

Expression of miR-376 in blood of pregnant women with preeclampsia and its effect on 25-hydroxyvitamin D

JINLAN LI^{1*}, JIANGDONG DU^{2*}, ZHAN WANG³, CHENGHONG WANG⁴, JIANHUA BAI⁵ and SU ZHANG¹

¹Department of Obstetrics and Gynecology, Jining First People's Hospital, Jining, Shandong 272111;

²Department of Laboratory, Yuhuangding Hospital of Yantai, Yantai, Shandong 264000;

³Department of Endoscopy Center, The Affiliated Central Hospital of Qingdao University, Qingdao, Shandong 266001;

Departments of ⁴Surgery and ⁵Obstetrics, People's Hospital of Zhangqiu District, Jinan, Shandong 250200, P.R. China

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Abstract. The study aims to investigate the clinical significance of regulating the expression of 25-hydroxyvitamin D (25-OH-VD) via microRNA (miRNA)-376c in the occurrence and development of preeclampsia (PE) in pregnant women. Peripheral blood and placental tissues were collected from pregnant women in 4 groups, including 67 normal pregnant women, 41 pregnant women with gestational hypertension, 40 pregnant women with mild PE and 51 pregnant women with severe PE. The expression of 25-OH-VD and miRNA-376c in peripheral blood were analyzed via reverse transcription-quantitative polymerase chain reaction (RT-qPCR); the protein expression of 25-OH-VD was analyzed via western blotting, and the clinical significance of its expression was also analyzed. The expression of miRNA-376c in peripheral blood in pregnant women was decreased ($P < 0.01$), and the expression of 25-OH-VD in peripheral blood was significantly decreased ($P < 0.01$); there was a significantly positive correlation between the expression of miRNA-376c and 25-OH-VD ($P < 0.01$). There was a significantly positive correlation between miRNA-376c and the protein expression of 25-OH-VD in placental tissues ($P < 0.01$). The downregulation of miRNA-376c expression in peripheral blood and placental tissues in pregnant women had significantly positive correlations with gestational age, plasma albumin level and fetal weight, but had significantly negative correlations with blood pressure and urinary protein level. ($P < 0.01$). The downregulation of 25-OH-VD expression in placental tissues also had such correlations. The low expression of miRNA-376c in PE patients is involved in the

occurrence and development of PE through downregulating the expression of 25-OH-VD.

Introduction

Hypertensive disorder complicating pregnancy (HDCP) refers to relevant diseases, in which pregnancy coexists with hypertension. It is estimated that the global incidence rate of HDCP is approximately 12.5%, and it has been >10% in pregnant women during pregnancy in China, among which patients with preeclampsia (PE) account for approximately 7%. PE is a symptom of pregnant women after 20 weeks of gestation, which is mainly characterized by new-onset hypertension and urinary protein, seriously threatening the maternal and infant health and safety. At present, it is also one of the major causes of increased mortality rates of pregnant women and perinatal infants (1-5). Studies have shown that PE is among the top three reasons for the death of pregnant women in developing countries, and severe HDCP will lead to serious complications in patients, such as heart failure, cerebral hemorrhage, placental abruption and coagulation disorders, and increase the risks of cardiovascular and cerebrovascular diseases in the long term after pregnancy (6-8). The pathogenesis of PE remains unclear so far, but it certain that the abnormal placental function plays an important role in the occurrence and development of PE.

As a newly-discovered ribonucleic acid (RNA) in recent years, microRNA (miRNA) is a kind of endogenous non-coding small RNA, which inhibits or promotes the cleavage of messenger RNA (mRNA) through the specific sequence translation to regulate the expression of target genes, resulting in the biological behaviors of diseases (9-11). Some studies have shown (12) that the differentially-expressed miRNA exists in PE in placental tissues of pregnant women, which may lead to the cell apoptosis and transcription in placental tissues, and some miRNAs are stably expressed in the blood and are easily detected. As mentioned in the articles of Mayor-Lynn *et al* (13), the comparison of miRNA and target gene expression between PE and normal placenta showed that miRNA and target gene expression are involved in the occurrence of PE. The high and low expression of 25-hydroxyvitamin D (25-OH-VD), an indispensable vitamin of human body, may directly reflect the storage level of VD (14). Wei *et al* (15) described in their articles

