MiR-887 Promotes the Progression of Hepatocellular Carcinoma via Targeting VHL

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Abstract
Background: MiR-887 has been proved to promote the tumorigenesis in diverse cancers, but its function and downstream mechanism in hepatocellular carcinoma remain obscure. Methods: Quantitative real-time polymerase chain reaction was performed to detect the expression levels of miR-887 in hepatocellular carcinoma tissues and cell lines. MiR-887 mimics and miR-887 inhibitor were transfected into Huh7 and MHCC97H to establish miR-887 overexpression or inhibition models. Cell Counting Kit-8 and colony formation experiment were conducted to monitor cell proliferation. Subcutaneous xenotransplanted tumor model and tail vein injection model in mice were also established to further verify the effect of miR-887 on hepatocellular carcinoma in vivo. The targeting relationship between miR-887 and von Hippel-Lindau tumor suppressor (VHL) was determined by quantitative real-time polymerase chain reaction, Western blot, and luciferase reporter gene assay. Results: miR-887 expression in hepatocellular carcinoma tissues was significantly upregulated. Compared with the control cells, the proliferation and metastasis of cancer cells were enhanced by miR-887 mimics and suppressed by miR-887 inhibitor. Compared with control mice, the volume and weight of subcutaneous tumors of mice in the miR-887 mimics group were significantly elevated, and the significant increase was found in the occurrence of lung metastasis. Moreover, bioinformatics tools showed that miR-887 and VHL had 2 binding sites. Luciferase activity assay demonstrated that miR-887 can inhibit the luciferase activity of VHL, and miR-887 mimics could reduce the expressions of VHL at both messenger RNA and protein levels to increase hypoxia-inducible factor expression. Conclusion: The upregulation of miR-887 could facilitate the proliferation and metastasis of hepatocellular carcinoma cells via targeting VHL.

Keywords
miR-887, VHL, hepatocellular carcinoma

Abbreviations
CCK-8, Cell Counting Kit-8; DFS, disease-free survival; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HIF, hypoxia-inducible factor; miR, microRNA; OS, overall survival; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time polymerase chain reaction

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Introduction
Liver cancer is a frequently diagnosed malignant tumor worldwide, ranking as the third common cause of cancer-related death, among which hepatocellular carcinoma (HCC) is the most common pathological type. It is worth noting that about 50% of newly diagnosed HCC cases and deaths worldwide occur in China, and only 25% of patients are eligible for curative resection. A line of evidences have reported that the 5-year overall survival (OS) rate of HCC is only about 18%. An in-depth study of the molecular mechanism of HCC

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tumorigenesis is conducive to finding new therapeutic molecular targets, which is of great significance to further improve the survival rate of patients.

The function of microRNA (miR) in tumorogenesis and development has been gradually clarified. MicroRNA can also be used for monitoring high-risk population with HCC, which emerges as a tumor marker for HCC diagnosis and prognosis. It is reported that miR-887 participated in the resistance of breast cancer cells to “nutritional deprivation,” which reduced the apoptosis of tumor cells and promoted the proliferation. These results suggested that miR-887 may have the effect of promoting tumor. However, the function and mechanism of miR-887 in HCC remains poorly understood.

*VHL*, a tumor suppressor gene named after Von Hippel-Lindau disease, is located on chromosome 3p25-26. Inhibition or mutation of *VHL* gene can cause the aggregation and activation of hypoxia-inducible factor (HIF) in cells, thus enhancing the expression of its downstream target gene to promote the occurrence and development of tumor vessels and inhibit the apoptosis of tumor cells. Previous studies have proved the function of VHL/HIF pathway in proliferation, metastasis, and drug resistance of HCC cells, but the mechanism of abnormal activation of VHL/HIF pathway in HCC tissues has not been fully elucidated.

In this study, we found that miR-887 was highly expressed in HCC tissues, which was proved to be closely linked to adverse prognosis of patients. Cell experiments showed that miR-887 can promote the proliferation and metastasis of HCC cells, while miR-887 inhibitor has the opposite function. Furthermore, *VHL*, as target gene of miR-887, can upregulate the expression level of HIF-1α via inhibiting VHL expression. By contrast, miR-887 inhibitor was verified to partially reverse the upregulation of HIF-1α expression in hypoxia culture. Our research provided a line of results to support the conclusion that miR-887 was a potential HCC marker and therapeutic target, which also rendered a reasonable explanation for activation of VHL/HIF pathway in HCC tissue.

