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Correlation between miR-1207-5p expression with steroid-induced necrosis of femoral head and VEGF expression

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression level of micro-ribonucleic acid-1207-5p (miR-1207-5p) in steroid-induced necrosis of femoral head (SNFH) and its correlation with SNFH. Meanwhile, we also aimed to analyze the relationship between miR-1207-5p expression and vascular endothelial growth factor (VEGF) in the femoral head.

PATIENTS AND METHODS: From May 2016 to December 2017, 60 patients aged (55.4±8.7) were selected in our hospital. All patients were diagnosed and confirmed as SNFH. Total RNA was extracted from the necrotic femoral head tissues and peripheral blood. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to detect the expression level of miR-1207-5p in tissues. At the same time, immunohistochemistry and Western blotting were adopted to detect VEGF expression in the bone tissue of patients with high or low expression of miR-1207-5p. 7 patients with femoral neck fracture aged (45.6±4.51) were enrolled in the control group. In the animal experiment, the rat SNFH model was established by intraperitoneal injection of lipopolysaccharide and methylprednisolone. Subsequently, the expression levels of miR-1207-5p and VEGF in necrotic femoral tissues were detected. Meanwhile, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was applied to detect cell apoptosis in bone lacunae of miR-1207-5p high expression group and miR-1207-5p low expression group, respectively.

RESULTS: The expression level of miR-1207-5p in the necrotic bone tissue of the SNFH group was significantly higher than that of the control group. The expression level of miR-1207-5p was inversely proportional to Harris Hip score ($p<0.05$). A higher expression of miR-1207-5p indicated a lower expression level of VEGF ($p<0.05$). The animal experimental results revealed that miR-1207-5p expression in the necrotic femoral head tissue of SNFH group was significantly higher than that of the control group. Furthermore, the number of apoptotic cells in bone lacunae was remarkably higher in miR-1207-5p high expression group ($p<0.05$).

CONCLUSIONS: MiR-1207-5p is significantly up-regulated in necrotic femoral head tissue and serum of SNFH patients. Meanwhile, its expression level is inversely proportional to Harris Hip score of patients. The possible underlying mechanism may be related to the inhibitory effect of miR-1207-5p on VEGF.

Key Words:
MiR-1207-5p, Steroid-induced necrosis of femoral head, VEGF.

Introduction

Since the 1960s, glucocorticoids have been widely used in the clinical treatment of various diseases, such as systemic lupus erythematosus, severe respiratory distress syndrome and various complications after organ transplantation[1,2]. In recent years, with the widespread application of glucocorticoids in clinical practice, the incidence rate of steroid-induced necrosis of femoral head (SNFH) is increasing. It has been reported that SNFH accounts for 51% of non-traumatic necrosis of the femoral head[3]. Due to the decreased number of blood vessels at the lesion site, blood supply of the local bone tissue is reduced. Meanwhile, ischemia and anoxia may occur. On the other hand, the shock of various hormones can result in an increase in the apoptosis of osteoblasts[4]. These are all important causes of femoral head necrosis. A large number of genes and proteins are involved in the occurrence and development of SNFH. However, the mechanism of SNFH has not been fully understood yet. Therefore, it is of great significance to clarify the pathogenesis of SNFH for future prevention and treatment[5].

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Micro-ribonucleic acids (miRNAs) are a group of single-stranded non-coding RNAs with 20-24 nt in length. Previous studies have found that miRNAs exist in eukaryotes. MiRNAs can regulate the expression of various genes through targeted combination with specific genes. They also exert crucial effects on cell proliferation, differentiation, apoptosis, angiogenesis and other physiological activities. Currently, several studies[6,7] have reported the important role of miRNAs in SNFH. For example, miR-145 can improve SNFH in rats by inhibiting the osteoprotegerin (OPG)/receptor activator of nuclear factor-kappaB ligand (RANKL)/RANK signaling pathway[8]. MiR-34a can target inhibit transforming growth factor beta-induced factor homeobox 2 and OPG/RANK/RANKL signals, ultimately reducing the progression of SNFH[9]. On the contrary, miR-206 can induce the apoptosis of osteoblasts by targeting programmed cell death protein 4 (PDCD4), eventually aggravating SNFH[10]. These studies have manifested that different miRNAs may play opposite roles in SNFH. However, the expression of miR-1207-5p in SNFH has not been reported. Furthermore, its correlation with SNFH is rarely elucidated.