Correspondence to: Dr Su Zhang, Department of Obstetrics and Gynecology, Jining First People's Hospital, 6 Jiankang Road, Jining, Shandong 272111, P.R. China
E-mail: us56eq@163.com

*Contributed equally

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that the serum 25-OH-VD level in PE patients is significantly decreased compared with that in normal pregnant women. Baker and Delles (16) reported that miRNA-376c is expressed in PE patients. Whether there is a relationship between the expression of 25-OH-VD and miRNA-376c remains unknown. Therefore, the relationship between miRNA-376c and 25-OH-VD was investigated in this study, so as to provide a basis for studying the occurrence and development of PE.

Patients and methods

Clinical data of patients. A total of 199 pregnant women in Jining First People's Hospital (Jining, China) from September 2014 to May 2016 were collected, including 67 normal pregnant women aged 28.4 ± 3.5 years old on average, 41 pregnant women with HDCP aged 30.1 ± 2.4 years old on average, 40 pregnant women with mild PE aged 29.4 ± 2.8 years old on average and 51 pregnant women with severe PE aged 30.8 ± 4.1 years old on average. All patients were diagnosed according to previous evidence (1). Clinical data of patients were collected for statistical analysis (Table I). All patients and their families signed the informed consent, and this study was approved by the Medical Ethics Committee of Jining First People's Hospital (Jining, China).

Inclusion criteria: Patients without familial genetic disease and immune deficiency; patients without congenital heart disease, hypertension, chronic nephritis and hepatitis and diabetes mellitus; patients with healthy diet and regular rest; patients without diseases of respiratory or digestive system.

Exclusion criteria: Patients with twin pregnancy or above; patients with a history of blood transfusion or application of special drugs during pregnancy; patients with severe infection during pregnancy; patients with limb defects or suffering major accidents before pregnancy; patients who were not cooperative in treatment or follow-up.

Specimen collection. During treatment, 3 ml plasma was drawn from pregnant women using an ethylenediaminetetraacetic acid (EDTA)-k anticoagulant tube, let stand at room temperature for 30 min, and centrifuged using a centrifugal machine at $2,500 \times g$ for 5 min. Then the supernatant was taken and transferred into an Eppendorf tube, and stored in an ultra-low temperature refrigerator at -80°C for the same-batch detection. Approximately 30-50 g placental tissues were collected within 5 min after delivery, rinsed repeatedly with 0.9% NaCl solution, sucked dry using the dry gauze, and stored in liquid nitrogen. Within 12 h, tissues were transferred into the ultra-low temperature refrigerator at -80°C for the same-batch detection.

Extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) detection of miRNA and mRNA. Total RNA was extracted from serum and placental tissues using TRIzol method (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in strict accordance with the instructions. The concentration of total RNA extracted was detected using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan), and its purity was detected via protein electrophoresis. Total RNA ($1 \mu\text{g}$) was taken to synthesize complementary deoxyribonucleic acid (cDNA) using the RT Revert Aid First

Strand cDNA Synthesis kit and Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (both from Thermo Fisher Scientific, Inc.). The cDNA synthesized was stored in a refrigerator at -20°C . Primer sequences of miRNA-376c and 25-OH-VD mRNA were designed by Shanghai GenePharma Co., Ltd. (Shanghai, China) (Table II). Quantitative analysis was carried out using the ABI 7500 fluorescence PCR amplification instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). Reaction conditions of miRNA-376c are as follows: 95°C for 45 sec, 95°C for 15 sec, 60°C for 25 sec and 72°C for 20 sec, a total of 45 cycles. Reaction conditions of 25-OH-VD mRNA were as follows: 95°C for 1 min, 95°C for 20 sec, 60°C for 25 sec, 72°C for 20 sec, a total of 40 cycles. The relative expression levels of miRNA-376c and 25-OH-VD mRNA were calculated using the $2^{-\Delta\Delta\text{C}_q}$ method (17).

