression level. Western blot analysis was used to identify alterations in wnt2 protein expression.

Results: The mRNA and protein expression levels of the *WNT2* gene were reduced in placentas from patients with preeclampsia when compared with placentas from healthy women. The average methylation level of the promoter of the *WNT2* gene was elevated in the placentas from patients with preeclampsia compared with the controls placentas from healthy women.

Conclusions: The findings of this study have shown that molecular mechanisms, including aberrant activation of the *WNT2* gene signaling pathway, may be involved in the pathogenesis of preeclampsia. Promoter hypermethylation and reduced expression of the *WNT2* gene requires further study to determine a potential role in the diagnosis and treatment of preeclampsia.

MeSH Keywords: Blotting, Far-Western • DNA Methylation • Pre-Eclampsia • Wnt2 Protein

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/903602

3277 2 2 39

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Background

Preeclampsia is a serious multisystem disorder of human gestation, affecting up to 10% of pregnant women worldwide, and results in maternal morbidity and mortality [1,2]. Preeclampsia is characterized by gestational hypertension as well as proteinuria and some patients present with renal failure, liver dysfunction, and neurological manifestations such as seizures [3]. Preeclampsia may progress to eclampsia due to late presentation, delayed diagnosis, and delayed treatment. Conventional monitoring for preeclampsia is performed by blood pressure measurement, urine protein detection, and clinical observation [4]. Therefore, there is a need to identify reliable hallmarks for the early diagnosis of preeclampsia and to develop efficient treatment strategies.

Because preeclampsia occurs in pregnancy, where the placenta is the an important component of the maternal-fetal interface, and clinical symptoms of preeclampsia rapidly abate after the placenta is delivered, the placenta may play a crucial role in the etiology of this preeclampsia [5]. Extravillous trophoblast cells, the major cell type in the placenta, acquire tumor-like capacities allowing cells to invade the endometrium and myometrium during early placentation, and are involved in remodeling of the maternal spiral artery during pregnancy [5,6]. Preeclampsia is associated with placental ischemia caused by trophoblast invasion and failure to fully develop uteroplacental spiral arteries [7,8].

Recent studies have shown that the *WNT2* gene signaling pathway in placentation and placental development in first trimester trophoblasts may be associated with promoter hypermethylation [9]. The *WNT2* gene signaling pathway is also implicated in extraembryonic development, placental vasculogenesis, chorioallantoic fusion, and placental function [10,11]. Preclinical studies in mice have shown that the Wnt receptor gene *FZD5* is essential for yolk sac and placental angiogenesis [11]. In 2010, Sonderegger and coworkers reviewed the role of the Wnt signaling pathway in modulating placental function and trophoblast differentiation during human reproduction [12]. Multiple members of the Wnt pathway have been shown to be dysregulated in recurrent miscarriage, and endometriosis [13–15]. Aberrant expression in β-catenin and Dickkopf-1 has been reported to be involved in recurrent spontaneous abortion [13]. 17 β-estradiol (E2) drives β-catenin, triggers up-regulation of VEGF and estrogen receptor α (ERα) in endometriosis through activating Wnt/β-catenin signaling [15]. Therefore dysregulated Wnt/β-catenin signaling pathway may be implicated in the pathogenesis of recurrent abortion and endometriosis.

A secreted Wnt ligand is a glycoprotein that has several key roles in the development different forms of malignancy, including in angiogenesis [16,17]. Exogenous Wnt2 confers the property of angiogenesis in Wnt2-deficient endothelial cells in vascularized tumors and is expressed in healing wounds [18]. In 1996, Monkley and colleagues showed that targeted disruption of the *WNT2* gene in mice resulted in placentation defects, including reduced birth weight and increased perinatal mortality [10]. Additionally, mutation in the *WNT2* gene resulted in reduced fetal capillaries and increased fibrinoid vascular change, as well as abnormal placental phenotype associated with defective placental angiogenesis [10].