In this work, the expression of miR-1207-5p in the necrotic femoral head tissue of SNFH patients and SNFH rat model was examined. The correlation between miR-1207-5p expression and Harris Hip score of SNFH patients was analyzed. Furthermore, the expression of VEGF in femoral head tissues and the apoptosis of osteoblasts in bone lacunae were detected. Our work aimed to elucidate the specific role of miR-1207-5p in SNFH and the underlying molecular mechanism.

Patients and Methods

Patients

60 SNFH patients aged (55.4±8.7) were collected in our hospital from May 2016 to December 2017. All patients were diagnosed and confirmed as SNFH according to the Expert Consensus on Diagnosis and Treatment Standards for Adult Femoral Head Necrosis (2012 Edition). Meanwhile, 7 patients with femoral neck fracture aged (45.6±4.51) were enrolled in the control group. All the above procedures were approved by the Ethics Committee of our hospital.

Main Instruments and Reagents

Total RNA isolation reagent TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), SYBR Green Real Time-Polymerase Chain Reaction (PCR) Master Mix Kit (Toyobo, Shanghai, China), reverse transcriptases and protein kinase K (Promega, Madison, WI, USA), primers (designed by BGI, Shenzhen, China), Real-Time fluorescence quantitative PCR instrument (Stratagene, La Jolla, CA, USA) and the Du-600 enzyme reader (Beckman, Miami, FL, USA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

(1) Total RNA in the femoral head tissue was extracted according to the instructions of TRIzol reagent. The concentration and purity of extracted RNA were detected by an ultraviolet spectrophotometer. When the ratio of the absorbance at 260 to 280 (A_{260}/A_{280}) was 1.8-2.0, the RNA could be used. (2) Messenger RNAs (mRNAs) were synthesized into complementary deoxyribonucleic acids (cDNAs) through RT and stored in a refrigerator at 80°C for subsequent use. (3) RT-PCR system: 2.5 μL 10× Buffer, 2 μL cDNAs, 0.25 μL forward primers (20 μmol/L), 0.25 μL reverse primers (20 μmol/L), 0.5 μL deoxy-ribonucleotide triphosphates (10 mmol/L), 0.5 μL Taq enzymes (2×10^6 U/L) and 19 μL double distilled water. The amplification systems of RT-PCR were the same. (4) Calculation of Ct value: the number of cycles that the fluorescent signal in each well plate experienced when it reached the set threshold was recorded. The expression level of miR-1207-5p in each group was calculated *via* the relative quantification method.

Enzyme-Linked Immunosorbent Assay (ELISA)

A specific procedure was as follows: (1) 3 mL blood samples were collected from patients. (2) Standards were prepared according to the kit instructions. (3) Standards and samples were added into each reaction well. (4) Streptavidin-horseradish peroxidase was added for incubation. (5) Washing and color development were conducted. (6) After adding the stop buffer, the absorbance was measured by an ultraviolet spectrophotometer.

Establishment of the Rat Model

Forty male Sprague-Dawley rats aged 10-12 weeks were randomly divided into SNFH group (n=20) and control group (n=20). Rats in the SNFH group were intraperitoneally injected with lipopolysaccharides (LPS) (20 μg/kg) twice, with a 1-day interval each time. Meanwhile, high-dose

of methylprednisolone (40 mg/kg) was injected intramuscularly 1 day later 3 consecutive times, with a 1-day interval each time. However, rats in the control group were given an equal amount of normal saline. One month later, materials were drawn for subsequent molecular biology experiments. This study was approved by the Animal Ethics Committee of Daqing Oilfield General Hospital Animal Center.

