CHAPTER THREE

New advances on the inhibition of Siwei Xiaoliuyin combined with Temozolomide in glioma based on the regulatory mechanism of miRNA21/221

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Abstract

Objective: To provide evidence for the mechanism of Chinese medicine to treat glioma. We observe the effects of Si wei xiao xiu yin combined with chemotherapy on the growth of subcutaneous xenografts in nude mice and the expression of miRNA-21 and miRNA-221 in tumor tissues.

Methods: The subcutaneous transplantation model of nude mice was established by subcutaneous inoculation of glioma U87 cell suspension. They were randomly divided into saline group, traditional Chinese medicine group, temozolomide group and traditional Chinese medicine combined with temozolomide group to observe the changes in body weight, and the tumor weight, length, short diameter, volume of mice. The relative expression levels of miRNA-21 and miRNA-221 in tumor tissues were detected by qRT-PCR, and the differences between groups were compared.

Results: After 28 days of gavage, the tumor growth of the other three groups was slower than that of saline group, and the difference was most significant in the combination group ($P = 0.008 < 0.05$), besides, the relative expression of the three groups of miRNA-21 and miRNA-221 was significantly inhibited compared with saline group, and the difference was significant in the combination group ($F = 8.918$, $P = 0.010 < 0.05$).

Conclusion: To some extent, Si wei xiao xiu yin combined with temozolomide can inhibit the growth of subcutaneous xenografts in glioma nude mice. The mechanism may be related to the inhibition of miRNA-21 and miRNA-221 expression.

1. Introduction

Glioma is the most common primary central nervous system malignancy, occurring at any age, more common in adults, with the highest mortality rate among all brain malignancies. At present, gliomas are treated in a variety of ways, including surgical resection, radiotherapy, chemotherapy, etc. But the prognosis of glioma patients is still poor, with a median survival rate of only 14.6 months (Ostrom, Gittleman, Stetson, et al., 2015). And the cause of glioma, research shows that (Yeru, Xiangmei, Min, et al., 2017) essence is the abnormality of many genes. Mutation loss of tumor suppressor gene and overexpression of proto-oncogene may explain the occurrence

and development of glioma at the molecular level. Therefore, scholars try to find an effective way to treat glioma from the perspective of glioma-related abnormal genes and their mechanisms of action.

miRNAs are endogenous single-stranded noncoding small RNAs of approximately 23 nucleotides in length. They bind to the 3′-untranslated region (3′-UTR) of the target gene mRNA, regulate multiple gene expression, and participate in cell proliferation, migration, differentiation and apoptosis. Studies have found that multiple miRNAs are abnormally expressed in gliomas (Ye, Wei, Zhang, et al., 2017). It affects the malignant grade of glioma and prognosis of patients by regulating antitumor factors, regulating angiogenesis, cell cycle and apoptosis (Ames, Halushka, & Rodriguez, 2017). The expression levels of miRNA-21 and miRNA-221 are closely related to the invasion, migration and treatment of glioma. Studies have confirmed that (Chan, Krichevsky, & Kosik, 2005) miRNA-21 is overexpressed in highly malignant brain tumors and is positively correlated with the pathological grade of glioma; another meta-analysis which focused on the diagnostic performance of miRNAs found that (Zhang, Pang, Xin, et al., 2016) plasma miRNA-221/222 levels were significantly up-regulated in glioma patients and correlated with prognosis.

Traditional Chinese medicine treatment of glioma emphasizes the overall concept. It is advocated to achieve the role of righting and suppressing tumor by regulating the overall state of the patient (Ruobing & Zhiyun, 2018). It has a good clinical effect on hair loss and gastrointestinal reactions caused by surgical trauma and radiotherapy and chemotherapy. Si wei xiao liu yin is a clinically effective prescription, which consists of curcuma, gecko, *Solanum nigrum*, tuckahoe. Previous studies found that (Zhang, Li, Tan, et al., 2017), curcuma have certain antiglioma effects, and its mechanism may be related to inhibition of tumor angiogenesis factors VEGF, Ang-2 and up-regulation of TSP-1 expression. Gecko extract can inhibit the proliferation of C6 cells, which may cause apoptosis by increasing the expression of caspase-9 and AIF protein (Wenjing, Wang, Ying, et al., 2015). Huang and others also found that (Huang, Zhang, Chen, et al., 2017) Gecko water extract inhibited the proliferation of hepatoma cells in a dose-dependent and time-dependent manner, and they found in vitro that it significantly inhibited the formation of tumor spheres and the proportion of tumor stem cells. *Solanum nigrum* can also inhibit tumor growth by inhibiting cell proliferation, inducing apoptosis, arresting cell cycle, inducing autophagy, inhibiting epithelial-mesenchymal transition, inhibiting tumor metastasis, sensitizing radiotherapy and chemotherapy, and inhibiting angiogenesis

(Xia, Zheng, & Hu, 2017). Experiment has shown that (Jun, Deng, Luo, et al., 2017) sulfated polysaccharides have a more pronounced inhibition rate on Hep G2 cells and MCF-7 cells, thus confirmed that it has good antitumor activity. However, the mechanism of traditional Chinese medicine formula treatment of glioma is still under investigation.