Western blotting. Total protein was extracted from placental tissues of patients in each group in strict accordance with the steps in the instruction. The protein was separated via 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene fluoride (PVDF) membrane, sealed with 5% skim milk and washed with phosphate-buffered saline (PBS) for a total of 3 times (3-5 min/time). Then rabbit monoclonal 25-OH-VD antibody (dilution, 1:500; cat. no. ab219464; Abcam, Cambridge, MA, USA) and rabbit polyclonal GAPDH antibody (dilution, 1:500; cat. no. ab37168; Abcam, Cambridge, MA, USA) were added for incubation in a refrigerator at 4°C overnight. After that, the protein was washed with 0.1% PBS 3 times (3-5 min/time), and added with horseradish peroxidase-labeled rabbit secondary antibody (diluted at 1:3,000) for 1 h. The membrane was washed with PBS 3 times, followed by color development using electrochemiluminescence (ECL) developing solution (Cell Signaling Technology, Danvers, MA, USA) and image development in the film holder. The film was cleaned and stored for analysis.

Statistical analysis. In this study, experimental results and collected clinical data of patients were analyzed using SPSS 20.0 software package (IBM Corp., Armonk, NY, USA), and images were made using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance followed by post hoc test (Least Significant Difference) was used for the expression of miRNA-376c and 25-OH-VD and clinical data. Spearman's rank correlation analysis was used for the correlation analysis between two groups, and Chi-square test was used for the comparison of rate. Measurement data were presented as mean \pm standard deviation (mean \pm SD). $P < 0.05$ suggested that the difference was statistically significant.

Results

Expression of miRNA-376c and 25-OH-VD mRNA in peripheral blood of pregnant women. miRNA-376c and 25-OH-VD in peripheral blood and placenta of patients in 4 groups were detected via RT-qPCR. The detection results of expression of miRNA-376c in the peripheral blood and placenta of pregnant women with severe PE, mild PE and HDCP and normal pregnant women showed that the expression levels in mild PE,

Table I. Clinical data of patients.

Groups	Normal group (n=67)	HDCP group (n=41)	Mild preeclampsia group (n=40)	Severe preeclampsia group (n=51)
Maternal age (years)				
<28	32	26	19	26
≥28	35	25	21	27
Urine protein (g/24 h)	No	0.15±0.09	1.24±0.87	3.21±2.14
Serum creatinine (g/l)	46.1±6.47	54.8±10.9	54.2±11.8	59.8±9.4
Gestational week (weeks)	39.1±0.6	38.1±1.2	38.5±0.9	35.1±3.1
Maternal weight (kg)	74.4±8.4	75.9±7.4	75.1±5.7	70.5±10.4
Fetal weight (kg)	3.47±0.58	3.27±0.68	3.37±0.55	2.31±0.49
Blood pressure (mmHg)				
Systolic pressure	119.1±9.8	138.2±14.8	138.4±10.5	158.4±17.6
Diastolic pressure	76.1±7.4	87.5±12.9	88.2±6.9	98.3±8.4
Exercise habit				
Yes	48 (71.64)	18 (43.90)	16 (40.00)	29 (56.86)
No	19 (28.36)	23 (56.10)	24 (60.00)	22 (43.14)
Place of residence				
Urban area	47 (70.15)	28 (68.29)	22 (55.00)	28 (56.86)
Rural area	20 (29.85)	13 (31.71)	18 (45.00)	23 (43.14)
Nationality				
Han	52 (77.61)	31 (75.61)	28 (70.00)	33 (54.90)
Minority	15 (22.39)	10 (24.39)	12 (30.00)	18 (45.10)
Educational level				
< Junior college	14 (20.90)	28 (68.29)	23 (57.50)	30 (64.71)
≥ Junior college	53 (79.10)	13 (31.71)	17 (42.50)	21 (35.29)

HDCP, hypertensive disorder complicating pregnancy.

Table II. Primer sequences.