Immunohistochemical Staining

The cut tissue sections were baked in an oven at 60°C for 30 min. Then the sections were dewaxed with xylene (5 min×3 times), followed by dehydration with 100%, 95% and 70% ethanol 3 times, respectively. The endogenous peroxidase activity was inhibited by 3% hydrogen peroxide methanol. Subsequently, the tissues were sealed with sheep serum for 1 h. Antibodies against the VEGF were diluted at 1:200 [phosphate-buffered saline (PBS)] and incubated at 4°C overnight. After washing with PBS 4 times in a shaker, the second antibody was added. Color development was performed with diaminobenzidine. 6 samples were randomly selected from each group, and 5 fields were randomly selected for each sample. Finally, photographing was performed under a 400× optical microscope.

Western Blotting

After the femoral head tissues of rats in each group were fully ground in lysis buffer, they were ultrasonically lysed and centrifuged. The supernatant was collected and split into Eppendorf tubes. The concentration of the extracted protein was measured *via* the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) by an ultraviolet spectrophotometry. Then the protein volume of all samples was set constant to equal concentration. After splitting, the tissues were placed in a refrigerator at -80°C. Subsequently, the extracted total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (PVDF) (Roche, Basel, Switzerland). After incubation with primary antibody at 4°C overnight, the membranes were incubated with goat anti-rabbit secondary antibody for 1 h in the dark. The protein band was scanned and quantified by the Odyssey membrane scanner. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control.

Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) Staining

The cut femoral head tissue sections were baked in an oven at 60°C for 30 min. Then the sections were dewaxed with xylene (5 min×3 times), followed by dehydration with 100%, 95% and 70% ethanol, respectively 3 times. After that, the sections were incubated with protein kinase K for half an hour and rinsed with PBS. TUNEL and Luciferase-labeled dUTP were added for reaction at 37°C for 1 h. Then, the horseradish peroxidase-labeled secondary antibody was added for reaction again at 37°C for 1 h. Subsequently, the sections were reacted at room temperature for 10 min, with 3,3'-diaminobenzidine as the substrate. Then the nucleus was stained with hematoxylin. Finally, photographing and counting were carried out under an optical microscope.

Statistical Analysis

Statistical Product and Service Solutions 22.0 analysis software (IBM, Armonk, NY, USA) was used for all statistical analyses. Measurement data were expressed as mean ± standard deviation. The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

Expression of MiR-1207-5p in Femoral Head Tissue of Patients

As shown in Figure 1, RT-PCR results manifested that the expression of miR-1207-5p in the femoral head tissue of SNFH patients was markedly increased, which was about 5.6 times higher than that of the normal control group ($p<0.05$).

Expression of MiR-1207-5p in Serum of Patients

MiR-1207-5p was not only expressed in tissues but also partially released into circulating peripheral blood. The content of miR-1207-5p in the serum of patients was further detected. The results were consistent with miR-1207-5p expression in tissues. The content of free miR-1207-5p in the serum of the SNFH group was about 2.5 times higher than that of the control group ($p<0.05$) (Figure 2).

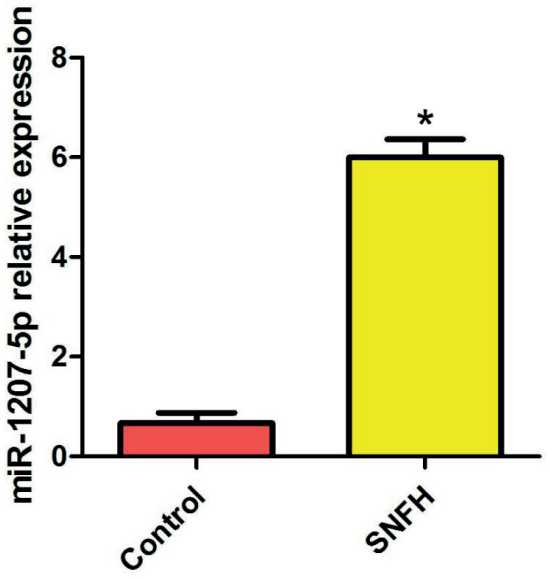

Figure 1. Expression of miR-1207-5p in femoral head tissue. Control: control Group, and SNFH: SNFH group. *p<0.05 represented a statistical difference compared with the control group.

