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Research on the expression of MRNA-518b in the pathogenesis of *placenta accreta*

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Abstract. – OBJECTIVE: To investigate the expression of micro ribonucleic acid miR-518b and its regulatory role in the pathogenesis of placenta accreta.

PATIENTS AND METHODS: A total of 50 parturient women in the Obstetric Department were collected and divided into observation group (placenta accreta, n=23) and control group (normal placenta, n=27). After the placental tissues were removed via surgery, the expressions of osteopontin (OPN) and vascular endothelial growth factor (VEGF) were detected using the immunohistochemical method. The relative expression levels of OPN and VEGF proteins were detected via Western blotting, and the relative expression levels of OPN messenger RNA (mRNA), VEGF mRNA and miR-518b were detected via quantitative polymerase chain reaction (qPCR). Moreover, the correlations of miR-518b with OPN mRNA and VEGF mRNA were studied via Pearson correlation analysis.

RESULTS: Compared with those in control group, the expressions of OPN and VEGF proteins in observation group were significantly increased, while the levels of OPN mRNA, VEGF mRNA and miR-518b in observation group were also significantly elevated ($p<0.05$). There were positive correlations between miR-518b and levels of OPN, VEGF mRNA.

CONCLUSIONS: The high expression of miR-518b may lead to the development of placenta accreta through upregulating the transcription and protein expression of downstream VEGF and OPN, providing insights for the future therapy against the pathogenesis of placenta accreta.

Key Words:
Placenta accreta, mRNA-518b, Osteopontin, Vascular endothelial growth factor.

Introduction

Placenta accreta represents a common pathological process of female reproductive system in clinic, which refers to the aberrant change caused by the invasion and even penetration of placenta into myometrium. It requires more nutrients and blood supply in the case of endometrial damage or maldevelopment at the site of placental implantation[1,2]. Recently, the incidence rate of *placenta accreta* has been significantly increasing. *Placenta accreta* causes great damage to patients, including severe symptoms such as postpartum hemorrhage, and even death. Therefore, the concerns on *placenta accreta* have been raised in every country currently, but its pathogenesis remains unclear yet. A micro ribonucleic acid (miRNA) belongs to a kind of non-coding RNA, which is currently considered to play an important regulatory role in biological function of life activities such as cell proliferation, differentiation and apoptosis[3,4]. It has been proved involved in the pathogenesis of a variety of diseases. Studies have demonstrated that miRNAs degrades or inhibits the downstream messenger RNA (mRNA) through partially or completely pairing with the corresponding sequences of untranslated region of downstream target genes. As one of the important members of the miRNA family, miR-518b is derived from the chromosome 19 and has close correlations with the reproductive system and placental tissues [5]. According to further studies[6,7], miR-518b is mainly expressed in placental tissues, and it has certain specificity in the placenta and a close correlation with the placental development, which is considered as one of the important

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indexes for evaluating the embryonic development and pregnancy status. However, the correlation between the miR-518b expression and *placenta accreta* is still unclear. In addition, vascular endothelial growth factor (VEGF) and osteopontin (OPN) are closely related to the *placenta accreta* and play important roles in its pathogenesis, but their correlations need also to be determined. This study was thus performed to clarify the expression and role of miR-518b in the pathogenesis of *placenta accreta*, and illustrate the possible relations of miR-518b with the expressions of VEGF and OPN proteins.

Patients and Methods

Patients

A total of 50 parturient women admitted to the Obstetric Department in our hospital from October 2017 to March 2018 were collected and divided into observation group (pathologically diagnosed with *placenta accreta*, n=23) and control group (normal placenta, n=27). In observation group, the puerperae were aged (33.61±4.54) years old with gravidity of (2.47±1.27) times and gestational age of (37.97±0.93) weeks. In control group, the puerperae were aged (31.98±5.66) years old with gravidity of (2.12±1.19) times and gestational age of (38.77±0.84) weeks. There were no differences in the age, gravidity and gestational age between the two groups (p>0.05). The diagnostic criteria for *placenta accreta* met those proposed by Miura et al[6], and puerperae complicated with gestational diabetes mellitus, gestational hypertension or premature rupture of membrane were eliminated. This study was pre-approved by the Ethical Committee of our hospital. All the individuals involved in this study agreed and signed the informed consent about the collection of placental specimens.