Several studies have provided evidence that the pathogenesis of preeclampsia is closely associated with the methylation status of relevant genes [19–21]. DNA methylation covalently adds a methyl group to a cytosine, usually in the context of cytosine-phospho-guanine dinucleotides (CpG) [22]. Previous studies have shown that epigenetic modification, including by methylation, contributes to the modulation of all gene expression by changing the accessibility of DNA to the actuators of gene activation such as transcription factors [22,23]. We speculated that the alteration in the methylation status of the *WNT2* gene promoter in the placental tissues might play important roles in the variation of the *WNT2* gene expression.

The aim of this study was to examine the hypothesis that methylation of the promoter of the *WNT2* gene in the placenta from patients with preeclampsia, when compared with placentas from normal pregnancies, could contribute to the dysregulation of *WNT2* gene expression. To test this hypothesis, we assessed the expression level of the *WNT2* gene in placental tissues from patients with preeclampsia and from normal pregnancies.

Material and Methods

Patienst studied

This study was conducted in the Department of Gynaecology and Obstetrics, Binzhou Medical University Hospital, Binzhou, Shandong, China, between March 2013 and September 2016. Pregnant women, aged from 24 to 33 years (n=16) who were diagnosed with preeclampsia, were enrolled in the study if they fulfilled the following criteria: (i) women had a blood pressure ≥140 mmHg systolic and/or ≥90 mmHg diastolic after 20 wk gestation on two occasions at least 4 h apart; (ii) proteinuria 1+ or ≥300 mg in 24 h or renal insufficiency (serum creatinine >0.09 mmol/l). Women without hypertension and without hypertension-related complications that presented for delivery at term (≥37 weeks gestation) were also studied as normal controls (n=20). All pregnancies with factors that may influence the methylation patterns, including prenatal smoking, drinking, long-term addiction to medicines, and assisted reproduction were excluded from this study. Pregnancies with chronic

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Table 1. Primers used in this study.

	Forward	Reverse
Wnt 2	5'-CGCATTTGTGGATGCAAAGGA-3'	5'-TCGCCCGTTTTCCTGAAGTCG-3'
GAPDH	5'-GCCTGGAGAAAGCTGCTAAGTA-3'	5'-CGTTGTCATACCAGGAAATGAG-3'

hypertension, renal disease, or pregnancy complications such as gestational diabetes were also excluded.

Written informed consents were provided by all the included patients. The study design, sample collection, as well as investigations were approved by the local ethics committee of Binzhou Medical University Hospital. This study conformed to the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) statement developed by the Enhancing the QUAlity and Transparency Of health Research (EQUATOR) network [22].

Sample collection

Immediately after delivery from the uterus (within 30 min), placental villous tissue samples (0.5×0.5 cm) were collected from the maternal side of the placenta at a depth of 5 mm beneath the outermost layer of the placenta, near the insertion of the umbilical cord. These samples were washed in sterile phosphate buffered saline (PBS) to remove the maternal blood, snap frozen in liquid nitrogen, and stored at –80°C for later use.

DNA isolation and methylation-specific polymerase chain reaction (MSP)

Genomic DNA was isolated from lysed placental villous samples using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. DNA concentration was checked using a Nanodrop spectrophotometer, while evaluating the quality of the DNA product. DNA samples with an $OD_{260/230}$ ratio between 1.8 and 2.0 were assumed to be of good quality. To assess the methylation status of the *WNT2* gene promoter in placental tissue, methylation-specific polymerase chain reaction (MSP) assay was performed. Briefly, human placental genomic DNA treated with SssI methyltransferase (M.SssI) (New England Bio-labs, MA, USA) was used as a 100% methylation standard, whereas an EpiTect® control DNA, purchased from Qiagen, was used as a 0% methylation standard. These methylation standards, and isolated genomic DNA from each patient were bisulfite-converted using the EZ DNA Methylation-Gold Kit (Zymo Research Corporation, Irvine, CA, USA) according to supplier's protocol.