In this study, we established a subcutaneous xenograft model of glioma U87 nude mice to observe the effect of Si wei xiao liu yin combined with TMZ on the expression of miRNA-21 and miRNA-221 in tumor tissues of nude mice. Try to explain the mechanism of action of Siwei xiao liu yin combined with TMZ suppress glioma.

2. Materials and methods

2.1 Animals and medicines

A total of 32 nude mice of the SPF clean grade were purchased from the Guangdong Medical Experimental Animal Center. All the experimental mice were 4-week-old male nude mice weighing 16–20 g saline: purchased from Guangzhou Baxter Medical Products Co., Ltd.; Si wei xiao liu yin: curcuma 15 g (Kangmei Chinese Herbal Pieces, Guangxi, 140303041), Gecko 10 g (Kangmei Chinese Herbal Pieces, Jiangsu, 140302171), *Solanum nigrum* 15 g (Kangmei Chinese Herbal Pieces, Guangdong, 140311081), tuckahoe 60 g (Kangmei Chinese Herbal Pieces, Guangxi, 140401421) purchased by Guangdong Provincial Hospital of Traditional Chinese Medicine.

The above drugs are crushed to coarse powder, add 500 mL of distilled water, soak for 30 min, boil for 30 min after boiling, take juice, add two distilled water to 300 mL, boil for 30 min after boiling, and keep about 200 mL after boiling. Save in 4 °C (the process is controlled by Chinese medicine decoction device, the amount of water and heat, to ensure the quality of each decoction). Temozolomide (TMZ): purchased from Jiangsu Tian shi li di yi Pharmaceutical Co., Ltd.; TMZ was dissolved in DMSO, and each nude mouse was intragastrically administered with 50 mg kg^{-1} d^{-1}, that is, the intragastric volume was 150 μL 10 g^{-1}.

2.2 Cells and main reagents

The human glioma U87 cell was provided by the 629 Laboratory of Cancer Center of Sun Yat-sen University. Fetal bovine serum (Gibco, USA), trypsin (Gibco, USA), DMEM (Gibco, USA), DMSO (Gibco, USA), PBS (Gibco, USA), penicillin/streptomycin (Shanghai Fumeng Gene

Biotechnology Ltd.), DEPC (Guangzhou Weijia Technology Co., Ltd.), Chloroform (Guangzhou Qiyu Chemical Instrument Co., Ltd.), Absolute Ethanol (Guangzhou Chemical Reagent Factory), TaKaRa PrimeScript RT reagent kit with Gdna Eraser (RR047A) (Beijing Zhi jie Fang yuan Technology Co., Ltd.), SYBR Premix Ex Taq II (RR820A) (Beijing Zhi jie Fang yuan Technology Co., Ltd.).

2.3 Construction of subcutaneous transplantation model of U87 glioma nude mice

The frozen cell was taken out from the liquid nitrogen, resuscitated, and cultured in an incubator at 37 °C under a saturated humidity of 5% CO_2, and the culture solution was changed in 24 h. The cells were passaged when the cells were attached and grew to over 80% on the walls of the culture flask. During the passage, the cells in the logarithmic growth phase were taken, and a cell suspension having a concentration of 10.3×10^5 cells/mL under a microscope was prepared with saline. 32 experimental mice were taken and the cell was subcutaneously inoculated into the right armpit of the mice, each inoculated with 0.2 mL each time. Tumor long diameter *a* (mm) and short diameter *b* (mm) were measured on the 7th, 14th, 21st and 28th day after inoculated. The tumor formation and tumor size changes in nude mice were observed. Here, it is considered that the human glioma cell U87 nude mouse subcutaneous transplantation tumor model is completed. The successfully constructed animal model is placed in a laminar flow rack, and the applied cage, and animal contact equipment are all used after autoclaved.

2.4 Dosing regimen for animal models

Thirty-two tumor-bearing mice were randomly divided into four groups of eight animals each. The first group was a control group, in which each mouse was intragastrically administered with 200 μL of saline; the second group was Si wei xiao liu yin group, the dosage was 200 μL of traditional Chinese medicine decoction, and continuous administration for 31 days; the third group was TMZ group, the dose was 50 mg kg^{-1} d^{-1}, and the drug was administered continuously for 7 days; the fourth group was the combination group (Si wei xiao liu yin group + temozolomide), and continued to be administered with traditional Chinese medicine for 21 days after continuous administration for 7 days. Each group of mice was intragastrically administered at a dose of 200 μL at 10:00 every day.