Experimental Reagents and Instruments

Anti-OPN antibody and anti-VEGF antibody were bought from Abcam (Cambridge, MA, USA). The immunohistochemistry kit was purchased from Maxim (Fuzhou, Fujian, China). AceQ quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix kit and HiScript II Q RT SμperMix for qPCR (+gDNA wiper) kit were from TaKaRa (Otsu, Shiga, Japan). The optical microscope was provided by Leica (DMI 4000B/DFC425C, Wetzlar, Germany). The fluorescence quantitative PCR instrument was ABI 7500 (Foster City, CA, USA). Image-lab image analysis system and Image-Pro image analysis system were obtained from Bio-Rad (Hercules, CA, USA).

Research Methods

After admission, puerperae underwent all relevant examinations. After the delivery, placental tissues were immediately taken and kept into the frozen pathological section. Placental tissues were taken at the site of placental adhesion or implantation from the puerperae with placenta accreta confirmed by pathological results, while placental tissues were taken at the center of placenta from the puerperae with normal placenta, labeled and washed with normal saline. Part of tissue specimens were randomly selected and fixed in 4% paraformaldehyde solution for 48 h. The paraffin sections were prepared for immunohistochemical detection. The other part of tissue specimens were placed into an Eppendorf (EP) tube and stored at -80°C for Western blotting and qPCR.

Immunohistochemistry

The 5 μm-thick paraffin tissue sections were routinely dewaxed and soaked in water, and the citric acid buffer was added for antigen retrieval in a microwave oven. After sections were washed with phosphate-buffered saline (PBS), the endogenous peroxidase blocker was added for incubation for 10 min. After sections were washed again with PBS, they were sealed with goat serum for 20 min. Next, the sealing solution was discarded, and IL-1 primary antibody (1:200) and VEGF primary antibody (1:200) were added at 4°C overnight, followed by reaction with secondary antibodies for 10 min and streptavidin-peroxidase solution for 10 min, color development with 3,3'-Diaminobenzidine, counterstaining with hematoxylin and sealing with neutral balsam.

Western Blotting Detection

The placental tissues stored were added with the lysis buffer for ice bath for 60 min. The protein was quantified using the bicinchoninic acid assay, the standard curve and absorbance were detected using a microplate reader, and the protein concentration was calculated. After protein denaturation, the protein was separated via sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis. The gel was then transferred onto a membrane, sealed for 1.5 h with tris-buffered saline and Tween-20 (TBST) containing 5% nonfat milk. The membrane was incubated with the primary antibody (1:1000), secondary antibody (1:1000) and washed with Tris-buffered saline and Tween-20. Next, the image was developed using the chemilumine-

Table I. Primer sequences.

Name	Primer sequence
OPN	Forward: 5'-AGGTCTCCTCTGGCTCTG-3' Reverse: 5'-ACTTGAATCGGGTGTCG-3'
VEGF	Forward: 5'-TGCCCACTGAGGAGTCCAAC-3' Reverse: 5'-TGGTTCCCGAAACGCTGAG-3'
miR-518b	Forward: 5'-GACAAAGCGCTCCCC-3' Reverse: 5'-CAGTGCGTGTCGTGGAG-3'
GAPDH	Forward: 5'-ACGGCAAGTTCAACGGCACAG-3' Reverse: 5'-GAAGACGCCAGTAGACTCCACGAC-3'

scence reagent at dark, followed by analysis using the gel scanning imaging system.

qPCR Detection

The total RNA was extracted from the placental tissues stored at -20°C using an RNA extraction kit, and reversely transcribed into complementary deoxyribonucleic acid using a reverse transcription kit. The reaction system was 20 μL in total, and the reaction conditions are as follows: reaction at 51°C for 2 min, pre-denaturation at 96°C for 10 min, denaturation at 96°C for 10 s, annealing at 60°C for 30 s, in a total of 40 cycles. The relative expression levels of OPN mRNA, VEGF mRNA and miR-518b were calculated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The primer sequences are shown in Table I.