**Materials and Methods**

**Tissue Specimens and Ethical Statement**

We randomly collected cancer tissues and adjacent normal tissues from 48 patients with HCC who underwent radical surgery in Xiangyang Central Hospital from January 2017 to January 2019. No patients received neoadjuvant therapy (chemotherapy or radiotherapy) before operation. The control specimens were procured from the paracancerous tissue from the same patient (at least 3 cm from the surgical margin), in which no cancer cells were found by the postoperative pathological examination. All collected specimens were immediately stored in liquid nitrogen for RNA extraction. Our study was approved by Research Ethics Committee of Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts (approval no. YXLL-2017-0035). All participating patients provided written informed consent prior to enrollment in the study.

**Cell Lines**

Human HCC cell lines Hep3B, Huh7, Bel-7402, and MHCC97H and normal hepatocyte line QSG-7701 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences. All cells were cultured in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin–streptomycin solution (Life Technologies), and incubated at 37°C with 5% CO2. The medium was replaced every 3 days. The cells were passaged when the bottom of the culture bottle was covered with cells, digested with 0.25% trypsin, and then subcultured. Cells in logarithmic growth phase were taken for experiments.

**Animals Experiment**

BALB/c female nude mice (specific pathogen Free [SPF] grade) were selected and fed in a laminar flow hood. Nude mice were given sterile fodder and water. The temperature of the animal room was kept at 23°C to 25°C, with 40% to 60% humidity. The mice were randomly divided into 2 groups with 10 mice in each group and inoculated with Huh7 cells and Huh7/miR-887 mimics cells, respectively. Huh7 cells in logarithmic growth phase were taken and washed twice with phosphate-buffered saline (PBS) solution after pancreatic digestion to prepare single cell suspension. The cell concentration was then adjusted to 1 × 10⁷/mL. Each nude mouse was inoculated with 0.2 mL single cell suspension (containing 2 × 10⁶ cells) in subcutaneous tissue near the back of one underarm. After the tumor was formed, tumor volume was measured every 3 days. Mice were killed and tumor weight was measured 20 days later to monitor lung metastasis.

**Cell Transfection**

The cells were cleaned with PBS buffer 3 times, digested with pancreatin for 2 minutes, and then transferred to a sterile 15-mL centrifuge tube. The cells were subsequently centrifuged and counted. The transfected cells were observed after 12 hours, and the supernatant was replaced with the complete medium. MiR-887 mimics were designed and integrated by GenePharma, empty plasmid was used as control, and miR-887 inhibitor was purchased from RiboBio Company. Stable transfected cells were selected by Geneticin (Sigma-Aldrich).
Enhanced CCK-8 solution (Beyotime Biotechnology) at 37 °C for 15 seconds, and an annealing at 60 °C. The fluorescence signal was obtained at 60 °C. With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 as reference genes, the relative expressions of miR-887 and VHL were calculated by 2−ΔΔCt method. The primer sequences were synthesized by Shanghai Sangon Biotech Co, Ltd.

Cell Counting Kit-8 Assay

Cell Counting Kit-8 (CCK-8) experiment was carried out according to the instructions of CCK-8 kit, and cell growth curve was determined. Cells were harvested in logarithmic phase, digested with trypsin (0.25%), and cell suspension was prepared with 10% FBS. Huh7 and MHCC97H cells (3000 cells per well) were cultured in 96-well plates for 1, 2, 3 and 4 days, respectively. Then cells were incubated with 10 μL enhanced CCK-8 solution (Beyotime Biotechnology) at 37 °C for 1 hour. The culture was continued at 37 °C for 2 hours and then terminated. The empty control wells were zeroed. The absorbance value (optical density [OD] value) of each well was measured at 450 nm on the microplate reader. The relative OD ratio was used to express the cell proliferation capacity. The average value of 3 wells in each group was taken, the proliferation curve was drawn, and the experiment was repeated 3 times.

