Trophoblast cellular adhesion molecule expression regulated by different genes and their correlation with pregnancy-induced hypertension

Yunshan Chen¹, Yue Fan¹, Xiuyu Pan¹, Yunsheng Liao¹, Guochun Xiang²*

¹ Department of Obstetrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, 510180, Guangdong Province, China
² School of Health Management, Southern Medical University, Guangzhou, 510515, Guangdong Province, China

It was to study trophoblast cell (TC) adhesion molecules regulated by different genes in the placental tissue (PT) of patients with pregnancy-induced hypertension (PIH), and the correlation with the severity of PIH. 42 patients with PIH (13 cases in the mild PIH group, 11 cases in the moderate PIH group, and 18 cases in the severe PIH group) and 40 patients with normal pregnancy (NP group) were included. mRNA and protein levels in matrix metalloproteinase (MMP)-9, MMP-2, tissue inhibitor of metalloproteinases (TIMP)-1, and TIMP-2 of all patients were determined by semi-quantitative polymerase chain reaction (PCR) and Western blotting (WB), respectively. Compared to the NP group, MMP-9 and MMP-2 mRNA levels as well as their proteins in PT significantly decreased in PIH groups (P < 0.05). MMP-9 mRNA was greatly lower in the severe PIH group than mild PIH group (P < 0.05). MMP-2 mRNA in moderate and severe PIH groups was much lower than NP and mild PIH groups, and that in the severe PIH group was considerably lower than the moderate PIH group (P < 0.05). TIMP-1 mRNA and its protein highly increased in PT in PIH groups than NP group (P < 0.05). TIMP-2 mRNA was remarkably higher in the severe PIH group than in the NP group (P < 0.05). mRNA and proteins of MMP-9 and MMP-2 decreased in PT of PIH patients, while TIMP-1 mRNA and its protein increased, which were correlated with the severity of PIH. MMP-9, MMP-2, and TIMP-1 were involved in the pathogenesis of PIH by regulating the infiltration of TCs.

MMPs are a category of homologous enzyme family that can degrade almost all extracellular matrix and basilar membrane components. Up to now, there are 26 known MMPs, among which MMP-9 and MMP-2 genes are associated with biological behaviors of TC infiltration (13-15). Genes that are expressed in the human placenta and antagonistic to MMP function consist of tissue inhibitors of metalloproteinases (TIMPs) and KISS-1 genes (16-18). TIMPs are the natural inhibitor of MMP-2 as well as MMP-9.

In this work, MMP-2, MMP-9, TIMP-1, and TIMP-2 in different TCs were detected by immunohistochemical methods, to explore the correlation between MMPs and TCs. On this basis, it was also to explore the roles of MMPs in the invasion of TCs and placenta formation. By looking for indicators to predict the deterioration of trophoblastic diseases and the metastasis potential of trophoblastic tumors, it gave a reliable basis for early clinical prediction of trophoblastic disease deterioration and preventive chemotherapy.

Materials and Methods

Research objects

The placental tissues (PT) of the PIH group were selected from 42 patients with PIH who were hospitalized in the obstetric ward of Guangzhou Women and Children's
Medical Center, Guangzhou Medical University from December 2021 to December 2022 and underwent elective cesarean section. Their gestational age range was 37-42 weeks. The diagnostic criteria and histological typing criteria of PIH patients were in accordance with the unified textbook Obstetrics and Gynecology (6th edition) published by People's Medical Publishing House (19). The patients with essential hypertension and diabetes during pregnancy were excluded. Included patients were sorted into 3 subgroups in accordance with the severity of their conditions: mild PIH group (13 cases), moderate PIH group (11 cases), and severe PIH group (18 cases).

PTs of the normal pregnancy (NP) group were collected from 40 cases of normal full-term pregnancy who were hospitalized for elective cesarean section in the obstetric ward of Guangzhou Women and Children's Medical Center, Guangzhou Medical University during the same period. The gestational age ranged 37-42 weeks. Their pregnancy was normal, the fetus developed normally, and there was no any comorbidity or complication. These expectant mothers did not have a previous history of adverse pregnancy before and were not accompanied by hypertension, diabetes, chronic nephritis, and other medical history.

This project was approved by the Medical Ethics Committee of Guangzhou Women and Children's Medical Center, Guangzhou Medical University. Patients and their families knew about this project and signed the informed consent.

Collection and treatment of PT specimens

In both PIH and NP groups, samples were collected immediately after the placenta delivery during cesarean section. The central part of the maternal placenta surface was selected. The membrane was then cut and stripped, and 0.3 g of tissue was taken. The blood was cleaned with normal saline-treated by diethylpyrocarbonate (DEPC, purchased from Beijing BaiAoLaiBo Technology Co., Ltd.). Then, the tissue was immediately placed in liquid nitrogen. After 24 hours, it was stored in a refrigerator at -80 °C for RNA extraction later.

