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ORIGINAL ARTICLE

Mesenchymal-to-epithelial transition in the placental tissues of patients with preeclampsia

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During early pregnancy in humans, mesenchymal-to-epithelial transition (MET) contributes to decidualization of endometrial stromal cells in the uterus. Defects in decidualization can interfere with placental formation and can lead to pregnancy complications, including preeclampsia. However, MET markers in preeclamptic placental tissues have not been characterized. To investigate the association between changes in MET and preeclampsia, we evaluated MET markers in preeclamptic placental tissues relative to normal placentas. Placentas were collected from 20 preeclamptic and 20 normotensive healthy women. Protein and mRNA levels of MET-related markers, including E-cadherin, N-cadherin (neural cadherin), vimentin, ZO-1 (zona occludens 1) and SLUG, were analyzed via western blot and quantitative real-time PCR (Q-PCR), respectively. E- and N-cadherin were localized in the placentas through immunohistochemistry. The mRNA and protein expressions of GLI1 and GLI2 were detected by Q-PCR and western blot. In preeclamptic placentas, the mRNA and protein levels of E-cadherin and ZO-1 were elevated relative to the controls, whereas the levels of N-cadherin, SLUG and vimentin were lower. The staining intensities of E- and N-cadherin were consistent with their protein levels detected by western blot. The mRNA and protein levels of GLI1 and GLI2 were significantly lower in preeclamptic placentas compared with that in control placentas. We conclude that MET in the placenta may be associated with the progression of preeclampsia.
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Keywords: GLI1 and GLI2; mesenchymal-to-epithelial transition; preeclampsia

INTRODUCTION

Preeclampsia is a life-threatening disease that affects 5 to 8% of pregnancies. Clinical symptoms include sudden onset of hypertension accompanied by proteinuria, edema and, often, fetal growth restriction.[1] Although the etiology of preeclampsia has not been clearly delineated, it may be that preeclampsia-associated placental defects are partially due to inadequate or incomplete trophoblast cell invasion.[2]

Changes in cell phenotype between the epithelial and mesenchymal states, that is, epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET), are crucial to the complex remodeling of the embryo and organ architecture during gastrulation and organogenesis and to the metastasis of many carcinomas.[3] EMT is a process in which epithelial cells lose polarity and adhesiveness, change to a mesenchymal phenotype and gain increased mobility.[4] The most important molecular markers of EMT are loss of E-cadherin and gain of N-cadherin (neural cadherin) and vimentin.[5] In the normal placenta, trophoblasts localized at the tip of the chorionic villi undergo a conversion that resembles EMT, changing from coherently attached to migratory and invading the endometrium.[6] However, in preeclampsia patients, E-cadherin levels are elevated in trophoblast cells, and E-cadherin levels appear to correlate negatively with trophoblast cell invasion.[7] N-cadherin is a mesenchymal classical type I cadherin with well-described effects on cell invasiveness in a variety of cancers.[8] N-cadherin upregulation is a well-documented feature of cells undergoing EMT.[9,10] Recently, studies have also indicated that N-cadherin is involved in trophoblast invasion.[11,12] Some researchers have observed MET-like changes in preeclamptic placentas,[13,14] but the detailed mechanisms have not been addressed.

Slug is a zinc-finger transcriptional repressor in the Snail family and is known to have an important role in EMT.[15] E-cadherin is considered one of the targets of Slug.[16] Moreover, Slug is known to repress the expression of other epithelial markers, such as ZO-1 (zona occludens 1, or tight junction protein 1 (TJP1)).[17] However, the expression of Slug in the placentas of preeclamptic patients is unclear.

The Hedgehog signaling pathway is involved in embryonic organogenesis, and GLI transcription factors are vital effectors of the

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Hedgehog pathway. Recently, the Hedgehog signaling pathway was found to orchestrate the reprogramming of cancer cells via EMT.[18] GLI1 was responsible for the expression of HH-induced key EMT regulators, including Snail1, Slug and Twist, and both GLI1 and GLI2 acted directly as transcriptional repressors of *CDH1* gene encoding E-cadherin.[19] However, the association between Hedgehog signaling and EMT/MET in preeclamptic placentas has not yet been investigated.

To investigate the association between changes in MET and preeclampsia, we compared the mRNA expressions and protein levels of the EMT/MET biomarkers E-cadherin, N-cadherin, vimentin, ZO-1 and Slug in preeclamptic placentas with those levels in normal placentas, and localized E- and N-cadherin in placental tissues through immunohistochemistry. We also evaluated the mRNA expressions of *GLI1* and *GLI2* in preeclamptic placentas relative to normal placentas.