Colony Formation Assay

The ability of cell colony formation was tested by colony formation assay. Cells in logarithmic growth phase were digested with 0.25% trypsin and dispersed into individual cells, and the cells were suspended in DMEM culture solution with 10% FBS for later use. The cell suspension was diluted in gradient multiples, and 50 cells were inoculated into each dish containing 10 mL of 37 °C preheated culture solution and gently rotated to disperse the cells evenly. The cell suspension was diluted by multiple gradient and inoculated in the culture dish (50 cells per dish) containing 10 mL 37 °C preheated medium. Afterward, we rotated the culture dish gently to disperse cells evenly. Cells were then cultured in an incubator with saturated humidity and 5% CO2 at 37 °C for 2 to 3 weeks. When there was a clone visible to the naked eye, the culture was terminated. The supernatant was subsequently discarded and the dish was carefully washed twice with PBS. Cells left was fixed with 4% paraformaldehyde for 15 minutes. Then the fixing solution was discarded and a proper amount of Giemsa stain (GIMSA) was applied to dye cells for 10 to 30 minutes. At the end, the dyeing solution was slowly washed off with running water. We inverted the plate and superimposed a transparent film with grid. The clone number with over 50 cells were calculated under a 40-fold microscope. Three multiple wells were set in each group. The experiment was repeated 3 times to take the average value. Ultimately, the clone formation rate was calculated: clone formation rate = (number of clones/number of inoculated cells) × 100%.

Transwell Assay

Transwell migration and invasion experiments were performed to monitor cell migration and invasion ability; 0.25% trypsin was then added to disperse Huh7 and MHCC97H cells. Cells were subsequently centrifuged, resuspended, and dispersed in a single well of a 24-well culture plate; 8 μm pore size Matrigel chamber (Corning) was used in the invasion experiment, but not in the migration experiment; 5 × 104 transfected cells were placed in the upper chamber, with Matrigel added. The medium containing 10% FBS was added in the lower chamber with 400 μL RPMI-1640 filled. After incubation at 37 °C for 24 hours, cells that failed to migrate were removed from the upper chamber. Transwell membrane was fixed with 4% paraformaldehyde for 10 minutes and then dyed with 0.5% crystal violet. In the end, the membrane was rinsed with tap water, and cells were counted under an inverted microscope. All experiments were done in triplicate and repeated 3 times.

Flow Cytometry

Cell cycle was analyzed by flow cytometry. Cells in logarithmic growth phase were inoculated into a 6-well plate with 1 × 10^6 cells/well. After digestion with pancreatin, cell suspension was collected and centrifuged at room temperature. Cells were collected by discarding supernatant, washed twice with PBS, and then centrifuged. Cells were resuspended with 100 μL buffer solution and mixed gently. After adding 5 μL Annexin V and 1 μL propidium iodide for staining, cells were incubated at room temperature in dark for 15 minutes. The flow cytometer was adopted to detect apoptosis, and the experiment was repeated 3 times.

Dual Luciferase Reporter Gene Assay

The luciferase reporter assay system (Promega) was used to carry out the luciferase reporter gene assay. The target fragments of wild-type VHL and mutant VHL were constructed and integrated into pGL3 vector (Promega) to construct pGL3-VHL-wilde type (VHL-wt) and pGL3-VHL-mutant (VHL-mut) reporter vector. VHL-wt or VHL-mut and miR-887 mimics or negative control were cotransfected into Huh7 cells. After 48 hours of transfection, luciferase activity was determined following the manufacturer’s protocol. All experiments were done in triplicate.
Western Blot

Hepatoma cell lysate was prepared with RIPA lysis buffer (Beyotime Biotechnology) containing phenylmethylsulfonyl fluoride or cocktail. Protein samples were added for SDS-PAGE and transferred to nitrocellulose membrane. After blocking the membrane with 5% calf serum, the membrane was probed with primary antibody VHL (1:1000; Cell Signaling Technology) and anti-GAPDH (1:2000; Santa Cruz). After washing, the membrane was incubated with horseradish peroxidase–conjugated secondary antibody (1: 2000; Santa Cruz Biotechnology) for 1 hour. Ultimately, an automatic developing device (ChemiDocXRS imaging system) was used to develop and calculate the gray value.