The procedure of total RNA extraction

Metal utensils were cleaned and soaked overnight in clear water. All glass utensils were soaked in concentrated sulfuric acid overnight, rinsed with water, and then soaked overnight in distilled water. The above utensils were roasted at 180 °C for 8 hours to inactivate RNase. Frozen storage tubes, Eppendorf tubes, Tip, etc. were soaked in 0.1% DEPC water at 37 °C overnight, then rinsed with sterilized water many times, dried, and sterilized by high-pressure steam for 20 min. RNase-free water was prepared by mixing 0.5 mL DEPC stock solution with 500 mL double distilled water in a shaker for 12 hours and standing at room temperature for several hours. Later, it was sterilized by high-pressure steam for 20 min. 75% RNase-free ethanol was prepared by adding RNase-free water to anhydrous ethanol. 100 mg PT was weighed and added with 1 mL TRIZOL (Shanghai Genmed Scientifics Inc.). After homogenate at 40 °C, 200 μL chloroform was added to shake and mix well. After standing for 2-3 min, it was centrifuged at 12,000 r/m, 4 °C, and for 15 min. The upper water phase was extracted, and isopropyl alcohol of equal volume was added for thorough mixing. After being placed at -70 °C for 2 hours, it was centrifuged again at 12,000 r/m at 4 °C for 15 min. The supernatant was discarded, then 1 mL 75% ethanol prepared above was added for centrifugation at 7,500 r/m for 5 min. The supernatant was also discarded; finally, the remains were dried, dissolved in 20 μL of RNase-free water, and then stored at -80 °C for later use.

Determination of RNA purity and RNA content

It was detected whether there was a degradation of RNA by 1% agarose gel electrophoresis with 0.5 μg/mL etidium bromide for 30 min (100 V). 3 bands of 28 s/18 s/5 s were clearly visible, and no DNA bands were observed, indicating that the extracted RNA was not degraded and relatively pure. The optical density (OD) at 260 nm and 280 nm were obtained by GeneQuant RNA/DNA analyzer using 1 μL RNA plus 99 μL RNase-free water. The OD260/OD280 ratio of RNA samples was also determined; the ratio 1.8-2.0 indicated that RNA extracted was pure and could be used for reverse transcription reaction.

RNA reagent concentration (μg/μL) = measured value (μg/μL)/10 [1]

Primer design

The synthetic internal reference genes β-actin, MMP-9, MMP-2, TIMP-1, and TIMP-2 were designed by Primer Express 3.0, all of which spanned two exons, thus avoiding false positives caused by a little DNA contamination. These primers were synthesized by Shanghai Bioengineering Co., Ltd., and the primer sequences were enumerated in Table 1.

Electrophoresis and semi-quantitative analysis of polymerase chain reaction (PCR) product

5 μL of PCR product was added with 1 μL 6× loading buffer and was subjected to 1.5% agarose-agar gel electrophoresis with 0.5 μg/mL etidium bromide for 60 min (120 V). UVI pro gel image analysis system was applied for observation and taking images, while UVI Band software was applied for scanning analysis. The ratio of the OD volume of the target gene band to that of the β-actin band was utilized as the relative mRNA content of the target gene.

Protein extraction from PTs

100 mg placental villi or PT was weighed, added with 1 mL TRIZOL for homogenate at 4 °C. 200 μL chloroform was then added for shaking and mixing well, left for 2-3 min and centrifuged at 13,000 r/m and 4 °C for 15 min. The upper water phase was discarded, and 0.3 mL ethanol in total was added to the precipitation and mixed thoroughly. After standing at room temperature for 2-3 min, centrifugation was performed again at 2,000 r/m and 4 °C for 5 min. The supernatant was then absorbed, added with 1.5 mL isopropyl alcohol, and placed aside at room temperature for 10 min. At 12,000 r/m and 4 °C, centrifugation was made again for 10 min. After, the supernatant was discarded; 0.3 M guanidine hydrochloride/95% ethanol was taken to rinse precipitation. It was put aside for standing at room temperature for 20 min, which was repeated 3 times. Afterward, it was centrifugated at 7,500 r/m, 4 °C, and for 5 min. The protein precipitation was suspended with 2 mL ethanol and left for 20 min at room temperature. Centrifugation for 5 min was conducted at 7,500 r/m and 4 °C, then the protein precipitation was dried and dissolved in 20 μL 1% sodium dodecyl sulfate. After a 10-min centrifugation
Western blotting (WB) method