2.5 Tumor volume measurement

On the 7th, 14th, 21st and 28th day after inoculation, the long diameter a (mm) and short diameter b (mm) of the tumor of each group of tumor-bearing mice were measured with calipers, tumor volume was calculated by $V_n = (a^2 \times b)\ 2^{-1}$, where "$n$" represents the number of days after administration. After 28 days of administration, all the four groups of tumor-bearing mice were sacrificed (cervical dislocation method), remove the intact subcutaneous tumor tissue and weigh it, the mean values of tumor weights of the 4 groups of mice were calculated separately.

2.6 Total miRNA extraction from glioma tissues

The total miRNAs of each group were extracted according to the miRcute miRNA extraction and isolation kit step; then 1 μL of the miRNA was aspirated to measure the concentration and purity of the total miRNA using a micronucleic acid analyzer.

2.7 Reverse transcription of miRNAs in glioma tissues

Take appropriate amount of total miRNA for reverse transcription reaction. The reverse transcription primers of miRNA-21 and miRNA-221 as follows. miRNA-21: reverse transcription: GTC GTA TCC AGT GCA GGG TCC GAG, GTA TTC GCA CTG GAT ACG ACT CAA CA. miRNA-221: reverse transcription: GTC GTA TCC AGT GCA GGG TCC GAG GTA, TTC GCA CTG GAT ACG ACG AAA CCC A; miRNA-21 and miRNA-221 were subjected to reverse transcription reaction under the action of reverse transcriptase to synthesize cDNA. The 25 μL reverse transcription reaction system includes 10 μL of template RNA, 2 μL of miRNA-21/18s reverse transcription primer, 15 μL of DEP, 4 μL of 5 × reaction buffer, 1 μL of ribonuclease inhibitor (20 U/μL), 2 μL of 10 mmol/L dNTP mix, and RevertAid M-MuLV Reverse Transcriptase (200 U/μL) 1 μL. Reaction conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min.

2.8 Detection of miRNA-21 and miRNA-221 expression levels by qPCR

PCR reaction system TaqMan MiRNA Assay (20 ×) 1 μL, reverse transcription reaction product (1:15 dilution) 1.33, TaqMan 20 × Universal PCR Master Mix II 10 μL, DEPC 7.67 μL. miRNA-21: upstream primer: ACG TTG TGT AGC TTA TCA GAC TG, downstream primer: GTG

CAG GGT CCG AGG T; miRNA-221: upstream primer: GTT CGT GGG AGC TAC ATT GTC TGC, downstream primer: GTG CAG GGT CCG AGG T. Predenaturation at 95 °C for 10 min, then predenaturation at 95 °C for 10 min, denaturation at 95 °C for 15S, annealing at 60 °C for 60S, 40 cycles, using 18s RNA as internal reference. Three replicate wells per sample were set, and $2^{-\Delta\Delta Ct}$ was calculated according to the formula $\Delta Ct = [Ct(\text{target miRNA})] - [Ct(\text{internal reference})]$ and $\Delta\Delta Ct = [\Delta Ct(\text{experimental group})] - [\Delta Ct(\text{control group})]$. That is, the relative expression level of the target gene.

2.9 Statistical analysis

The data were statistically processed using statistical SPSS 19.0. The count data were analyzed by χ^2 test, the measurement data were analyzed by t test, and the comparison between grades was performed by rank sum test.

3. Results

3.1 Changes in mouse body weight within 28 days after administration

Body weight was measured every 7 days for all mice, and the measurement was as of 28 days after administration. The body weight of the four groups of tumor-bearing mice showed an increasing trend within 28 days after administration. Within 21 days after administration, the body weight of the four groups of tumor-bearing mice was roughly the same, and there was no significant difference between the groups. When the body weight of the mice was measured on the 28th day, the difference between the saline group and the temozolomide group, the saline group and the combination group was significant ($P = 0.0000$, $0.002 < 0.05$), and the difference between the other groups was not significant (Fig. 1).

3.2 Tumor volume increase in tumor-bearing mice within 28 days after administration

All tumor-bearing nude mice were used to measure the tumor volume of the mice once every 7 days, and the tumor volume increment was calculated, which was cut off to the 28th day after administration. The researchers found that within 7 days of treatment, the tumor volume of each group of mice did not differ significantly within and between groups. By day 14, between the two groups, there was a difference in tumor volume between the saline and temozolomide, the saline and the combination group ($P = 0.013$,

