

miR-124 participates in the proliferation and differentiation of brain glioma stem cells through regulating Nogo/NgR expression

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Abstract. The effect of miR-124 on the proliferation and differentiation of brain glioma stem cells and Nogo/NgR signaling pathway were investigated. miR-124 mimic, miR-124 inhibitor and miR-control expression vector were designed and produced to transfect U87 glioma stem cells. The results of transfection were tested via RT-qPCR and the expression of protein was detected by western blot analysis. Cell proliferation was detected by MTT proliferation and the proportion of CD133⁺ cells was detected by immunomagnetic beads to determine cell differentiation. The correlation between miR-124 and Nogo-A, and NgR protein expression was analyzed by Spearman correlation analysis. The relative expression of miR-124 in cells of miR-124 mimic group was significantly higher than that of miR-124 inhibitor and miR-control groups ($P < 0.05$). The relative expression of Nogo-A and NgR protein in cells of the miR-124 mimic group was significantly lower than that of miR-124 inhibitor and miR-control groups ($P < 0.05$). Absorbance values of the cells in the miR-124 mimic and miR-control groups were significantly lower than those in the miR-124 inhibitor group at each time point ($P < 0.05$), while the values of the cells in the miR-124 mimic group were significantly lower than that in miR-control group ($P < 0.05$). The level of CD133⁺ cells in miR-124 mimic group was significantly lower than that in miR-124 inhibitor and miR-control groups ($P < 0.05$), while the level of CD133⁺ cells in miR-124 inhibitor group was higher than that in miR-control group ($P < 0.05$). Correlation analysis revealed that there was a negative correlation between miR-124 and the expression of Nogo-A and NgR protein ($P < 0.05$). miR-124 may participate in the differentiation of brain glioma stem cells through the Nogo/NgR pathway, which may bring a new direction for the clinical treatment of brain glioma.

Introduction

Brain glioma is the most common primary malignant tumor of intracranial tumors, accounting for approximately 45% of all intracranial tumors. The prognosis of brain glioma patients is generally poor, and with the increase of malignancy, the prognosis is worse (1,2). Findings have shown that the median survival time of patients with grade IV glioblastoma multiform is only 1 year, and the 5-year survival rate is less than 5%, being one of the malignant tumors with the highest human mortality (3). Surgical treatment is the most effective treatment for brain gliomas. With the development of molecular-targeted therapy, the survival of brain glioma patients has been improved effectively, but the overall therapeutic effect is still not ideal and the occurrence and development mechanism of brain glioma is not clear yet (4,5). Therefore, it is very important to continue to study the changes of molecular level in brain gliomas and to find new therapeutic targets for the clinical treatment of gliomas.

In 2008, it was reported that miR-124 could inhibit the proliferation of glioblastoma multiform by affecting CDK6 to promote the differentiation of brain glioma stem cells (6). However, little further research has been carried out on this issue. Thus, the mechanism of miR-124 regulating brain glioma stem cell differentiation is not fully understood, and its role has not been further verified. The Nogo family has three subtypes, Nogo-A, Nogo-B and Nogo-C, which can inhibit the growth and reconnection of synapses in central nervous system (CNS) diseases. Nogo-A is the longest isomer, enriched in CNS, while NgR is the receptor of Nogo (7). Nogo/NgR signaling pathway has been reported to be related to brain injury, and the inhibition of its activation can reduce the death of microglial cells (8). Effect of miR-124 on the proliferation and differentiation of U87 glioma stem cells and its effect on Nogo/NgR signaling pathway were analyzed in this study to explore its mechanism of action.

Materials and methods

Cell sources. Brain glioma cell U87 MG-Luc2 was purchased from ATCC (ATCCHTB-14-LUC2), hereinafter referred to as U87 cells and has been authenticated by STR profiling (Beijing Microread Genetics Co., Ltd.). The cells were cultured in DMEM complete medium containing 10% fetal calf serum

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(Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd.) with a culture condition of 37°C, 5% CO₂ and 95% relative humidity.

This study was approved by the Ethics Committee of Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Art and Science (Xiangyang, China).