METHODS

The local Ethics Committee approved the study protocol. All subjects provided written informed consent.

Patients and sample collection

The placental tissues from 20 healthy women at full-term delivery and from 20 preeclamptic patients at delivery were collected at the Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong Province, China. Preeclampsia was diagnosed in accordance with the recommendations of the American Society of Hypertension. Placenta samples exclusive of calcified areas were divided into two parts: one part was quickly frozen in liquid nitrogen, and the other was fixed in 10% formalin and embedded in paraffin, as in the conventional protocols.

There were no significant differences in maternal age, parity, alanine transaminase or fetal gender ratio between the preeclamptic and control groups. Relative to the healthy control subjects, in the preeclampsia patients the following parameters were all significantly higher ($P<0.05$): body mass index, gravida, systolic and diastolic blood pressure, uric acid, aspartate transaminase, creatinine and urine protein. However, in the preeclampsia group, the neonates' birth weights were significantly lower ($P<0.01$; Supplementary Table 1).

Western blot

The placental tissues were homogenized using RIPA buffer containing a protease inhibitor cocktail, and the protein concentrations were quantitated using a BCA Kit (Thermo Scientific, Tewksbury, MA, USA). Forty micrograms of total protein was resolved via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were blotted in 5% nonfat milk/Tris-buffered saline and Tween-20 and incubated serially with different primary antibodies (Table 1), overnight at 4 °C. After two washes, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence assay. The data were analyzed using the Image J software, which is public-domain software developed by the National Institute of Health (NIH, Bethesda, MD, USA). Each sample was analyzed in triplicate. The levels of E-cadherin, N-cadherin, vimentin, Slug and ZO-1 were normalized to β-actin.

Table 1 Antibodies used for western blot

Targets	*Species*	*Manufacturer*	*Dilution*
β-Actin	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	1:1000
E-cadherin	Rabbit	Cell Signaling Technology	1:1000
N-cadherin	Rabbit	Cell Signaling Technology	1:1000
ZO-1	Rabbit	Cell Signaling Technology	1:1000
Slug	Rabbit	Cell Signaling Technology	1:1000
Vimentin	Rabbit	Cell Signaling Technology	1:1000
GLI1	Rabbit	BosterBio (Wuhan, China)	1:200
GLI2	Rabbit	Bioss antibodies (Beijing, China)	1:200

Abbreviation: ZO-1, zona occludens 1.

Immunohistochemistry

Immunohistochemistry was performed using a Goat ABC Staining System (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, 6 μm sections were deparaffinized completely and immersed in citric acid buffer (10 mmol l^{-1} of sodium citrate, 10 mmol l^{-1} of citric acid) and boiled in a microwave oven at 92–98 °C for 15 min to expose the antigens. The sections were cooled to room temperature and sequentially incubated at room temperature with 3% H_2O_2 in methanol for 15 min to quench endogenous peroxidase; blocked with non-immune serum for 1 h; incubated with rabbit polyclonal anti-human E-cadherin, N-cadherin (Cell Signaling Technology; 1: 500 dilution), GLI1 (Boster-Bio, Wuhan, China; 1:100) and GLI2 (Bioss antibodies; Bioss, Beijing, China; 1:100) at 4 °C overnight; incubated in biotinylated secondary antibody for 1 h; and finally treated with AB reagent for 1 h. Intervening washes with phosphate-buffered saline were performed after incubation when necessary. The sections were stained with diaminobenzidine, counterstained with hematoxylin and mounted. As a negative control, the sections were stained by omitting the primary antibody. The signals were recorded with an Olympus digital camera system (Tokyo, Japan), and the digital images were processed by Adobe PhotoShop (Version 7.0; Adobe, San Jose, CA, USA).

Quantitative real-time PCR

Total RNA from the placental tissues was extracted using TRIzol (Invitrogen, Life Technologies, Grand Island, NY, USA) in accordance with the manufacturer's instructions. A total of 1.0 μg RNA was reversely transcribed to cDNA in a final volume of 25 μl using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time; Takara, Dalian, China) in accordance with the manufacturer's instructions. Oligonucleotide primers were synthesized by Invitrogen (Shanghai, China). The sequences of the specific primers for E-cadherin, N-cadherin, vimentin, ZO-1, SLUG, GLI1 and GLI2 are listed in Table 2. All PCR reactions were performed using SYBR Premix Ex Taq (Tli RNaseH Plus; Takara) and a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an endogenous control. The data were analyzed using the delta C_t (ΔC_t) method, where C_t is the cycle threshold, and $\Delta C_t = C_{t\text{gene of interest}} - C_{t\text{GAPDH}}$. All reactions were performed in triplicate for each gene.