The polymerase chain reaction (PCR) was performed using HotStart PCR enzymes (TAKARA, JAPAN) and carried out for 35 cycles consisting of denaturation at 98°C for 15 sec, annealing at 60°C for 15 sec, and extension at 72°C for 45 sec, followed by a final 3-min extension at 72°C for all primer sets.

RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from placentas by using the Trizol reagent (TaKaRa, Japan) according to the manufacturer's instructions, and subsequently digested with RNase free DNase I. After the concentration and purity of the RNA were evaluated, the cDNA was generated by reverse transcription using 1 µg RNA as template. Subsequently, quantitative real-time polymerase chain reaction (RT- PCR) was carried out in a Mx3000PTM Real-Time PCR System (Stratagene, La Jolla, CA, USA) with SYBR Green. The relative mRNA expression levels of the *WNT2* gene were evaluated by using the 2–ΔΔCt method and normalized to an internal control GAPDH.

The sequences of forward and reverse primers (synthesized at Sangon Biotech, Shanghai, China) used in this study are listed in Table 1.

The amplification profile was one cycle at 95°C for 5 min, followed by 38 cycles (36 cycles for GAPDH) of denaturation 98°C for 10 s, hybridization annealing at 62°C (60 °C for GAPDH) for 30 s, and extension at 72°C for 45 s, followed by one extension cycle of 10 min at 72°C. The mRNA levels were determined by measuring absorbance value using 'Quantity one 4.6' software. Experiments were performed in triplicate.

Western blot assay

Total proteins were extracted from placental samples cells by homogenizing on ice in lysis buffer containing 10 mM Tris–HCl (pH 6.8), 100 mM NaCl, 2 mM $MgCl_2$, 4% SDS, 15% (v/v) glycerol, 0.2 M dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were centrifuged at 4°C (14,000 g, 5 min) before the supernatant was collected. Protein extracts (50 µg per lane) were then subjected to 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes at 60 V for 1 h. After blocking the membranes with 5% skimmed milk in Tween 20 and tris buffered saline (TBST), the target protein was probed with the goat anti–human Wnt2 primary antibody (diluted at 1: 1000; Santa Cruz Biotech. Inc., Santa Cruz, CA) and incubated overnight at 4°C, rinsed with PBST for three times (10 min

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Table 2. Clinical data of pregnancies complicated with PE and uncomplicated peers.

	PE (n=16)	Normal (n=20)
Maternal age (years)	29.50±4.02	29.0±3.09
Gestational age at recruitment (weeks)	35.4±0.7	36.0±1.1
Gestational age at delivery (weeks)	36.8±0.9	37.2±0.7
BMI (kg/m^2)	27.6±0.2	26.8±0.8
Gravidity (median, range)	2 (1–4)	2 (1–3)
Multiparity, n (%)	5 (31%)	7 (35%)
SBP (mm Hg)	159.7±2.7	109.3±1.4
DBP (mm Hg)	101.8±2.0	72.8±1.3
Proteinuria* (g/24 h)	2.67±1.04	0.26±0.29
Fetal gender (male/female)	9/7	11/9
Neonatal birthweight (kg)	2.5±0.1	2.8±0.9
Apgar score	8.8±0.2	9.0 ±0.2

per time), and incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG secondary antibody (1: 3000, Beijing Solarbio Science & Technology Co., Ltd. Beijing, China) at room temperature for 1 hour. Then, the membrane was washed with PBST for three times (10 min per time), followed by developed using enhanced chemiluminescence (ECL) reagents (Millipore, Merck KGaA, Germany). The membrane was then rinsed in stripping buffer (62.5 mM Tris-HCl, 100 mM 2-mercaptoethonal, 2% SDS, pH 6.7) to remove the bound antibodies and re-incubated with mouse monoclonal primary antibody against human beta-actin (1: 600, Santa Cruz Biotech. Inc) and goat anti-mouse IgG (1: 2000) Beijing Solarbio Science & Technology Co., Ltd).