Genes	Forward primers	Reverse primers
U6 (internal reference)	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
miRNA-376c	5'-AACATAGAGGAAATTCACG-3'	5'-CGCAAGGATGACACGCAAATTC-3'
25-OH-VD	5'-CAGAGCATGGACAGGGAGCAA-3'	5'-GCAACTCCTCATGGCTGAGGTCTC-3'

miRNA, microRNA; 25-OH-VD, 25-hydroxyvitamin D.

HDCP and severe PE groups were decreased, and there were statistically significant differences compared with that in the normal group ($P<0.05$). With the progression of the disease, the expression of miRNA-376c in the peripheral blood and placental tissues was decreased. The expression of 25-OH-VD mRNA showed a decreasing trend with the aggravation of the disease, and there were statistically significant differences in the mild PE, HDCP and severe PE groups compared with the normal group ($P<0.05$) (Fig. 1).

Correlation analyses of expression of miRNA-376c and 25-OH-VD mRNA in peripheral blood and placental tissues

with clinical data. In this study, the correlations of the expression of miRNA-376c and 25-OH-VD mRNA with maternal age, urine protein, fetal weight and blood pressure in mild PE, HDCP and severe PE groups were analyzed. Results showed that the decreased expression of miRNA-376c in peripheral blood and placental tissues was positively correlated with maternal age and fetal weight ($P<0.01$), but negatively correlated with blood pressure and urine protein level in patients ($P<0.01$). The expression of 25-OH-VD mRNA in placental tissues was positively correlated with maternal age and fetal weight ($P<0.01$), but negatively correlated with blood pressure and urine protein level in patients ($P<0.01$) (Table III).

Table III. Correlation analyses of expressions of miRNA-376c and 25-OH-VD mRNA with clinical data.

Variables	n	miRNA-376c in peripheral blood		miRNA-376c in placental tissues		25-OH-VD in placental tissues	
		r_s	P-value	r_s	P-value	r_s	P-value
Age	199	0.731	<0.01	0.713	<0.01	-0.614	<0.01
Fetal weight	199	0.647	<0.01	0.434	<0.01	-0.517	<0.01
Blood pressure	199	-0.541	<0.01	-0.461	<0.01	0.521	<0.01
Urine protein	132	-0.614	<0.01	-0.467	<0.01	0.519	<0.01

miRNA, microRNA; 25-OH-VD, 25-hydroxyvitamin D; mRNA, messenger RNA.