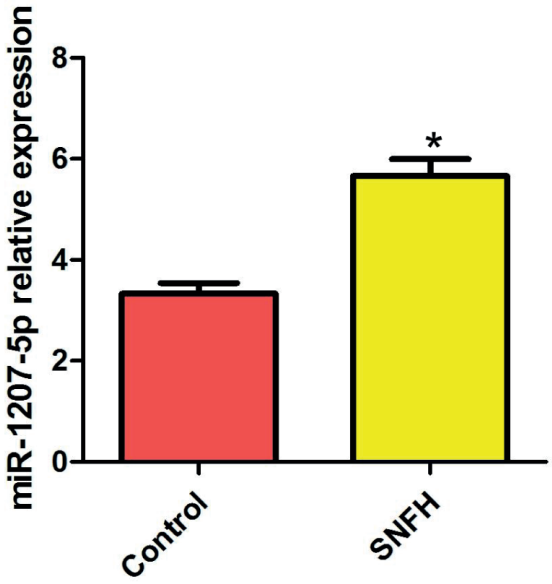

Figure 2. Expression of miR-1207-5p in serum of patients. Control: Control group, and SNFH: SNFH group. *p<0.05 represented a statistical difference compared with the control group.

Correlation Between MiR-1207-5p Expression in Femoral Head Tissue and Harris Hip Score of SNFH Patients

According to the average expression level of miR-1207-5p in the femoral head tissue (6.728±1.392), SNFH patients were divided into two groups, including miR-1207-5p high expression group and miR-1207-5p low expression group. Firstly, the morphology of bone tissues in the high-expression and low-expression groups were observed using hematoxylin and eosin (H&E) staining. As shown in Figure 3, the morphology of osteoblasts in miR-1207-5p high expression group became abnormal. However, the morphological changes in miR-1207-5p low expression group were relatively slight. The relationship between miR-1207-5p expression level and Harris Hip score of patients was further analyzed (Table I). It was found that Harris Hip score in miR-1207-5p high expression group was notably lower than that of the miR-1207-5p low expression group (p<0.05).

Relationship Between MiR-1207-5p Expression and VEGF Expression in Femoral Head Tissue of SNFH Patients

Considering that VEGF played an important role in the pathogenesis of SNFH, VEGF expression in miR-1207-5p high-expression and low-expression groups was measured *via* Western blotting. The results revealed that VEGF expression in miR-1207-5p low expression group was significantly higher than that of miR-1207-5p high expression group (p<0.05) (Figure 4).

Immunohistochemical Staining Results of VEGF in the Femoral Head Tissue of SNFH Patients

To visualize better the expression and distribution of VEGF in the femoral head tissue, the immunohistochemical technique was used to stain VEGF in bone tissues. The results demonstrated that VEGF expression in miR-1207-5p high expression group was significantly higher than that of the miR-1207-5p low expression

Table I. Relationship between the miR-1207-5p expression level and the Harris Hip score of SNFH patients.