Statistical Analysis

Statistical Product and Service Solutions 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis in this study. Continuous data are presented as means ± standard deviation (SD), and were analyzed by using one-way ANOVA, with the Tukey's post-hoc test. Pearson correlation analysis was adopted for the correlation analysis. $p<0.05$ suggested that the difference was statistically significant.

Results

Immunohistochemical Detection of OPN and VEGF Expression

As shown in Figure 1, the positive expressions of OPN and VEGF were indicated as dark brown color, and there were a few positive expressions of OPN and VEGF in control group compared with those in observation group. The results showed that expressions of OPN and VEGF in observation group were statistically increased compared with those in control group ($p<0.05$) (Figure 2).

Relative Expression Levels of OPN and VEGF Proteins Detected via Western Blotting

The Western blotting data indicated that levels of OPN and VEGF proteins were significantly elevated in observation group compared to those in control group ($p<0.05$) (Figure 3, 4).

MRNA Expression Level Detected via qPCR

Likewise, at mRNA level, the relative expressions of OPN, VEGF and miR-518b were significantly higher in observation group than that in control group ($p<0.05$) (Figure 5).

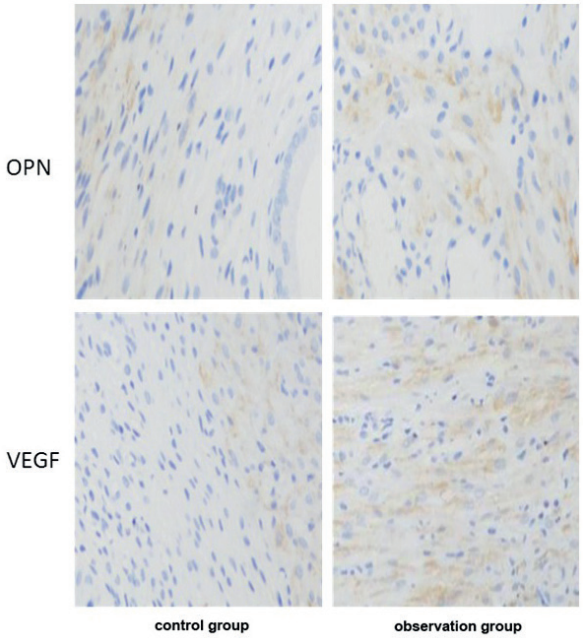

Figure 1. Immunohistochemical detection of OPN and VEGF expressions (×200).

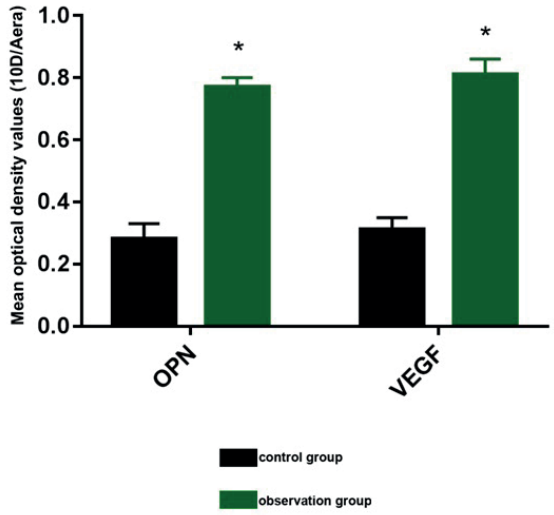

Figure 2. Mean optical density values of OPN and VEGF expressions.