Cell passage. When cell attachment growth density of U87 reached 90%, the cells were digested with 0.25% trypsin. U87 cells were transferred into DMEM medium and cultured in incubators at 37°C, 5% CO₂ then synaptic retraction was observed under a microscope. The culture was collected for the third generation use.

Isolation of U87 brain glioma stem cells. U87 cells with an attachment growth density of 90% were collected and digested with 0.25% trypsin. The U87 brain glioma stem cells were sorted by adding tumor stem cell culture medium (Jiangsu Promocell Biotechnology Co., Ltd.). The medium was replaced every two days, centrifugation was performed at 800 x g for 5 min at 4°C before replacement, and then half of the medium was replaced. After 3 weeks, the suspension cells were collected and passaged in accordance with the above method.

Construction and transfection of miR-124 expression vector. The miR-124 mimic, miR-124 inhibitor and miR-control expression vectors were designed and produced by Shanghai Gene Pharmaceutical Technology Co., Ltd. The cells were digested by trypsin for 24 h before transfection, and U87 stem cells were transfected when the cells were fused to approximately 80%, the specific steps referred to the specification of the kit. The cells were cultured in 37°C, 5% CO₂ incubator for 48 h, and the medium was changed every 6 h. The transfection results were detected by RT-qPCR. Lipofectamine™2000 transfection kit was purchased from Shanghai Yanjin Biotechnology Co., Ltd.

RT-qPCR. The concentration of cell suspension was adjusted to 1x10⁷, and the total RNA was extracted by adding suspension and TRIzol lysate at 3:1. After extraction, 1.5% agarose gel electrophoresis was used to analyze the integrity of RNA, micro-amount of nucleic acid analyzer was used to detect the purity of extracted RNA. A260/A280 value between 1.8 and 2.0 was considered to meet the experimental requirements. RT-qPCR reaction was performed after the completion of RNA extraction. Reverse transcription system: oligo dt primer 1.0 µl, 5X PrimeScript Buffer 4.0 µl, 2.5 U/µl polyA polymerase 1 µl, dNTP mixture 1.0 µl, total RNA 2 µg, RNase-free distilled water was added to 10 µl. Reaction condition was: 40°C for 15 min, 85°C for 5 sec; PCR amplification was carried out after the completion of reverse transcription reaction. PCR amplification system was: cDNA template 2 µl, SYBR-Green Mix 25 µl, upstream primer 0.5 µl, downstream primer 0.5 µl, double-steamed water was added to 50 µl. PCR reaction program was: 30 cycles of predenaturation at 95°C for 3 min, denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 60 sec, elongation at 72°C for 5 min after the cycle was complete. *GAPDH* was used as the internal parameter of the reaction. Samples were set in triplicate for each test, and 2^{-ΔCq} was used to analyze the results (9).

The primer sequence was designed and produced by Hepeng (Shanghai) Biotechnology Co., Ltd. (Table I).

Western blot analysis. The expression of Nogo-A and NgR protein was detected by western blot analysis. The total protein was extracted from the cell by RIPA total protein lysate (Wuhan Aspen Biological Company) and the concentration of extracted protein was determined by BCA, and the BCA protein quantitative detection kit was purchased from Beyotime Institute of Biotechnology. Then, 40 µg extracted protein solution was used for SDS-PAGE electrophoresis, and buffer was added at a 1:4 dilution. β-actin protein was used as an internal reference, the concentration gel electrophoresis was performed at a constant 80 V for 40 min, and separation gel electrophoresis was performed at a constant 120 V for 90 min. Trans-membrane was conducted at a constant 100 V for 100 min and blocked at 37°C for 60 min after the completion of electrophoresis. After trans-membrane, the immunological reaction was carried out. Incubation was performed with primary rabbit anti-human Nogo-A polyclonal antibody (1:200; cat. no. 10740-1-AP; ProteinTech Group, Inc.) at 4°C for 16 h. It was washed the next day in lukewarm water 3 times, 10 min each time with PBS. Then, incubation was performed with secondary goat anti-rabbit polyclonal IgG (1:500; cat. no. SA00001-2; ProteinTech Group, Inc.) at 37°C for 60 min. ECL luminescent reagent was used for visualization and fixation. QuantityOne software was used to analyze the strip after film scanning. Protein relative expression level was calculated as: band gray value/internal parameter gray value. Western blot detection kit was purchased from Beyotime Institute of Biotechnology.