Group	Case (n)	score	χ^2	p
MiR-1207-5p high expression group	28	12.4±2.1	12.112	0.003
MiR-1207-5p low expression group	32	17.1±1.8		

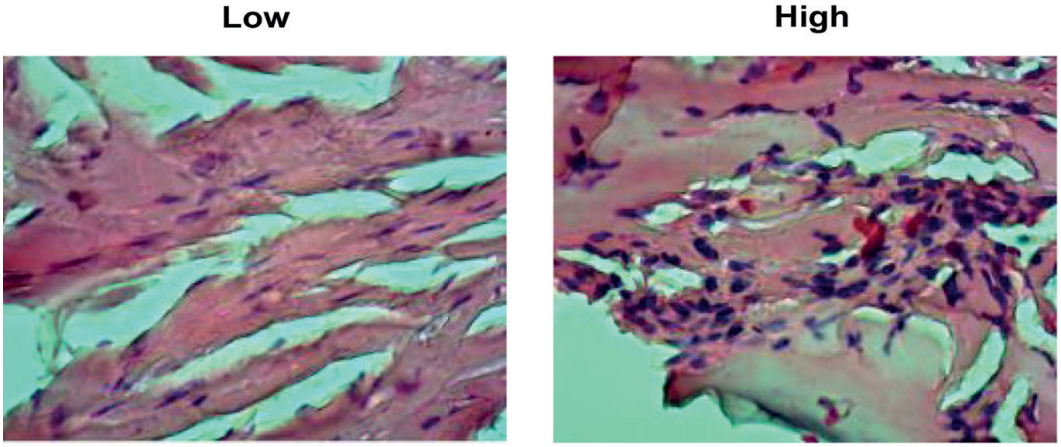

Figure 3. H&E staining of femoral head tissues of SNFH patients in miR-1207-5p high-expression group and miR-1207-5p low-expression group. Low: low expression group, and High: high expression group (Magnification × 200).

group. This confirmed again that miR-1207-5p regulated femoral head necrosis by mediating VEGF (Figure 5).

Expression of MiR-1207-5p in Femoral Head Tissue of SNFH Rats

Moreover, the SNFH rat model was established to further verify our hypothesis. The results demonstrated that the expression pattern of miR-1207-5p in the femoral head tissue of rats was the same as that in the SNFH patients. As shown in Figure 6, the expression level of miR-1207-5p in the femoral head tissue of SNFH rats was remarkably higher than that of the healthy control rats ($p<0.05$).

Influence of MiR-1207-5p Expression on VEGF in Femoral Head Tissue of Rats

Similarly, 20 SNFH rats were divided into high-expression group and low-expression group according to the expression level of miR-1207-5p. The influence of miR-1207-5p expression on VEGF was detected. H&E staining results showed that bone tissue damage in miR-1207-5p high expression group was much more serious than that of the miR-1207-5p low expression group (Figure 7). Western blotting results also manifested that VEGF expression in the necrotic femoral head tissue of rats in miR-1207-5p high expression group was significantly lower ($p<0.05$).

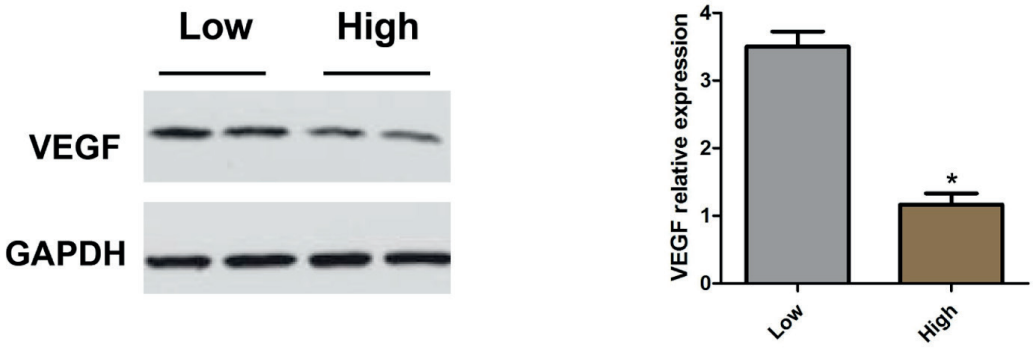

Figure 4. VEGF expression in femoral head tissue of SNFH patients in miR-1207-5p high-expression group and miR-1207-5p low-expression group. Low: low-expression group, and High: high-expression group. *$p<0.05$ represented a statistical difference compared with the low-expression group.