Statistical Analysis

All data were described as the mean ± SD. SPSS 22.0 was applied for statistical analysis with Student t test. The correlation between 2 different parameters was analyzed by χ² test. P < .05 is viewed as significant difference.

Results

MiR-887 Was Highly Expressed in HCC Cells

We detected miR-887 expressions in 48 HCC tissues and adjacent normal tissues by qRT-PCR. It was indicated that compared with normal tissues, the expression of miR-887 in HCC tissues was significantly upregulated (P < .05; Figure 1A). Subsequently, we detected the expression of miR-887 in normal liver cells and HCC cell lines and obtained similar results. The expression of miR-887 in HCC cell lines was significantly higher than that in normal liver cells (P < .05; Figure 1B).

Highly Expressed miR-887 Was Closely Related to Pathological Features and Prognosis of Patients With HCC

To explore the correlation between miR-887 expression and HCC, 48 HCC samples were divided into miR-887 high expression group and low expression group. We analyzed the correlation between miR-887 expression and clinicopathological characteristics of patients with HCC (Table 1). In detail, the high expression of miR-887 was related to tumor size and lymphatic invasion (P < .05). Kaplan Meier analysis (http://www.kmplot.com/analysis) revealed that there was no difference between the OS and disease-free survival (DFS) of patients with HCC with high expression and low expression of miR-887. It was worth mentioning that OS and DFS of patients with HCC with highly expressed miR-887 were significantly lower than those with low expression (Figure 2A-C). Collectively, these data implied that miR-887 was oncogenic in HCC and could be a potential unfavorable biomarker for patients.

Highly Expressed miR-887 Can Facilitate the Proliferation and Metastasis of HCC Cells

To determine miR-887 role in HCC, we performed the inhibition and overexpression on miR-887 in HCC cells. We adopted miR-887 mimics to overexpress miR-887 in Huh7 and miR-887 inhibitor to inhibit miR-887 in MHCC97H (Figure 3A). The CCK-8 and colony experiment revealed that overexpressed miR-887 can accelerate the proliferation of Huh7, while inhibition of miR-887 can repress the proliferation of MHCC97H (P < .05; Figure 3B-D). Transwell assay indicated that the overexpression of miR-887 significantly facilitated the invasion and migration of Huh7, while the inhibition of miR-887
restrained the invasion and migration of MHCC97H (P < .05, Figure 3E and F). Flow cytometry demonstrated that the apoptosis of Huh7 was impeded after the overexpression of miR-887, while the inhibition of miR-887 promoted the apoptosis of MHCC97H cells (P < .05; Figure 3H).

**High Expression of miR-887 Accelerated HCC In Vivo**

To further verify the cancer-promoting effect of miR-887 in HCC, we conducted tumor-forming experiment in nude mice. The results showed that the tumor volume and quality in the overexpressed miR-887 group were significantly higher than those in the control group (P < .05; Figure 4A and B). The lung metastasis of mice after overexpression of miR-887 was significantly higher than that in the control group (P < .05; Figure 4C). Moreover, qRT-PCR and Western blot showed that VHL expression in miR-887 overexpression group was significantly lower than that in the control group, while HIF-α expression was significantly higher than that in the control group (P < .05; Figure 4D).

**MiR-887 Can Directly Bind to VHL at miRNA Recognition Sites to Promote HCC**

TargetScan revealed that miR-887 and VHL had 2 binding sites (Figure 5A and B). We further verified by dual luciferase activity assay that miR-887 reduced the luciferase activity of wild-type VHL but had no significant effect on VHL mutant (Figure 5C and D). Moreover, we detected the expressions of miR-887 and VHL in 48 HCC tissue samples and found that their expression was significantly negatively correlated (P < .05; Figure 5E). The above results indicated that VHL could bind to miR-887 in a targeted manner. Both qRT-PCR

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**Table 1.** Correlation Between miR-887 Expression and Clinicopathologic Features of Patients With Hepatocellular Carcinoma.