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 20 (SPSS Inc., Chicago, IL, USA). The SPSS 20.0 software. Quantitative data are expressed as mean ± s.d. Statistical analysis was performed using the two-tailed unpaired Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

RESULTS

mRNA level of MET-related genes in placentas

To investigate MET-like changes in preeclamptic placentas, the mRNA levels of the MET biomarker E-cadherin, N-cadherin, Vimentin, ZO-1 and Slug. were detected by quantitative real-time PCR (Q-PCR) (Figure 1). Compared with the control placentas, in preeclamptic placentas, the mRNA expression of *ECAD* (coding E-cadherin) and *ZO-1* was significantly elevated and that of *SLUG* and *VIM* (coding vimentin) were significantly lowered ($P<0.05$). *NCAD* (coding N-cadherin) mRNA level was lower in preeclamptic placentas compared with that in the controls, but the difference did not reach significance (Figure 1; $P>0.05$).

Table 2 Primer sequences

Gene	*PCR product (bp)*	*Forward primer sequence (5′–3′)*	*Reverse primer sequence (5′–3′)*
ECAD	168	TTCCCTCTTTCATCTCCTG	ATAGTTCCGCTCTGTCTTTG
NCAD	176	GGTAATCCTCCCAAATCAA	CCACAAAACATCAGCACAA
VIM	218	GCGTGAAATGGAAGAGAA	TTGGAAGAGGCAGAGAAA
SLUG	115	ATGTCGGTTGTCTGGTTG	TCTCCTGTGTTTTGTTCTTGT
GLI1	100	TCCTTACCTCCCAACCTCTG TCTAC	CCCAACTTCTGGCTCTTCCTGT
GLI2	156	GTGGGTTAGGGATGGACTGAGG	GTTTTTTGGTGGTAAAGTGGGTGG
GAPDH	218	GGAGTCAACGGATTTGGTCG	TCCTGGAAGATGGTGATGGG

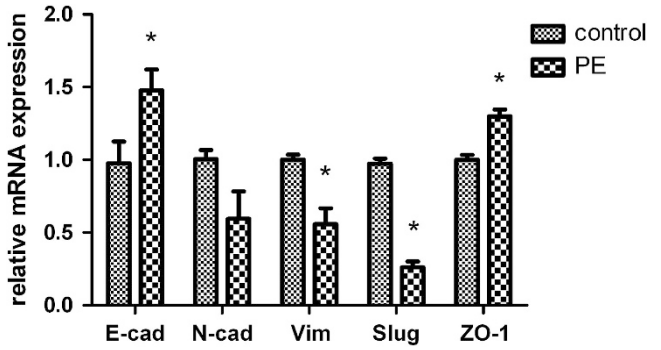

Figure 1 The mRNA levels of E-cadherin (E-cad), N-cadherin (N-cad), vimentin (Vim), Slug and ZO-1 in the placental tissues from preeclamptic patients and healthy pregnant subjects. $^*P<0.05$, $n=20$.

MET-related protein level in placentas

To verify the results of the mRNA analysis, we further examined the protein levels of E-cadherin, N-cadherin, vimentin, ZO-1, and Slug (with reference to β-actin) via western blot. Relative to the control placentas, in the preeclamptic placentas, the protein levels of E-cadherin and ZO-1 were significantly higher ($P<0.05$; Figures 2a and b), whereas those of N-cadherin, Slug and vimentin were significantly lower ($P<0.05$; Figures 2a and b).

The immunohistochemistry staining results revealed that, in both the preeclamptic and control placentas, E-cadherin and N-cadherin proteins were primarily located in the trophoblasts at similar levels (Figure 3). The protein levels of E-cadherin and N-cadherin detected by western blot were consistent with the immunohistochemistry staining intensities (Figures 2b and 3).

GLI1 and GLI2 mRNA and protein levels in placentas

To explore the association between MET and the Hedgehog signaling pathway, we investigated the mRNA and protein expressions of GLI1 and GLI2 in preeclamptic and normal placentas using Q-PCR. The mRNA levels of both *GLI1* and *GLI2* in the preeclamptic placentas were significantly lower compared with those of the control placentas (Figure 4a). The immunohistochemistry staining results revealed that, in both the preeclamptic and control placentas, GLI1 and GLI2 proteins were primarily located in the trophoblasts (Figure 4b). The protein levels of GLI1 and GLI2 detected by western blot were consistent with the mRNA levels (Figure 4c).