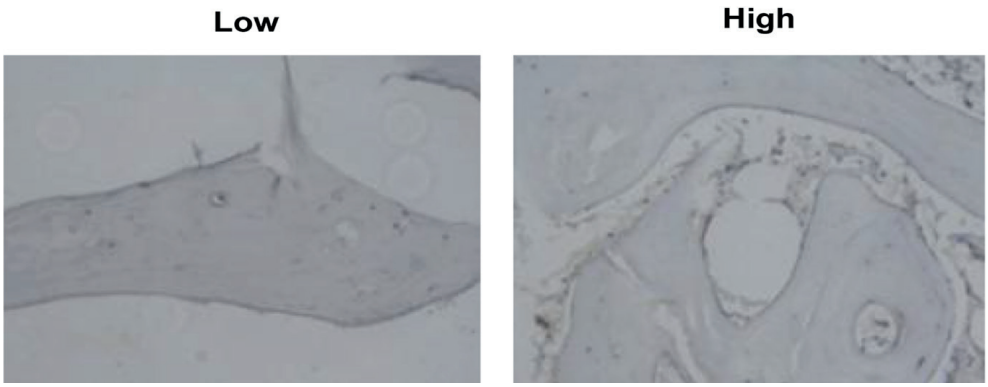

Figure 5. Immunohistochemical staining of VEGF in femoral head tissue of SNFH patients in miR-1207-5p high-expression group and miR-1207-5p low-expression group**.** Low: low-expression group, and High: high-expression group (Magnification × 40).

Influence of MiR-1207-5p Expression on the Apoptosis of Osteoblasts in Femoral Head Tissue of Rats

Since osteoblast apoptosis is an important pathological change of SNFH, we wondered whether the expression level of miR-1207-5p could affect the apoptosis of osteoblasts. TUNEL technique was adopted to detect the apoptosis of osteoblasts in the necrotic femoral head tissue of SNFH rats. It was found that the number of osteoblasts in the miR-1207-5p high expression group was 3.21 times higher than that of the miR-1207-5p low expression group ($p<0.05$) (Figure 8).

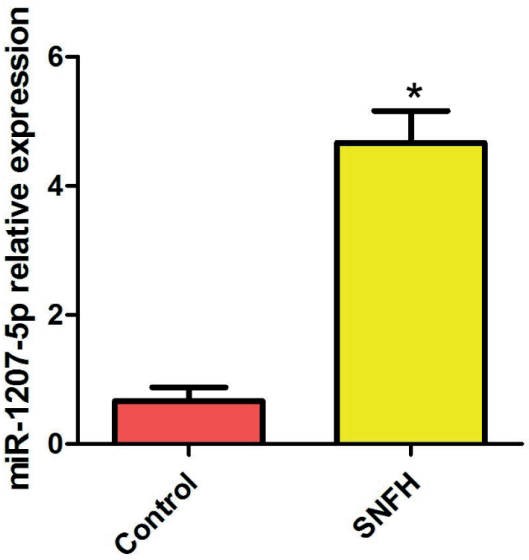

Figure 6. Expression of miR-1207-5p in femoral head tissue of SNFH rats. Control: Control Group, and SNFH group: steroid-induced femoral head necrosis group. *$p<0.05$ represented a statistical difference compared with the control group.

Discussion

In recent years, the incidence rate of SNFH has increased year by year due to the massive use of exogenous glucocorticoids. Currently, multiple studies are trying to reveal genes or proteins that are crucial to the occurrence and development of SNFH. However, the precise molecular mechanism of SNFH is still unclear. Meanwhile, there is a lack of effective and precise prevention and control of SNFH in clinic[11,12]. With the rapid development and innovation of molecular biology technology, people have formed a deeper understanding of the pathogenesis of SNFH. Many researchers have gradually shifted from the original focus on pathogenic genes to non-coding RNAs, including miRNAs and long non-coding RNAs. MiRNA is an endogenous and highly conserved RNA with a length of about 19-22nt. It has no protein-coding function[13]. MiRNAs can inhibit the translation of messenger RNAs or degrade them directly by incomplete or complete binding to the 3'-untranslated region of mRNAs[14,15]. At present, about 600 miRNA species have been discovered. Most of them have been proved to be closely related to the occurrence and development of diseases.