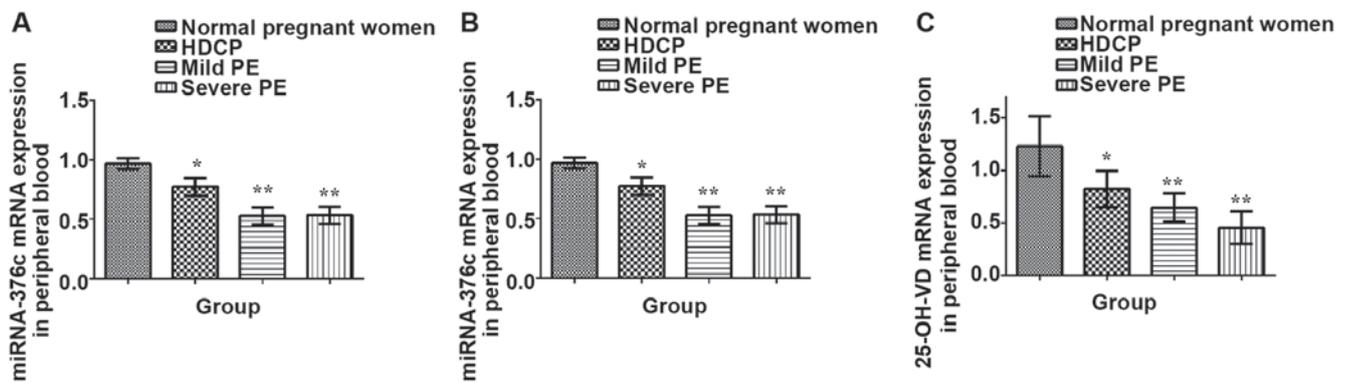


Figure 1. Expressions of miRNA-376c and 25-OH-VD mRNA in peripheral blood of pregnant women. (A) The expression of miRNA-376c in peripheral blood; it can be seen from the figure that it is significantly decreased with the progression of disease. (B) The expression of miRNA-376c in placental tissues is the same as that in peripheral blood. (C) The expression of 25-OH-VD mRNA in placental tissues is significantly decreased, indicating that miRNA-376c may promote the downregulation of 25-OH-VD mRNA. miRNA, microRNA; 25-OH-VD, 25-hydroxyvitamin D; mRNA, messenger RNA; HDCP, hypertensive disorder complicating pregnancy; PE, preeclampsia. Compared with normal pregnant women, * $P < 0.05$; Compared with normal pregnant women, ** $P < 0.01$.

Changes in expression of miRNA-376c in peripheral blood and placental tissues and expression of 25-OH-VD mRNA in placental tissues. Changes in the expression of miRNA-376c in the peripheral blood and placental tissues and the expression of 25-OH-VD mRNA in the placental tissues of pregnant women in the 4 groups were studied. Results revealed that the expression of miRNA-376c and 25-OH-VD mRNA were decreased. There were significantly positive correlations between miRNA-376c in peripheral blood and 25-OH-VD mRNA in placental tissues, and between miRNA-376c in placental tissues and 25-OH-VD mRNA ($P < 0.01$), suggesting that with the decrease of miRNA-376c expression in the peripheral blood or placental tissues and the expression of 25-OH-VD mRNA in the placental tissues is also decreased. Therefore, miRNA-376c may affect the expression of 25-OH-VD gene and play an important role in the occurrence and development of hypertension. Besides, the expression of miRNA-376c in peripheral blood and placental tissues was consistent and showed a positive correlation ($P < 0.01$), indicating that miRNA-376c in peripheral blood may reflect the expression level of miRNA-376c in placental tissues.

miRNA-376c and 25-OH-VD mRNA and protein expression in placental tissues and their correlations. Finally, 10 patients were randomly selected from each group to analyze the miRNA-376c and 25-OH-VD mRNA and protein expression

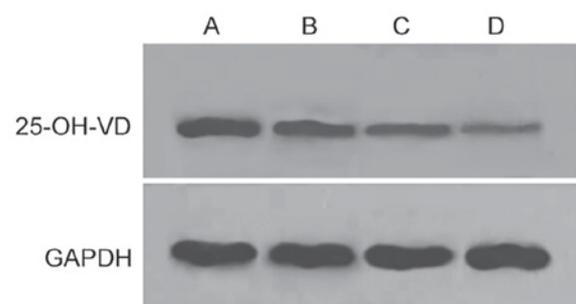


Figure 2. Western blotting detection. (A) 25-OH-VD protein expression in normal people. (B) 25-OH-VD protein expression in HDCP. (C) 25-OH-VD protein expression in mild PE. (D) 25-OH-VD protein expression in severe PE. As can be seen from the figure, the 25-OH-VD protein expression is gradually decreased with the progression of disease. 25-OH-VD, 25-hydroxyvitamin D; HDCP, hypertensive disorder complicating pregnancy; PE, preeclampsia.

levels in placental tissues. Results revealed that the 25-OH-VD protein expression were significantly downregulated in the 4 groups (Fig. 2). The expression of miRNA-376c in placental tissues was positively correlated with the expression of 25-OH-VD protein, but negatively correlated with the expression of 25-OH-VD mRNA, suggesting that miRNA-376c promotes the production of 25-OH-VD protein through regulating 25-OH-VD mRNA in tissues.

Discussion

As a kind of non-coding RNA molecule with 19-23 nucleotides in length, miRNA regulates the different expression of molecules at the post-transcriptional level, thus playing an important regulatory role in the body (18). miRNA is widely distributed in animals and plants. The regulation of miRNA can affect the cell proliferation, apoptosis, differentiation and other processes, and miRNA is also involved in the occurrence and development of tumors (19). At present, the relationship between miRNA and human diseases has gradually gained people's attention. As a member of miRNA-379 and miR-656 clusters, miRNA-376c is located on a large miRNA cluster in the DIO3 region of human chromosome 14.33. In this cluster, most miRNAs are differentially expressed in the placenta, but its mechanism remains unclear yet at present (20).