MTT proliferation in vitro. U87 brain glioma stem cells were prepared into a single cell suspension of 4x10⁶/ml, performed routine vaccination, and cultured in 96-well cell culture plate. Then, 20 µl MTT solution (5 mg/ml) was added after 6 h, and cells were cultured at 37°C for 4 h continuously. Supernatant containing impurities was removed, dimethylsulfoxide was added, and the plate was placed on a horizontal vibration table. The absorbance values at wavelength 570 nm were measured at 12, 24, 48 and 72 h. The MTT test kit was purchased from Shanghai LMAI Bioengineering Co., Ltd.

Detection of differentiation of U87 brain glioma stem cells by immunomagnetic beads. U87 brain glioma stem cells were prepared into a single cell suspension of 1x10⁶/ml. CD133⁺ cells were separated by immunomagnetic beads, washed, re-suspended, counted in serum-free medium, and the cell morphology was observed. The immunomagnetic beads kit was purchased from Shanghai Chem Biotechnology Co., Ltd.

Statistical analysis. SPSS19.0 [AsiaAnalytics (formerly SPSS China)] was used for statistical analysis. Measurement data were expressed as mean ± standard deviation. ANOVA and the LSD post hoc test were used for multigroup comparison. The t-test was used for comparison between two groups and χ² test was used for rate comparison. The correlation between miR-124 and Nogo-A, and NgR protein expression was analyzed by Spearman's correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Table I. Primer sequences.

Gene name	Forward primer	Reverse primer
miR-124	5'-GCTAAGGCACGCGGTG-3'	5'-GTGCAGGGTCCGAGGT-3'
GAPDH	5'-CGGAGTCAACGGATTGTGTCGTAT-3'	5'-AGCCTTCTCCATGGTGGTGAAGAC-3'

Table II. Expression of Nogo-A and NgR protein in U87 brain glioma stem cells.

Item	Nogo-A protein	NgR protein
miR-124 mimic	0.392±0.011	0.533±0.012
miR-124 inhibitor	0.569±0.013 ^a	0.711±0.014 ^a
miR-control	0.483±0.012 ^{a,b}	0.601±0.014 ^{a,b}
F	162.463	135.470
P-value	<0.05	<0.05

^aCompared with miR-124 mimic group, P<0.05; ^bCompared with miR-124 inhibitor group, P<0.05.

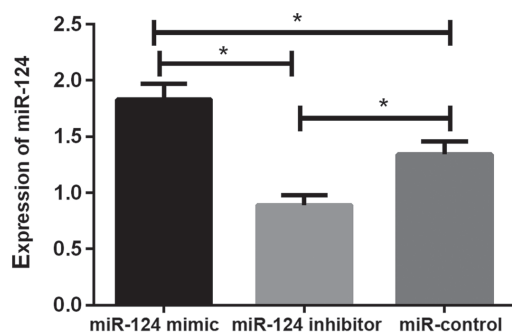


Figure 1. Relative expression of miR-124 in U87 brain glioma stem cells after transfection. The RT-qPCR results showed that the relative expression of miR-124 in cells of miR-124 mimic group was significantly higher than that of miR-124 inhibitor and miR-control groups, while miR-124 inhibitor group was lower than miR-control group. *P<0.05.

Results

Relative expression of miR-124 in U87 brain glioma stem cells after transfection. The relative expression levels of miR-124 in the miR-124 mimic, miR-124 inhibitor and miR-control groups were 1.83±0.14, 0.89±0.09 and 1.34±0.12, respectively. There was statistical difference between the three groups (P<0.05). The relative expression of miR-124 in cells of miR-124 mimic group was significantly higher than that of miR-124 inhibitor and miR-control groups (P<0.05), while the relative expression of miR-124 in cells of miR-124 inhibitor group was lower than that of miR-control group (P<0.05; Fig. 1).