Since the morbidity and mortality rates of SNFH have increased year by year worldwide, the role of miRNAs in SNFH has also attracted more and more attention. For example, connexin43 and miR-206 expression levels in SNFH patients are significantly higher than those of controls. Meanwhile, higher expression levels indicate worse prognosis of patients[16]. Another study has found that miR-320 expression is sig-

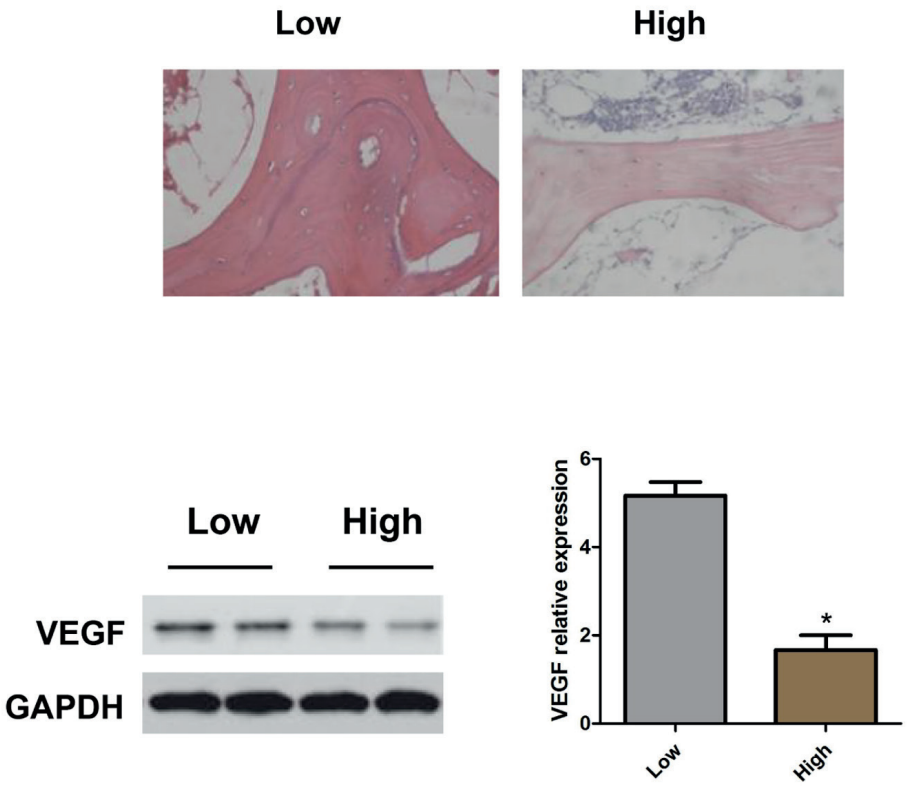

Figure 7. H&E staining of femoral head tissue and Western blotting of VEGF in SNFH rats of miR-1207-5p high-expression group and miR-1207-5p low-expression group. Low: low-expression group, and High: high-expression group. *p<0.05 represented a statistical difference compared with the control group (Magnification: 40 ×).