PE, as one of HDCP, is closely related to the health and safety of pregnant women. It has been reported that the damage and dysfunction of vascular endothelial cells (VEC) may be the central part of the pathogenesis, eventually leading to eclampsia. After VEC injury, tissue hypoxia, hemoconcentration, enhanced permeability, increased expression of coagulation factors and decreased expression of anticoagulant protein fibrinolysis factor will occur, leading to imbalance of the coagulation and fibrinolytic systems, ultimately resulting in thrombosis (21). Under the prethrombotic state, atherosclerosis in placental vasculature, placental ischemia and hypoxia as well as dysfunction may happen. As a result, the maternal body will secrete a large number of plasma cytotoxic substances, damaging the VEC function of maternal body and resulting in PE. It has been found in recent studies that miRNA has some differences in the expression in PE placental and normal placental tissues, which may indicate that miRNA is involved in the occurrence and development of PE, helping us acquire a better understanding of the pathogenesis of PE (22).

This study was performed strictly in accordance with the inclusion and exclusion criteria. The expression of miRNA-376c and 25-OH-VD mRNA in peripheral blood and placental tissues in the normal pregnant women and those with HDCP, mild PE and severe PE were detected, and it was found that the expression level of miRNA-376c was significantly decreased with the gradual aggravation of the disease, and it was positively correlated with the expression of 25-OH-VD mRNA and protein, suggesting that miRNA-376c may participate in the occurrence and development of PE through regulating the expression of 25-OH-VD gene in tissues. Fu *et al* (23) said in his article that the miRNA-376c expression levels in placental tissues of Canadians and Chinese with severe PE are significantly decreased compared with that in the normal group in the same period, and the expression level in patients with advanced PE also shows a decreasing trend compared with that in normal subjects, which is basically consistent with the results obtained in this study, further proving the experimental results. The correlations between miRNA-376c expression and clinical data were analyzed, and it was found that the miRNA-376c expression was positively correlated with maternal age and fetal weight, but negatively correlated with blood pressure and urine protein of pregnant women. With the aggravation

of the disease, the expression of miRNA-376c in patients with severe PE was significantly decreased compared with those in other groups, the fetal weight was small and most fetuses were premature. It has been reported in the literature (24) that the risks of neonatal defect disease and premature delivery are higher in PE patients.

Finally, the correlations of miRNA-376c expression in placental tissues and peripheral blood of pregnant women in the 4 groups were compared, and results showed that there was a positive correlation between them, suggesting that significant changes in miRNA expression can be observed in both peripheral blood and placental tissues. Recently, some studies have shown (25) that miRNA can be detected in serum and plasma and it can be used as a diagnostic tool. According to the results of this study, it was found that the expression of miRNA-376c in peripheral blood was similar to that in placental tissues, and the collection of peripheral blood was characterized by convenience and small trauma compared with that of peripheral tissues. Therefore, it is suitable for pregnant women.

At the same time, this study had some deficiencies. Only the miRNA-376c expression in placental tissues and peripheral blood were detected and investigated preliminarily, but its occurrence mechanism and process were not studied in depth. Besides, our study was not representative due to the insufficient samples, and whether the race and geographical differences may lead to biased results, remains unknown. In future studies, the sample size should be increased to better prove our views, laying a foundation for clinical diagnosis.

In conclusion, the expression of miRNA-376c in placental tissues and peripheral blood in varying degrees of HDCP were detected in this study. Results showed that the miRNA-376c expression was significantly decreased with the progression of the disease. The expression of 25-OH-VD mRNA and protein in placental tissues were also obviously decreased and they were positively correlated. Finally, changes in miRNA-376c and 25-OH-VD were significantly correlated with blood pressure and urine protein in pregnant women, indicating that miRNA-376c and 25-OH-VD interact with each other and participate in the occurrence and development of HDCP.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JL and JD designed the study and performed the experiments. CW, ZW and JB collected the patient data. JL and SZ analyzed the patient data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Jining First People's Hospital (Jining, China). All patients and their families signed the informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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