| Clinicopathologic factors | Patients number (n = 48) | miR-887 expression | P value | \( \chi^2 \) |
|---------------------------|--------------------------|--------------------|---------|---------|
| Gender                    |                          | Lower (n = 24)     | Higher (n = 24) |         |
| Male                      | 36                       | 16                 | 20      | 1.778   | .182   |
| Female                    | 12                       | 8                  | 4       |         |        |
| Age                       |                          |                    |         |         |
| \( \leq 50 \)              | 22                       | 10                 | 12      | 0.336   | .562   |
| >50                       | 26                       | 14                 | 12      |         |        |
| Liver cirrhosis           |                          |                    |         |         |
| Yes                       | 40                       | 18                 | 22      | 1.350\# | .245\a |
| No                        | 8                        | 6                  | 2       |         |        |
| Tumor size (cm)           |                          |                    |         |         |
| <5                        | 30                       | 10                 | 20      | 8.889\b | .003\b |
| \( \geq 5 \)              | 18                       | 14                 | 4       |         |        |
| Microvascular invasion    |                          |                    |         |         |
| Present                   | 18                       | 8                  | 10      | 0.356   | .551   |
| Absent                    | 30                       | 16                 | 14      |         |        |
| Tumor differentiation     |                          |                    |         |         |
| I-II                      | 10                       | 4                  | 6       | 0.505   | .477   |
| III-IV                    | 38                       | 20                 | 18      |         |        |
| Lymphatic invasion        |                          |                    |         |         |
| Present                   | 26                       | 9                  | 17      | 5.371\c | .020\c |
| Absent                    | 22                       | 15                 | 7       |         |        |

\( ^\text{a} \)Yates correction.  
\( ^\text{b} \)P < .01.  
\( ^\text{c} \)P < .05.

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**Figure 2.** High expression of miR-887 was associated with prognosis of patients with hepatocellular carcinoma (HCC). A and B, Differences in overall survival rate (OS) between patients with HCC with highly expressed miR-887 and patients with lowly expressed miR-887 were analyzed by Kaplan-Meier analysis. C, Difference in disease-free survival (DFS) between patients with HCC with highly expressed miR-887 and patients with lowly expressed miR-887 was analyzed by Kaplan-Meier analysis.
and Western blot were used to detect the effect of miR-887 overexpression and inhibition on VHL expression. The results showed that VHL expression was downregulated and HIF-α expression was upregulated after miR-887 overexpression (P < .05; Figure 5F), while VHL and HIF-α expression showed the opposite side after miR-887 inhibition (P < .05; Figure 5G).

**Discussion**

Although encouraging advance in diagnosis and therapy has been achieved in HCC, the OS rate remains unfavorable. It has been well-documented that the tumorigenesis and progression of HCC are correlated with the expressions of oncogenes and tumor suppressor genes.\(^{11,12}\) Hence, to unravel the underlying pathological mechanisms of HCC is important for the improvement of therapeutic effect and the prognosis. This study showed the upregulation of miR-887 in HCC tissues and cells. Further, overexpressed miR-887 can promote proliferation, invasion, and metastasis of tumor cells and inhibit apoptosis. Besides, we found that VHL can target miR-887, specifically activate VHL/HIF pathway to enhance the progression of HCC, which provides a new therapeutic target for the research and treatment of HCC.

VHL is a key component of E3 ligase, which can ubiquitinate and degrade HIF-α. Therefore, inactivation of VHL protein inhibits the function of E3 ligase, which leads to the upregulation of HIF-α, thus leading to disease deterioration.\(^{13}\) The expression of HIF-α will be increased in normal cells only when exposed to hypoxia, while abnormal VHL (mutation or decreased expression) can lead to increased HIF expression in cells in any environment.\(^{14-16}\) The process of tumor formation is also an adaptive response to hypoxia, one is to form corresponding multivessel system and the other is to increase glycolytic metabolic rate.\(^{17,18}\) When HIF-α accumulates, the expressions of its downstream target genes VEGF, GLUT1, and so on, also increase, thus promoting angiogenesis, cell energy
metabolism, and further facilitating the occurrence and development of tumors. Hypoxia-inducible factor 1α is considered as a factor that can speed up tumor growth. When solid tumors grow, genes downstream of HIF perform their respective functions, making tumors full of chaotic blood vessels and invasive. VHL/HIF pathway can regulate the proliferation of tumor cells and has been considered as a key target for tumor therapy. VHL/HIF-1 signaling pathway plays a role in renal cell carcinoma. Hypoxia-inducible factor 1α represses mitochondrial activity and consumption in VHL-deficient renal cell carcinoma by restraining the activity of c-Myc. The decrease in VHL expression can induce the increase of HIF-1α expression, which accelerates the proliferation of renal cell carcinoma cells and the progression of the disease.