100μg protein sample was mixed with 4× loading buffer, shaken and mixed well, boiled for 10 min, and centrifuged. The sample was drawn and dropped into the adding well with a Hamilton sample injector (Shanghai Yuyan Instruments Co., Ltd.), with a protein molecular weight of 5 μL as standard loading. After the power supply was connected, the gel voltage was 70 V. When the front end of the gel entered the separation glue, the voltage was increased to 120 V. Electrophoresis was continued until bromphenol blue slightly got out from the separation glue, which lasted about 2 hours. Protein was transferred from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel to the nitrocellulose membrane (Amersham Biosciences Corporation, UK). The transparent surface of the membrane transfer clip, gasket, filter paper, nitrocellulose membrane, gel, filter paper, gasket, and graphite surface of the membrane transfer clip was placed in turn. These were immersed in a transfer buffer for a 30-min balance, then all the air bubbles were squeezed out; the transfer was performed at 4 °C and 60 V for 3 hours. After the transfer, the nitrocellulose membrane was removed, and 5% calf serum albumin was added for a 2-hour sealing at room temperature. The primary antibody was added to the sealing solution and the dilution was 1:50, overnight at 4 °C. The sealing solution containing the primary antibody was discarded, and the nitrocellulose membrane was placed in a plate and then washed with an appropriate volume of Tris Buffered Saline Tween (TBST) for 10 min. TBST was replaced and the membrane was washed, again and again, repeated 5 times. The secondary antibody (goat anti-rabbit IgG-HRP) was added in a dilution of 1:100; after 1 hour at 37 °C, the secondary antibody was discarded, and TBST was utilized for a 10-minute washing, which was also repeated 5 times. For electrochemiluminescence development, Reagent A and Reagent B were prepared and mixed in 1: 1, evenly coated on a nitrocellulose membrane, which was then oscillated for 1 min. X-ray film was put on the membrane when it was air-dried, and images were developed at an exposure time of 30 seconds.

Rabbit anti-human antibodies, including MMP-2 protein antibody, MMP-2 unilateral antibody, TIMP-1 protein antibody, TIMP-2 protein antibody, and β-actin protein antibody, were all purchased from Wuhan Boster Biological Technology Co., Ltd.

WB semi-quantitative analysis

The ratio of OD volume of the target protein band to that of the β-actin band was taken as the relative content of the target protein, which was processed using the ScnImage system after scanning of X-ray films.

Methods of statistics

SPSS 23.0 (IBM, New York, USA) was applied for statistical analysis in this work. The measurement data according to normal distribution were represented in $\bar{x}\pm s$, and analyzed using an independent sample t-test. Enumeration data were described in percentage (%), as the chi-square test was adopted. In the results, a difference was considered to be statistically significant as $P<0.05$.

Results

Natural conditions of full-term NP and PIH patients

40 cases of normal full-term pregnancy in the NP group had a mean age of (27.38±2.73) years old. For 42 patients with PIH, the mean age was worked out as (28.67±5.64) years old. These PIH patients were divided into 3 subgroups in line with the severity of the disease: mild PIH group (13 cases), moderate PIH group (11 cases), and severe PIH group (18 cases). The natural conditions of NP and PIH patients were compared in Figure 1.

Correlation between target gene expression in PT and the severity of PIH

Compared with the NP group, the MMP-9 mRNA level in PT of the PIH group was greatly decreased, with a
difference statistically significant ($P<0.05$). The mild PIH group was not observably different from the NP group ($P=0.05$). It in moderate and severe PIH groups was highly lower than in the NP group, and that was also considerably lower in the severe PIH group than mild PIH group, with statistical significance ($P<0.05$) as displayed in Figure 2.

In terms of MMP-2 mRNA expression, it was much decreased as well in the PT of the PIH group compared with the NP group, with a difference having statistical significance ($P<0.05$). There was no significant difference between the mild PIH group and the NP group ($P=0.05$). Moderate and severe PIH groups were significantly lower than NP and mild PIH groups, and that in the severe PIH group was also distinctly lower than the moderate PIH group, showing statistical significance ($P<0.05$) as in Figure 3.

Compared with the NP group, TIMP-1 mRNA expression in PT in the PIH group was greatly increased, as the difference was statistically significant ($P<0.05$). There was no significant difference between the mild PIH group and the NP group ($P=0.05$). The severe PIH group was significantly higher than the NP group and mild PIH group ($P<0.05$) as presented in Figure 4.

PHI group had not any significant difference in TIMP-2 mRNA level in PT from the NP group ($P>0.05$). The TIMP-2 mRNA in the severe PIH group was observably higher than the NP group with a difference of statistical significance ($P<0.05$), but not any significant difference was shown among the three PIH groups as displayed in Figure 5.