Expression of Nogo-A and NgR protein in U87 brain glioma stem cells after transfection. The relative expression levels of Nogo-A protein in the miR-124 mimic, miR-124 inhibitor and miR-control groups were 0.392±0.011, 0.569±0.013 and 0.483±0.012, respectively, the relative expression levels of NgR protein was 0.533±0.012, 0.711±0.014 and 0.601±0.014,

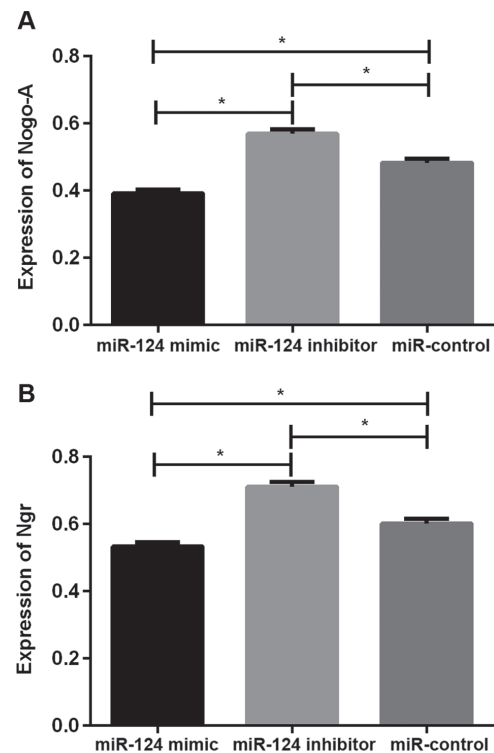


Figure 2. (A) Expressions level of Nogo-A protein in U87 brain glioma stem cells after transfection. The relative expression of Nogo-A protein in cells of miR-124 mimic group was significantly lower than that of miR-124 inhibitor and miR-control groups, while the miR-124 inhibitor group was higher than miR-control group. (B) Expression level of NgR protein in U87 brain glioma stem cells after transfection. The relative expression of NgR protein in cells of miR-124 mimic group was significantly lower than that of miR-124 inhibitor and miR-control groups, while the miR-124 inhibitor group was higher than miR-control group. *P<0.05.

respectively. There was significant difference in Nogo-A and NgR protein between the three groups (P<0.05), the relative expression of Nogo-A and NgR protein in cells of miR-124 mimic group were significantly lower than those of miR-124 inhibitor and miR-control groups (P<0.05), while the relative expression of Nogo-A and NgR protein in cells of miR-124 inhibitor group were higher than those of miR-control group (P<0.05; Table II and Fig. 2).

Detection results of U87 brain glioma stem cells by MTT proliferation in vitro. The results of MTT proliferation in vitro showed that the absorbance values in the three groups were significantly different at each time point (P<0.05). The absorbance values of the cells in the miR-124 mimic and miR-control groups were significantly lower than those in the miR-124 inhibitor group at each time point (P<0.05), while the values of the cells in the miR-124 mimic group was significantly lower than that in miR-control group (P<0.05; Fig. 3).

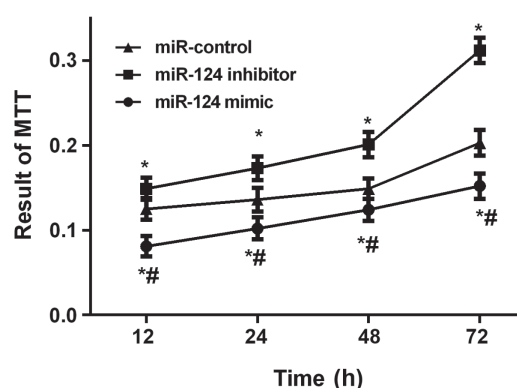


Figure 3. Detection results of U87 brain glioma stem cells by MTT proliferation *in vitro*. The absorbance values of the cells in the miR-124 mimic and miR-control groups were significantly lower than those in the miR-124 inhibitor group at each time point, while the miR-124 mimic group was significantly lower than miR-control group. * $P < 0.05$, compared with miR-control group; # $P < 0.05$, compared with miR-124 inhibitor group.