Accumulating studies have reported that miR-331-3p can target VHL to promote HCC. However, the study on the mechanism of VHL/HIF-1 signaling pathway activation in HCC is still insufficient. In general, miRNA capable of promoting tumor will target mRNA encoding tumor suppressor protein, whereas miRNA showing tumor suppressor characteristics will target mRNA encoding cancer protein to participate in the occurrence and development of tumor. Changes in miRNA expression have been confirmed in mounting studies to participate in the development of various diseases. MicroRNAs mainly participate in the occurrence, development, and metastasis of tumors by regulating the expression of signal molecules such as cytokines, growth factors, proapoptosis, and antiapoptosis genes. Some scholars have pointed out that miR-429 can suppress cancer by binding to RAB23 in HCC, and its low expression can cause the inhibition on the proliferation and metastasis of HCC cells. MiR-888-5P is upregulated in HCC tissues and cells. Transfection of its mimics can promote the proliferation and metastasis of HCC cells and then increase the expression of MMP-2 and MMP-9 proteins. At present, the research on miR-887 in HCC has not been involved, but previous studies have validated that the expression of miR-887-5p in serum of patients with endometrial cancer is significantly increased compared with healthy people. In breast cancer, miR-887,

Figure 4. Highly expressed miR-887 can promote the progression of hepatocellular carcinoma (HCC). A, The subcutaneous tumor volume was measured every 3 days. B, The subcutaneous tumor quality was measured after the mice were killed. C, Lung metastasis was observed after the mice were killed. D, The expressions of VHL and hypoxia-inducible factor α (HIF-α) in tumor cells of miR-887 overexpressed mice and control mice were detected by real-time polymerase chain reaction (RT-PCR) and Western blot (compared with pc-miRNA, *P < .05; **P < .01; ***P < .001; NS P > .05).
miR-203, miR-3619, and miR-182 are highly expressed, which are proved to inhibit PLD-mediated cancer cell invasion caused by vimentin.\textsuperscript{30} We confirm for the first time that miR-887 is highly expressed in HCC and plays a role as a cancer-promoting factor in HCC, which is consistent with the above research reports and provides a new target for the treatment and diagnosis of HCC in the future. In addition, we confirmed the target binding relationship between miR-887 and VHL, further improving the mechanism of VHL/HIF-1 signaling pathway activation in HCC.

In conclusion, miR-887 plays a role as a cancer-promoting gene in HCC. In detail, upregulated miR-887 can activate VHL/HIF-1 signaling pathway, thus promoting HCC progression.

Authors' Note
The data used to support the findings of this study are available from the corresponding author upon request.

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Declaration of Conflicting Interests
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Figure 5. MiRNA-887 can promote hepatocellular carcinoma (HCC) via VHL. A and B, TargetScan indicates there are 2 binding sites between miR-887 and VHL. C and D, miR-887 reduced the luciferase activity of wild-type VHL. E, Real-time polymerase chain reaction (RT-PCR) was carried out to detect the expressions of miR-887 and VHL in the tissues of 48 HCC samples, respectively, and the results showed that their expressions were significantly negatively correlated. F and G, The effects of miR-887 overexpression and inhibition on VHL and hypoxia-inducible factor α (HIF-α) expressions were detected by quantitative RT-PCR and Western blot (compared with normal or control group, *$P < .05$; **$P < .01$; ***$P < .001$; NS $P > .05$.)
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