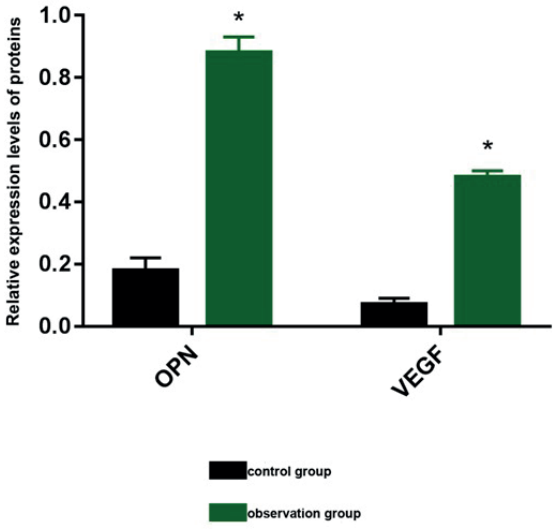

Figure 4. Relative expression levels of OPN and VEGF proteins.

Correlation Analysis

To further determine the relations between miR-518b and OPN, VEGF levels, we performed Pearson correlation analysis. The data showed that the level of miR-518b was positively associated with the expressions of OPN and VEGF mRNAs (r=0.719 and 0.821) (Figure 6, 7).

Discussion

It is known that villous trophoblasts are closely related to the pathological changes in *placenta accreta*. The trophoblasts are located on the contact region of placenta and maternal body, which is a kind of endothelial cell with extremely strong invasion. Under normal conditions, villous trophoblasts can invade the maternal body to a certain degree and are considered to play an important role in the utero-placental blood circulation [8]. In the case of endometrial damage or maldevelopment; however, villous trophoblasts will be implanted into the myometrium, and even penetrate the myometrium and serosal layer to invade the surrounding organs, leading to *placenta accreta* [9]. At present, studies have found that the prevalence rate of *placenta accreta* accounts for about 0.05-0.11% in pregnancy and shows an increasing trend. Moreover, artificial abortion, advanced age, fecundity and induced labor, are considered as major risk factors for *placenta accreta*[10,11]. VEGF is closely related to the repair of endometrial damage and involved in the placental angiogenesis[12,13]. It has been proposed that the abundance of endometrial blood flow affects the repair towards endometrial damage[14]. Therefore, the VEGF expression directly reflects the volume of endometrial blood flow. Accumulative evidence revealed that VEGF has a specific effect on endometrial vascular endothelial cells, which can promote the proliferation and migration of vascular endothelial cells and increase the vascular permeability, demonstrating an important role in endometrial angiogenesis [15,16]. At the same time, VEGF also contributes to the regulation of villous trophoblasts, for instance, enhancing cells invasion, which is of great importance in the occurrence of *placenta accreta*. According to the research, the positive rate of VEGF is approximately 25% in normal placental tissues, but up to 70% in *placenta accreta* tissues, indicating the close correlation between VEGF and *placenta accreta*. Moreover, the rise of maternal serum VEGF concentration is related to re-

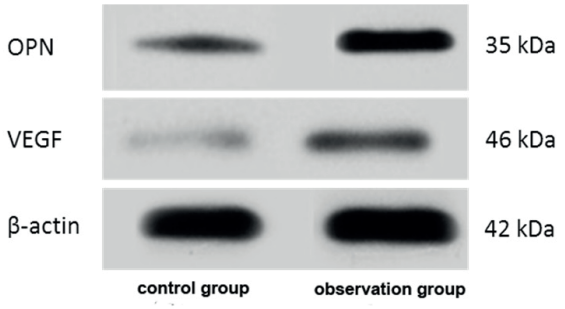

Figure 3. Protein expression detected via Western blotting.