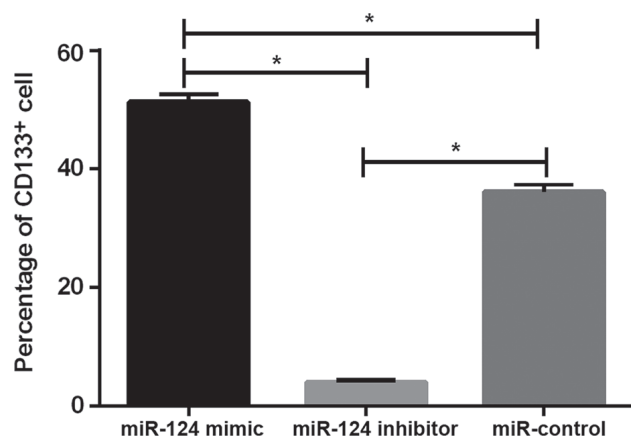


Figure 4. Detection results of CD133⁺ U87 brain glioma stem cells. The level of CD133⁺ cells in miR-124 mimic group was significantly lower than that in miR-124 inhibitor and miR-control groups, while the miR-124 inhibitor group was higher than miR-control group. * $P < 0.05$.

Detection results of CD133⁺ U87 brain glioma stem cells. The levels of CD133⁺ cells in the miR-124 mimic, miR-124 inhibitor and miR-control groups were 3.98 ± 0.45 , 36.12 ± 1.34 and $51.25 \pm 1.48\%$, respectively. There was statistical difference between the three groups ($P < 0.05$), the level of CD133⁺ cells in miR-124 mimic group was significantly lower than that in miR-124 inhibitor and miR-control groups ($P < 0.05$), while the level of CD133⁺ cells in miR-124 inhibitor group was higher than that in miR-control group ($P < 0.05$; Fig. 4).

Correlation analysis. The results of correlation analysis showed that there was a negative correlation between miR-124 and the expression of Nogo-A ($r = -0.855$, 95% CI: -0.9908 to -0.7954 , $P < 0.001$) and NgR ($r = 0.825$, 95% CI: -0.9845 to -0.6776 , $P < 0.001$; Fig. 5).

Discussion

With the development of molecular biology and related treatments, an increasing number of scholars have paid attention to the role of miRNA in tumors. miR-124 plays an important role

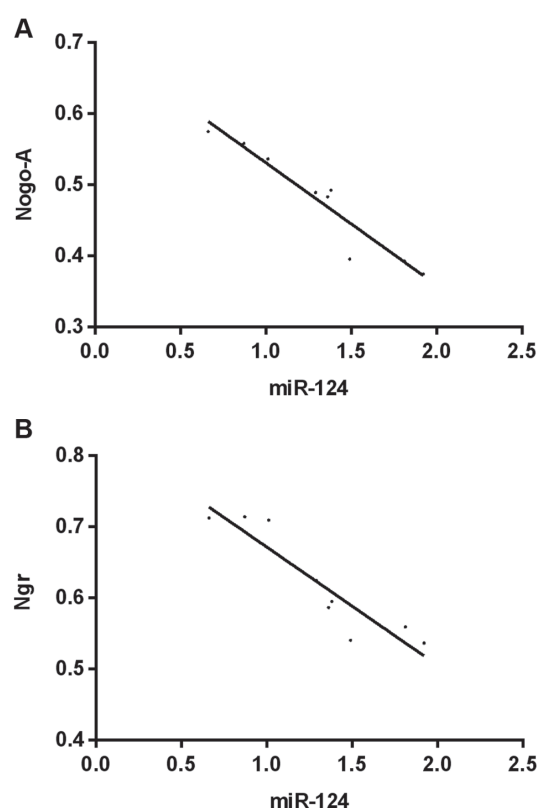


Figure 5. (A) Correlation between miR-124 and Nogo-A protein expression level. $r = -0.855$, 95% CI: -0.9908 to -0.7954 , $P < 0.001$; (B) Correlation between miR-124 and NgR protein expression level. $r = 0.825$, 95% CI: -0.9845 to -0.6776 , $P < 0.001$.

in neural development, but is rarely reported in gliomas. Some studies have found that the downregulation and upregulation of miR-124 expression in brain glioma can play the role of tumor suppressor gene (10), but the mechanism of miR-124 is not fully understood yet. There have been few reports on the Nogo/NgR pathway, reporting that reducing Nogo-A expression can protect brain damage and promote neuronal regeneration (11,12), while brain gliomas develop from carcinogenesis of spinal cord neurons (13). Therefore, whether the Nogo/NgR pathway plays a role in brain glioma and whether it is a target for miR-124 in the differentiation of brain glioma stem cells were investigated in this study in order to provide a new direction for clinical treatment.