Statistical analysis

All experimental data were presented as the mean ± standard deviation (SD). Significance between various samples was calculated using a two-tailed Student's t-test. P-values <0.05 were interpreted as statistically significant. All statistical analysis were performed with the SPSS 13.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the preeclamptic and normal pregnancies

The clinical data of 36 pregnancies (16 patients with preeclampsia; 20 uncomplicated pregnancies) were retrospectively analyzed (Table 2). There was an increase in the systolic blood pressure of the patients with preeclampsia when compared with the healthy controls (159.7±2.7 *vs.* 109.3±1.4 mm Hg, p<0.05). The diastolic blood pressure of the patients with preeclampsia was substantially greater when compared with the healthy control patients (101.8±2.0 *vs.* 72.8±1.3 mm Hg, p<0.05). Pregnant women with preeclampsia had an elevated urinary protein level compared with the normal controls (2.67±1.04 *vs.* 0.26±0.29 g/24 h, p<0.05). There was no significant difference in the number of previous pregnancies, maternal body mass index (BMI), maternal age, gestational age at delivery, gender and birth weight of the newborn babies, and neonatal Apgar score between the two groups. Patients with preeclampsia were treated with magnesium sulfate in order to reduce the risk of developing eclampsia. Steroids were used to accelerate the antenatal development of premature babies.

Decreased *WNT2* mRNA expression in the placentas of patients with preeclampsia

To investigate whether the expression of the *WNT2* gene was altered in patients with preeclampsia, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to characterize the *WNT2* mRNA expression patterns in different placental tissues from both patients with preeclampsia and healthy cohorts. Highly expressed endogenous *WNT2* gene was found in normal human placentas, with a decrease in *WNT2* gene expression in patients with preeclampsia when compared to the normotensive controls (Figure 1A, 0.92±0.12 *vs.* 2.28±0.17, P<0.05). This finding suggests that down-regulation of the *WNT2* gene is correlated with the development of preeclampsia, irrespective of the clinical characteristics of the pregnancies including age, BMI, gravidity, or fetal phenotypes, such as gender and birth weight.

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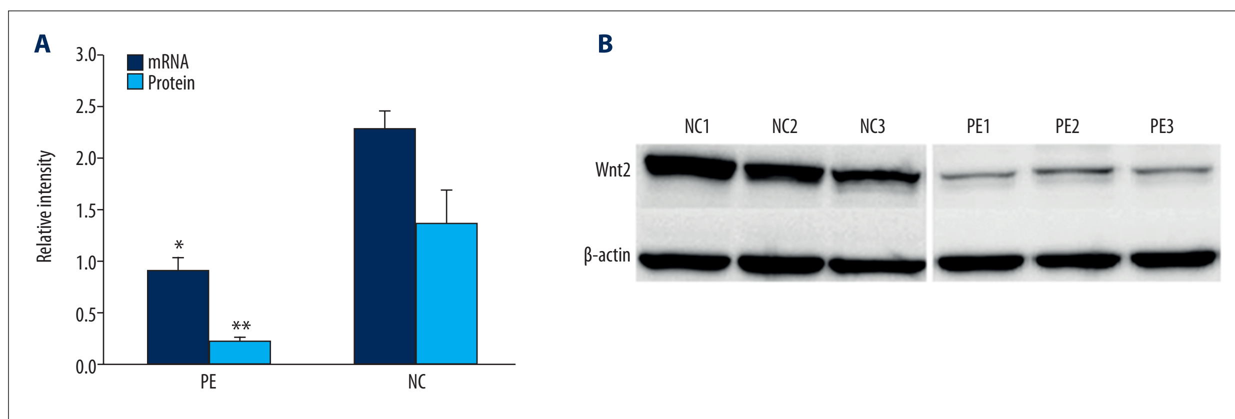

Figure 1. The expression of the *WNT2* gene was significantly lower in the placentas from pregnancies with preeclampsia (PE) compared with the normal control pregnancies (NC). (**A**) The relative mRNA and protein levels of the *WNT2* gene in placentas were respectively measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot assay and normalized to GAPDH. All of the experiments were performed in triplicate. * P<0.05. (**B**) Representative Western blot analysis of Wnt2 protein expression in NC and PE.