MMP-9, MMP-2, TIMP-1, and TIMP-2 protein expressions in PT of each group

WB analysis showed that MMP-9 protein (92 kDa) in the PT of the PIH group was remarkably lower than that of the NP group, having a statistically significant difference ($P<0.05$). There was not any significant difference between mild PIH and NP groups in MMP-9 protein expression ($P>0.05$), but those in moderate and severe PIH groups were lower than mild PIH group, with statistically
significant differences ($P<0.05$) as displayed in Figure 6 for details.

In WB analysis, MMP-2 protein (72 kDa) was also much lower in the PT of the PIH group than the NP group, with statistical significance ($P<0.05$). There were no significant differences between the mild PIH group and NP group ($P>0.05$), but those in moderate and severe PIH groups were lower than NP and mild PIH groups, showing differences statistically significant ($P<0.05$). These comparisons were displayed in Figure 7 for details.

WB analysis illustrated that TIMP-1 protein (28 kDa) in the PT of the PIH group was greatly higher than the NP group, showing a difference being statistically significant ($P<0.05$). In the severe PIH group was higher than those in NP and mild PIH groups, with differences having statistical significance ($P<0.05$). However, there was not any significant difference among other groups, which could be observed in Figure 8 for details.

It was demonstrated in WB analysis that TIMP-2 protein (21 kDa) in the PT of the PIH group was not remarkably different from the NP group ($P>0.05$). Figure 9 showed the details.

**Discussion**

PIH is a series of syndromes after 20 weeks of pregnancy, characterized by hypertension, proteinuria, and edema. PIH is a cause of increased perinatal mortality and has been a hot topic in pathologic obstetrics for many years. After many years of extensive researches, there has been sufficient development, however, its cause is not clear yet. Previous studies have indicated that serine proteolytic enzymes, cathepsin, and metalloproteinases are involved in TC infiltration, among which MMPs are most closely related to TC infiltration (20-22). MMP-9 and MMP-2 are produced by TCs during the period of maximum infiltration ability.

This work illustrated that the gene transcription levels and protein expressions of MMP-9 and MMP-2 in PT of the PIH group were memorably lower than those of the NP group, and their expressions also showed a downward trend with the aggravation of the disease. Sahay et al. (23) adopted Northern blotting to research women with preeclampsia and full-term NP, and their results showed that the MMP-9 mRNA level in PTs of women with preeclampsia was remarkably lower than those with full-term NP. Nascimento et al. (24) applied ELISA to detect PT extracting solution, from which it was discovered that MMP-9 protein in patients with preeclampsia was highly decreased. The above researches were consistent with the findings of this work, indicating that gene and protein expressions of MMP-9 and MMP-2 in placental TCs of PIH group, and their expressions also showed a downward trend with the aggravation of the disease.
pregnant women with PIH were greatly decreased, which perhaps was caused by the decreased infiltration ability of TCs when PIH occurred.

The function and activity of MMPs are regulated by TIMPs (25-27). The results of this study demonstrated that TIMP-1 mRNA in the PT of the PIH group was increasing, and the expression became more obvious with the aggravation of PIH. WB illustrated that TIMP-1 gene and protein levels in TCs increased with an increase in the severity of PIH. Romanowicz et al. (28) revealed that the TIMP-1 level in the plasma of patients with PIH increased, which was consistent with the outcomes of this work. In addition, this study also confirmed none of the significant differences in mRNA and protein levels of TIMP-2 between PIH and NP groups, only TIMP-2 mRNA level was higher in the severe PIH group than NP group. This indicated that TIMP-1 could inhibit the resistance to MMPs in the interaction between invasion and anti-invasion at the maternal-foetal interface. When PIH overexpression inhibits TC infiltration, TIMP-2 is not mainly involved in the inhibition of MMPs, but in other aspects possibly, such as vascular network formation.

In summary, MMP-9 and MMP-2 gene expressions, which promoted TC infiltration, decreased when PIH occurred; the more severe PIH, the more significant the decrease. TIMP-1 gene expression, which inhibited invasion, increased as PIH occurred; the more severe PIH, the more significant the expression increased. The gene expression between inducing and inhibiting infiltration was imbalanced during PIH, which had a relation with the severity of PIH.

Conclusion

MMP-9 and MMP-2, as their mRNA and protein levels promoted infiltration, decreased in PT of patients with PIH, while TIMP-1 mRNA and its protein increased. These were all correlated with the severity of PIH. MMP-9, MMP-2, and TIMP-1 got involved in the pathogenesis of PIH by regulating the infiltration of TCs. The limitation of this work was that only TIMP-1 and TIMP-2 among TIMPs were studied, while the effect of TIMPs on MMPs was not limited to inhibition. The mechanism of TIMPs in TC infiltration should be further explored later.

Fundings

The research is supported by: Guangzhou Municipal School (College) Joint Funding Project (No.: 202102010163); Guangzhou Education Science Planning Project (No.:202113547).

References

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