U87 brain glioma cells were used for screening of brain glioma stem cells because of their ability to grow into spheres, which can be used to screen stem cells that meet the experimental needs (14,15). Then the overexpression vector (miR-124 mimic), underexpression vector (miR-124 inhibitor) and blank vector (miR-control) of miR-124 were constructed to transfect U87 brain glioma stem cells and to regulate the expression level of miR-124. The RT-qPCR results showed that the relative expression of miR-124 in cells of miR-124 mimic group was significantly higher than that of miR-124 inhibitor and miR-control groups, while the relative expression of miR-124 in cells of miR-124 inhibitor group was lower than that of miR-control group, which indicated that the transfection was successful. Then the expression levels of Nogo, and NgR protein were detected and found that with the increase of miR-124 expression level, the expression level of NgR protein decreased,

and correlation analysis also revealed that there was a negative correlation between miR-124 and the expression level of Nogo and NgR protein, which suggested that there might be some regulatory relationship between miR-124 and Nogo/NgR pathway. CD133 is recognized as a surface marker of tumor stem cells, which is not expressed on the surface of differentiated tumor cells (16). Therefore, expressing the quantitative proportion of CD133 cells can reflect the differentiation of stem cells to some extent. Analytic results of the proliferation of U87 brain glioma stem cells showed that cells with high miR-124 expression level had a low proliferative ability. The analytic results of the differentiation of U87 brain glioma stem cells showed that U87 brain glioma stem cells with high miR-124 expression had a low proportion of CD133⁺ cells, indicating a high degree of the differentiation. These results are similar to those reported in related studies: miR-124 can promote the differentiation of brain glioma stem cells (6,10).

Brain glioma stem cells, a special subgroup of cells, are the source of brain glioma formation with a potential to differentiate into brain glioma cells (17). Differentiation is also an important feature of brain glioma stem cells and brain glioma cells. Brain glioma stem cells have no tumorigenic ability and their infinite proliferative ability also becomes limited after differentiation into brain glioma cells (18,19). Therefore, promoting the differentiation of brain glioma stem cells is also an important method for the treatment of brain glioma. On the other hand, brain glioma stem cells are more tolerant than brain glioma cells in the face of radiotherapy and chemotherapy, and their DNA repair ability is also stronger than that of brain glioma cells. Therefore, brain glioma stem cells are a new research direction in the treatment of brain glioma (20). At present this kind of treatment is still in the exploratory stage. miR-124 was first reported to participate in the differentiation of glioma stem cells in 2008, it was found that increased expression of miR-124 promoted the differentiation of brain glioma stem cells (6), but there are few reports on miR-124 and brain glioma stem cell differentiation since then. It has been reported that miR-124 can regulate the differentiation of astrocytes and other nerve cells (21). The finding verified the regulation effect of miR-124 on the differentiation of brain glioma stem cells to some extent. Nogo-A has been reported to inhibit the invasion and migration of brain glioma cells (22). Consequently, there is a close relationship between Nogo, NgR and tumor formation. The above results also improve the credibility of our conclusions to some extent.

However, this study also has some shortcomings. Brain gliomas are diverse (23). In addition, U87 brain glioma cells are only one of these subtypes; thus, the establishment of a study in other gliomas is needed for further verification. Furthermore, *in vitro* experiments cannot simulate the complex tumor microenvironment *in vivo*. Therefore, we hope that this study can promote further research in this field.

In conclusion, miR-124 may participate in the differentiation of brain glioma stem cells through the Nogo/NgR pathway, which may bring a new direction for the clinical treatment of brain glioma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YM was responsible for construction and transfection of miR-124 expression vector and PCR, as well as for drafting the manuscript. FS and YZ helped with western blot and MTT assay. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Art and Science (Xiangyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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