Decreased Wnt2 protein expression in patients with preeclampsia

The findings from RT-PCR were further validated by assessing Wnt2 protein expression levels using the Western blot assay. As shown in Fig. 1B, the Wnt2 protein with a molecular weight of 34 kDa was strongly expressed in the normal placentas, while protein expression was reduced in the placental tissues from preeclamptic pregnancies (Figure 1A, 1B) (0.23±0.03 *vs.* 1.37±0.32, p<0.01). This finding was in accordance with the qRT-PCR results that showed reduced *WNT2* mRNA expression. The decreased placental expression of the Wnt2 protein in patients with preeclampsia suggested a possible association with the pathogenesis of preeclampsia.

Expression of the *WNT2* gene promoter in placenta from patients with preeclampsia

We assessed the overall methylation status of the *WNT2* gene promoter separately in the placentas from patients with preeclampsia and matched normal controls by using DNA isolation and methylation-specific polymerase chain reaction (MSP) as a method. The degree of methylation of the *WNT2* gene promoter was elevated in the placentas from patients with preeclampsia, compared with the placentas from normal controls (0.576±0.190 *vs.* 0.238±0.112, p<0.05 (Figure 2).

These data indicated that *WNT2* gene expression was reduced, but an approximately 2.5-fold increase in the degree of promoter methylation was found in placental specimens from patients with preeclampsia relative to the normotensive individuals. Therefore, there was a potential inverse relationship between the overall the *WNT2* gene promoter methylation status and gene expression in preeclampsia.

Discussion

The canonical Wnt/beta-catenin signaling pathway is a conserved pathway composed of cell-surface receptors, multiple secreted ligands, and intracellular effectors that comprise an extremely interactive network. The Wnt pathway governs multiple physiological and pathological processes including proliferation, differentiation, apoptosis, homeostasis, and cell migration [24,25]. Moreover, abnormal activation of Wnt pathway is responsible for the development and progression of several forms of human malignancy [26]. The majority of the 19 known Wnt ligands and ten human frizzled (FZD) receptors have been discovered in human placenta and expressed in a gestation-dependent manner, suggestive of a significant role for Wnt signaling pathways in early placental function and differentiation [27]. Furthermore, primary extravillous trophoblast strongly expresses not only canonical, but also non-canonical Wnt, which suggests a c role of different Wnt signal transduction cascades in trophoblast maturation [28].

The *WNT2* gene was originally isolated by Wainwright and colleagues in 1988 while searching for the defective gene in cystic fibrosis [29]. Since then, several studies have shown its role as an important member of WNT gene family. The human *WNT2* gene is involved in multiple complicated signal transduction processes and, for this reason, its versatile function is still being investigated.

As a glycoprotein binding to the frizzled receptors, the wnt2 protein stimulates downstream gene expression via Wnt/beta-catenin in signaling. In the present study, we provided direct evidence that of a decrease in the expression level of the *WNT2* mRNA in placentas from patients with preeclampsia compared with normotensive counterparts (0.92±0.12 *vs.*