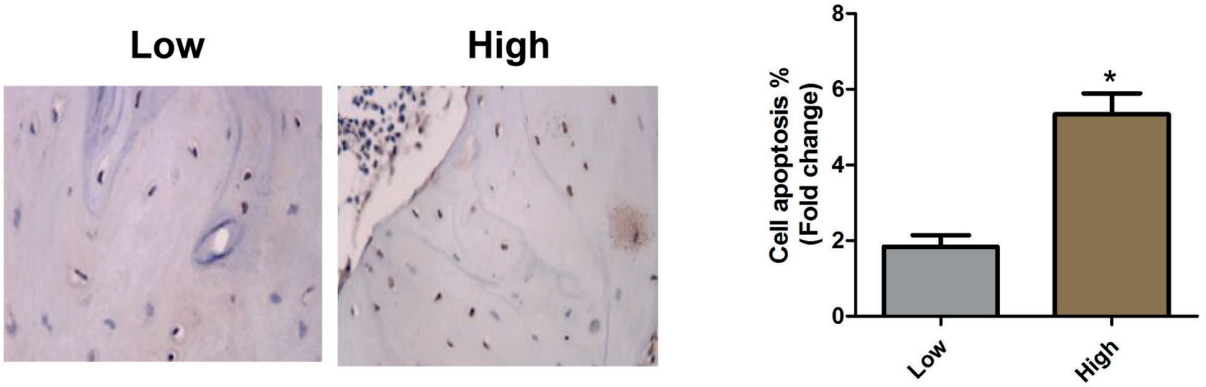

Figure 8. TUNEL staining of osteoblast apoptosis in femoral head tissue of SNFH rats in miR-1207-5p high-expression group and miR-1207-5p low-expression group. Low: low-expression group; and High: high-expression group. *p<0.05 represented a statistical difference compared with the low-expression group (Magnification: 100 ×)

nificantly decreased in SNFH patients, which is accompanied by increased CYP1A2 expression and activity. Furthermore, Luciferase reporter gene detection has found that cytochrome P450 1A2 (CYP1A2) is the downstream target gene of miR-320. Hemodynamic and microcirculatory results indicate that up-regulated CYP1A2 can greatly promote the occurrence and development of SNFH. However, the increase in miR-320 expression can inhibit the progression

of SNFH. These results all suggest that up-regulation of miR-320 may reduce the risk and development of SNFH by targeted inhibition of CYP1A2 expression[17].

The role of miR-1207-5p in the progression of various diseases has gradually been revealed. For example, in human gastric cancer tissues, miR-1207-5p and miR-1266 are confirmed as human telomerase reverse transcriptase inhibitors. By targeted inhibition of telomerase reverse transcriptase, miR-1207-5p and miR-1266 can significantly inhibit gastric cancer cell proliferation, cell cycle, migration and invasion[18]. MiR-1207-5p also plays a regulatory role in the malignant behavior of tumor cells by regulating tumor microenvironment. MiR-1207-5p is capable of suppressing the proliferation, differentiation and migration of lung cancer A549 cells. Meanwhile, it also inhibits the activation of signal transducer and activator of transcription 3 and protein kinase B signaling pathways. Additionally, miR-1207-5p overexpression can also suppress angiogenesis of human umbilical vein cells and regulate M2 phenotype of macrophages. MiR-1207-5p inhibits the metastasis of A549 cells in nude mice. Further studies have found that the regulatory effects of miR-1207-5p on lung cancer cells, endothelial cells and macrophages may be related to its targeted inhibition of colony stimulating factor 1 (CSF1)[19]. In this work, it was demonstrated for the first time that miR-1207-5p was highly expressed in necrotic femoral head tissue and peripheral blood of SNFH patients. Moreover, its expression level was inversely proportional to Harris Hip score. The SNFH rat model further revealed that the regulatory effect of miR-1207-5p on SNFH was related to its effect on epidermal growth factor receptors and cells. However, there were still some shortcomings in this research: (1) It was not verified by cell experiments, and (2) the direct target of miR-1207-5p in the SNFH model was not explored.

Conclusions

We found that the miR-1207-5p expression in SNFH was significantly increased. Meanwhile, its effect on the femoral head tissue of SNFH patients might be related to targeted inhibition of VEGF. In addition, miR-1207-5p was expected to become a new target for clinical prevention and treatment of SNFH.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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