DISCUSSION

This is the first report of the detailed expression profiles of EMT/MET markers and GLI1 and GLI2 in placentas from preeclamptic patients and healthy pregnant women. Our findings show that the mRNA and protein levels of E-cadherin and ZO-1 in preeclamptic placental tissues were elevated relative to those in placentas from healthy women, whereas those of N-cadherin, Slug and vimentin were lower. Moreover, mRNA levels and protein levels of GLI1 and GLI2 were also lower in preeclamptic placentas compared with that in the controls.

The migration and invasion of trophoblasts into the maternal spiral arteries and uterine tissue are pivotal events in placentation. Preeclampsia is characterized by shallow trophoblast invasion and unconverted narrow spiral arteries.[20] Lyall *et al.*[21] observed a major defect in myometrial spiral artery remodeling in preeclampsia patients. The importance of altered balance of angiogenic and antiangiogenic factors in the pathogenesis of preeclampsia indicates that soluble fms-like tyrosine kinase-1 and angiotensin II type 1 receptor autoantibody may be useful early screening markers for the prediction of preeclampsia.[22,23] Naicker *et al.*[24] demonstrated restricted invasion of trophoblastic cells in preeclamptic placentas. However, the mechanisms underlying restricted invasion of the trophoblastic cells in preeclamptic placentas are not clear.

EMT has crucial roles in the development of many tissues and organs and contributes to tissue repair and carcinoma progression,[25] including cancer cell migration, invasion and metastatic dissemination.[26] E-cadherin has been shown to reduce trophoblast cell invasion, and increased levels of E-cadherin have been detected in the trophoblasts of preeclampsia patients.[7] Moreover, Duzyj *et al.*[27] observed that the level of E-cadherin in the extravillous trophoblasts adjacent to the placental–myometrial interface was less than that in the myometrium. This finding suggested that the invasive phenotype of placenta accreta extravillous trophoblasts is associated with loss of E-cadherin. In the present study, we also observed elevated levels of E-cadherin in the preeclamptic placentas, which is consistent with previous results and suggests that higher E-cadherin levels may be related to restricted invasion by trophoblasts. Recent data have shown that N-cadherin levels were much higher in highly invasive HTR8/SVneo human EVT cells than in poorly invasive BeWo and JEG-3 choriocarcinoma cells and that N-cadherin promoted invasive behavior in HTR8/SVneo cells.[28] Peng *et al.*[12] found that increased levels of TWIST subsequently induced N-cadherin expression, which promoted human trophoblastic cell invasion *in vitro*. Consistent with these results, lower levels of N-cadherin were detected by western blot analyses in the PE placentas compared with that in the normal controls, although there was no difference in the expression of N-cadherin between preeclamptic placentas and normotensive placentas in Li's study.[29]

Slug, also known as Snail2, was initially found to regulate epithelial–mesenchymal plasticity during embryonic morphogenesis and to be capable of inducing EMTs when expressed in epithelial cells.[30] Slug is

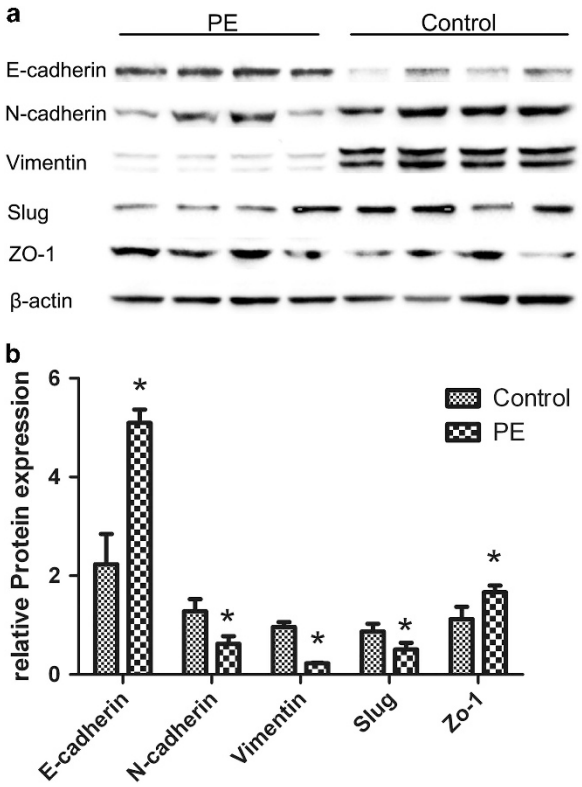

Figure 2 Protein levels of E-cadherin, N-cadherin, vimentin, Slug and ZO-1 in the placental tissues from preeclamptic patients and healthy pregnant subjects. (**a**) Representative blots from four random samples in each group. (**b**) Statistical analysis of protein levels. $*P<0.05$, $n=20$.