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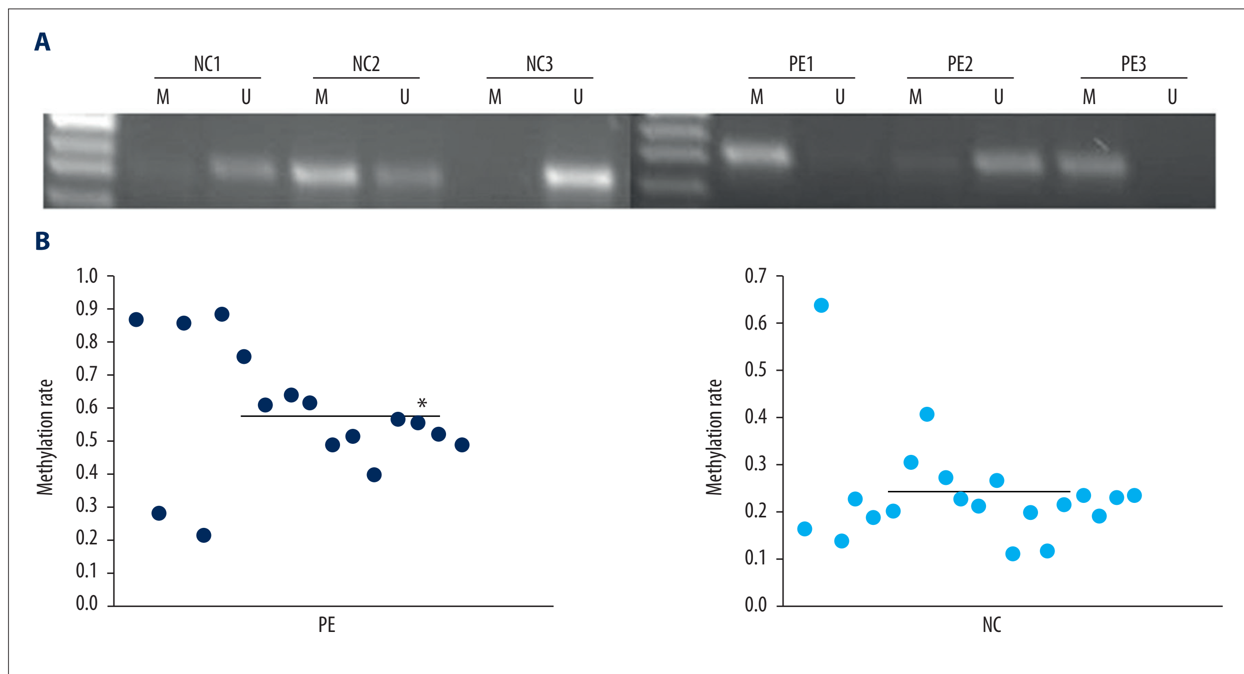

Figure 2. The extent of methylation in the *WNT2* gene promoter region was significantly enhanced in the placentas from patients with preeclampsia compared with the matched control group. (**A**) Methylation-specific polymerase chain reaction (MSP) analysis of the *WNT2* gene promoter in the placentas from patients with preeclampsia (PE) and normal control placentas (NC). NC and PE show the presence of both unmethylated and methylated DNA in the placental tissues, respectively. The bands detected with the methylated primer represent methylated *WNT2* gene (M), and those detected by using the unmethylated primer signify unmethylated *WNT2* gene (U). (**B**) Scatter plot depicting the increased the *WNT2* gene methylation extent in the PE *vs.* NC. The methylation rate was calculated using the following formula: [intensity of methylation/(intensity of methylation +intensity of unmethylation)]. * P<0.05.

2.28±0.17, P<0.05), using quantitative RT-PCR. Also, we used Western blot to show that the development of preeclampsia induced reduced expression levels of the wnt2 protein which otherwise was highly expressed in placentas of normotensive women (0.23±0.03 *vs.* 1.37±0.32, p<0.01). This finding was consistent with previously published studies, where enhanced Wnt2 protein expression was observed during the normal development of the placenta, while the *WNT2* gene expression level was reduced in third-trimester placentas in patients with severe preeclampsia [30,31]. Thus, our results suggested that the reduced expression of the *WNT2* gene was possibly associated with the development of preeclampsia, although the mechanism underlying the role of the *WNT2* gene in the pathogenesis of preeclampsia is unclear.