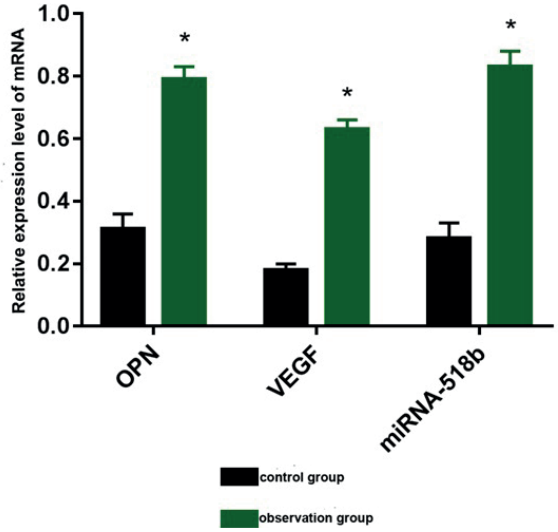

Figure 5. Relative expression level of mRNA.

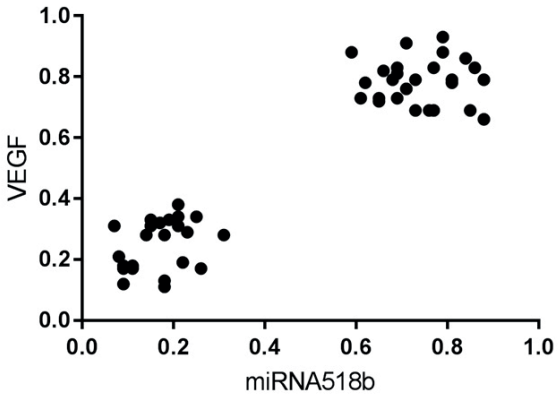

Figure 7. Correlation between miR-518b and VEGF.

current pregnancy loss[17]. Consistently, the results of this study confirmed that both VEGF mRNA and protein expression levels were significantly increased in *placenta accreta* tissues compared with those in normal placental tissues, validating the correlation between VEGF and occurrence of *placenta accreta*. As a secretory phosphorylated glycoprotein, OPN plays an important role in the cell migration, adhesion and extracellular matrix. Current studies[18-20] have showed that OPN exerts an important effect in the occurrence and development of tumor, and regulates the immune and tissue damage repair function. The expression level of OPN is higher in the endometrium in the implanting stage, which is further increased in the villus tissues in early pregnancy. Therefore, it is speculated that OPN has a close correlation with embryonic implantation, as well as placental formation, signal transduction and immunoregulation. Of note, our result found that both OPN mRNA and protein expression levels were significantly increased in *placenta accreta* tissues compared with those in normal placental tissues, and identified the correlation between OPN and occurrence of *placenta accreta*.

It has been demonstrated that miR-518b was closely implicated to the embryonic development, in placental tissues [21]. MiR-518b is correlated with the proliferation, apoptosis and invasion of villous trophoblasts, and participates in embryonic development and growth. The results of this study manifested that the miR-518b expression was significantly increased in *placenta accreta* tissues and presents positive correlations with VEGF mRNA and OPN mRNA expressions.

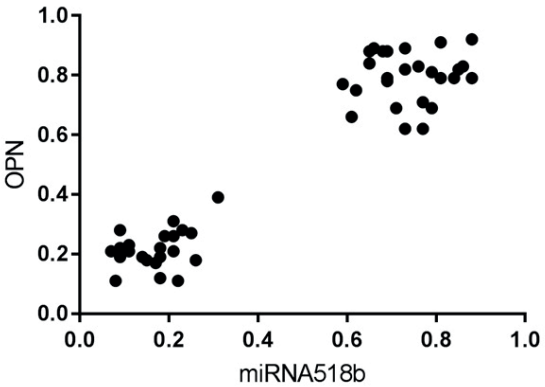

Figure 6. Correlation between miR-518b and OPN.

Conclusions

We showed that high expression of miR-518b enhances the invasion of villous trophoblasts and ultimately causes *placenta accreta*, which correlates with the levels of downstream VEGF and OPN.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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