thought to repress E-cadherin expression, leading to EMT in cancer cells.[31–33] Snail1 protein levels were lower and E-cadherin levels were higher in human preeclamptic placentas compared with control placentas.[34] Similar to Snail1, in the present study, we also observed lower levels of Slug in preeclamptic placentas compared with the controls. Recent data have shown that N-cadherin levels in melanoma cells are downregulated by E-cadherin expression induced by transfection of either full-length E-cadherin or the E-cadherin cytoplasmic domain, suggesting that N-cadherin is directly regulated by E-cadherin content.[35] Palma-Nicolás JP's study[36] found that thrombin induced E-cadherin repression by SLUG transcription factor expression and the concomitant upregulation of N-cadherin in retinal pigment epithelium cells. Consistent with these results, lower levels of N-cadherin were detected by western blot analyses accompanied by lower Slug and elevated E-cadherin in preeclamptic placentas compared with the controls.

The link between EMT and Hedgehog signaling has been previously established within many pathological conditions, in which Hedgehog/GLI signals regulate EMT in tumors and fibrosis.[37,38] Tang *et al.*[19] reported that in the physiologically mature placenta, Hedgehog signaling induced the transcription of key EMT/MET biomarkers, including Snail1, Slug and Twist, through GLI1 but inhibited E-cadherin through both GLI1 and GLI2. However, there is no literature about the relation between GLI transcription as a vital effector of the hedgehog signaling pathway and the risk of preeclampsia. In the present study, the mRNA levels and protein levels of GLI1 and GLI2 were lower in preeclamptic placentas, which is consistent with the observed reduced Slug and increased E-cadherin levels also detected in preeclampsia placentas. These data indicate that MET-like changes in preeclamptic placentas may be caused by downregulation of the Hedgehog signaling pathway. However, the mechanisms

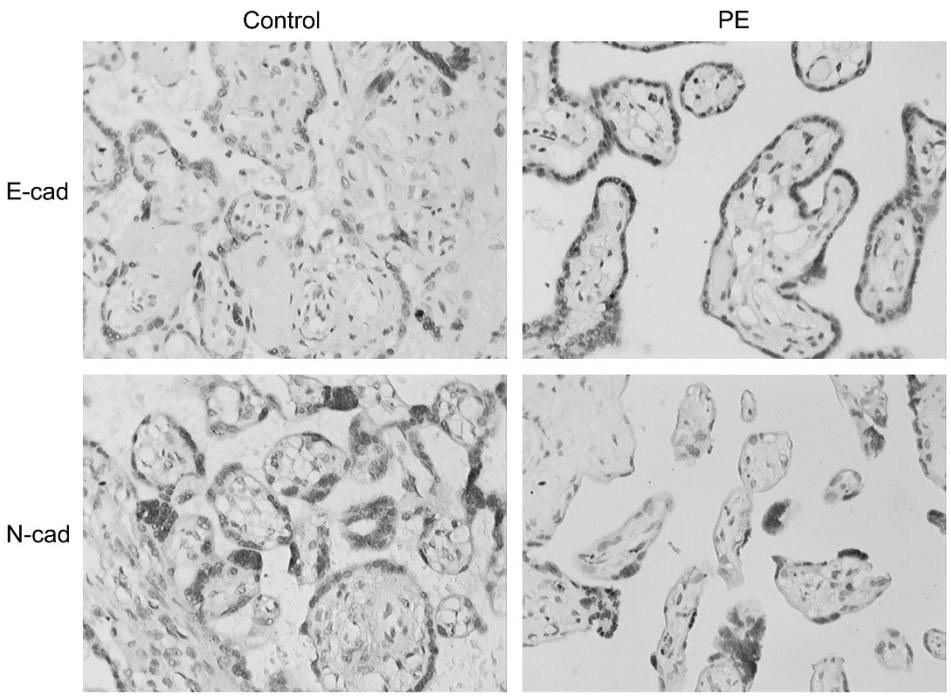

Figure 3 Localization of E- and N-cadherin in the preeclampsia and control placental tissues. Bar = 100 μm. A full color version of this figure is available at the *Hypertension Research* journal online.