The etiology of preeclampsia, and similar placenta-related pregnancy complications, are likely to be partly due to modulation of angiogenesis and immune factors at the maternal-fetal interface [31–33]. Women who have a reduced level of endothelial progenitor cells (EPCs) and increased natural killer (NK) cells in peripheral blood are more likely to develop preeclampsia [31]. Also, in the peripheral blood of women who subsequently had spontaneous miscarriage, decreased PAPP-A and CD34+cells have been observed [32]. Aberrant methylation of several angiogenic genes have been found in the placenta, including vascular endothelial growth factor (VEGF) and FMS-related tyrosine kinase 1 (FLT1), and have been shown to be involved in the development of preeclampsia [33]. Mice carrying a *WNT2* gene loss-of-function mutation showed reduced vascularization and structural defects in the placenta, causing the invasion of spiral arteries, which was responsible for perinatal death of the offspring [10]. Moreover, over-expression of the *WNT2* gene in endothelial cells has been shown to facilitate their angiogenic abilities *in vitro*; this finding has been corroborated by *in vivo* results showing that the *WNT2* gene was found to be highly expressed in the densely vascularized malignant murine tumors and in wound healing tissues in proximity to endothelial cells [18]. Taken together with these previous studies, our data may suggest that the *WNT2* gene functions as an angiogenic factor to promote the invasive competency of extravillous trophoblast and maternal spiral artery remodeling via inducing placental vascularization. Consequently, the down-regulation of the *WNT2* gene in placentas potentially contributes to the pathophysiology of preeclampsia. However, this speculation requires verification by further investigation in the context of the effects of exogenous Wnt2 on the invasive properties of

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human extravillous trophoblast cell lines *in vitro*. These studies are in progress in our laboratory.

Epigenetic mechanisms are essential in the regulation of gene expression by altering the accessibility of genomic DNA to transcription factors, thereby influencing gene activities [24,25]. miRNAs can bind to the 3'-untranslational region (3'-UTR) of target mRNAs, thereafter regulating gene expression through translational repression or degradation [24]. Accumulating evidence suggests a pivotal role of miRNAs in a variety of pregnancy-related complications, including preeclampsia and fetal growth restriction [34–36]. However, aberrant epigenetic patterns in human placentas, in particular altered DNA methylation of preeclampsia-susceptible gene promoters, are implicated in the pathophysiology of preeclampsia [37]. Pregnancy complicated by preeclampsia has been shown to be susceptible to placental global hypomethylation, which was responsible for higher systolic and diastolic blood pressure, as well as preterm and term preeclampsia [38]. An increase in the expression of VEGF caused by hypomethylation may act as a compensatory mechanism to restore blood flow as a response to impaired arterial remodeling during development of preeclampsia, as VEGF is considered to be a stimulator for endothelial cell proliferation [39]. We, therefore, propose that the suppressed the *WNT2* gene expression may result from the abnormal methylation status of the *WNT2* gene promoter in the placentas from patients with preeclampsia.

In this study, we performed DNA isolation and methylation-specific polymerase chain reaction (MSP) on placental tissue samples from cases of preeclampsia and normal pregnancies, to examine the placental methylation patterns of the *WNT2* gene promoter. We detected more pronounced methylation of the *WNT2* gene promoter (nearly 2.5-fold higher) in preeclampsia placentas relative to the normal controls. The up-regulation of the *WNT2* gene promoter methylation in preeclampsia was also validated by 450K methylation data obtained from GSE44712 [39]. The enhanced *WNT2* gene promoter methylation levels corresponded to the suppressed the *WNT2* gene expression at both mRNA level and the protein level. These findings suggest that methylation potentially plays a role in the modulation of the *WNT2* gene expression that may lead to trophoblast dysfunction, which will influence trophoblast invasion and spiral artery remodeling, hence the development of preeclampsia.

The results of this study showed that the *WNT2* gene promoter was hypermethylated in placentas from patients with preeclampsia and that the epigenetic variant is related to reduced Wnt2 protein expression, indicative of a role for altered placental methylation profiles in the placental dysfunction in preeclampsia. We have identified the *WNT2* gene as a potential therapeutic target and highlighted its functional consequences in the pathogenesis of preeclampsia. However, *in vitro* and animal studies to further investigate these findings are recommended

Conclusions

The findings of this study have shown that molecular mechanisms, including aberrant activation of the *WNT2* gene signaling pathway, may be involved in the pathogenesis of preeclampsia. Promoter hypermethylation and reduced expression of the *WNT2* gene requires further study to determine a potential role in the diagnosis and treatment of preeclampsia.

Conflict of interests

None.

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