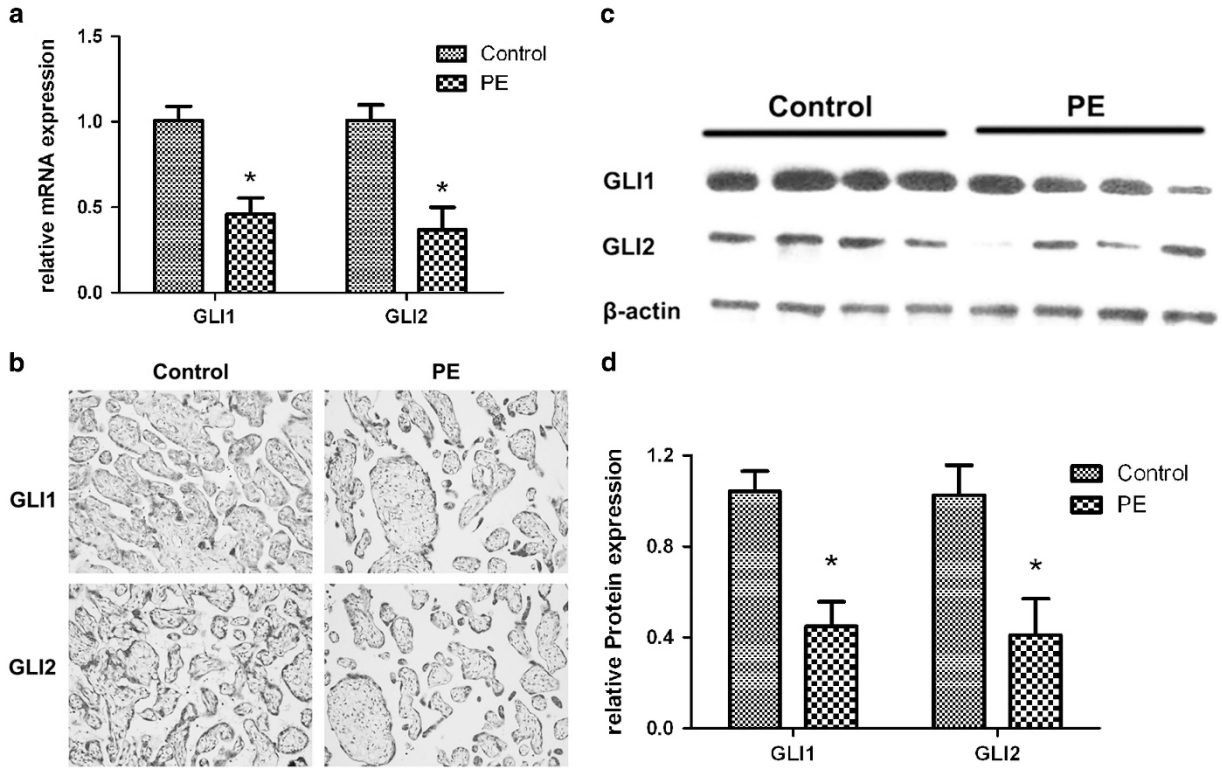

Figure 4 The expression levels of GLI1 and GLI2 in the placental tissues from preeclamptic patients and healthy pregnant subjects. (**a**) The mRNA expression levels of GLI1 and GLI2 in each group. (**b**) Localization of GLI1 and GLI2 in the preeclampsia and control placental tissues. (**c**) Representative blots from four random samples in each group. (**d**) Statistical analysis of protein levels. $*P<0.05$, $n=20$. A full color version of this figure is available at the *Hypertension Research* journal online.

governing how the Hedgehog signaling pathway contributes to the regulation of MET-like changes in preeclamptic placentas requires further investigation. In addition, epigenetic regulation, such as the regulation of microRNA, is involved in the pathogenesis of preeclampsia.[39] Yu F's study[40] demonstrated that microRNA-200a suppresses EMT in rat hepatic stellate cells via GLI2. Thus, further studies are warranted to investigate the micoRNA biomarkers targeting the hedgehog signaling pathway in maternal serum or in placenta to determine if they act as predictors or could be potential therapeutic targets for PE.

In summary, we found that MET-like changes are associated with the pathogenesis of preeclampsia and may be caused by the downregulation of GLI1 and GLI2.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Hypertension Research website (http://www.nature.com/hr)