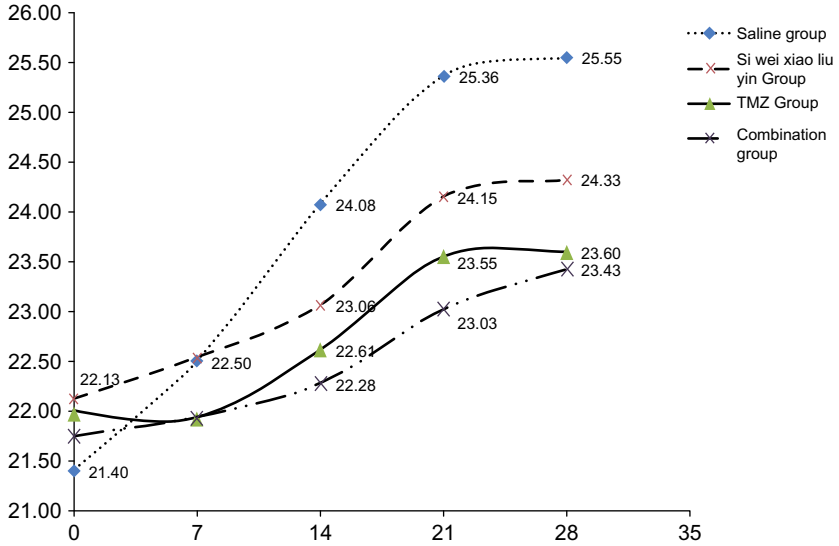

Fig. 1 Changes in body weight of nude mice at each time point in each group.

$0.008 < 0.05$). By day 21, the volume difference between the saline group and the other groups was significantly different ($P = 0.048$, 0.017, $0.012 < 0.05$). By the end of the experiment, comparison between the two groups, the saline group was significantly different from the other groups; the difference between the Si wei xiao liu yin group and the temozolomide group was not obvious; the tumor volume growth was significantly inhibited in the combination group (saline group and Si wei xiao liu yin group $P = 0.047 < 0.05$, saline group and temozolomide group $P = 0.044 < 0.05$, saline group and combination group $P = 0.031 < 0.05$, Si wei xiao liu yin group and temozolomide group $P = 0.086 > 0.05$, Si wei xiao liu yin group and combination group $P = 0.045 < 0.05$, temozolomide group and combination group $P = 0.012 < 0.05$) (Fig. 2).

3.3 Comparison of relative expression levels of miRNA-21 between groups

Independent *t*-tests were performed between the two groups. The results showed that compared with saline group, the relative expression of miRNA-21 was down-regulated in each group ($F = 4.934$, $P = 0.043 < 0.05$ compared with Si wei xiao liu yin group; $F = 9.063$, $P = 0.009 < 0.05$ with temozolomide group; and the combination group

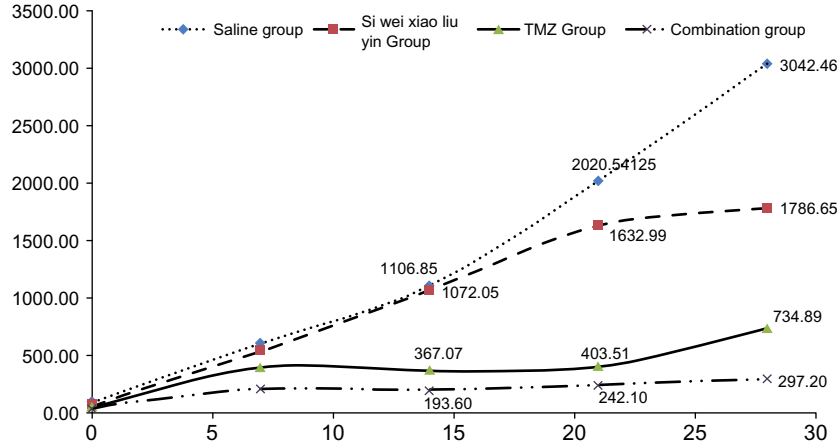

Fig. 2 Change in tumor volume at each time in each group.

F = 11.369, $P = 0.005 < 0.05$). Comparing the Chinese medicine group, the temozolomide group and the combination group, the researcher found that the relative expression of miRNA-21 was the smallest in the combination group (Si wei xiao liu yin group and temozolomide group: F = 5.914, $P = 0.029 < 0.05$; the Si wei xiao liu yin group and the combination group: F = 15.062, $P = 0.002 < 0.05$; temozolomide group and combination group: F = 6.311, $P = 0.025 < 0.05$).

3.4 Comparison of relative expression of miRNA-221 between groups

Independent *t*-tests were performed between the two groups. The results showed that compared with saline group, the relative expression of miRNA-221 was down-regulated in each group (with Si wei xiao liu yin group F = 4.705, $P = 0.048 < 0.05$; With the temozolomide group, F = 7.631, $P = 0.015 < 0.05$; and the combination group, F = 8.918, $P = 0.010 < 0.05$). Comparing Si wei xiao liu yin group, the temozolomide group and the combination group, the researchers found that the relative expression of miRNA-221 was the smallest in the combination group (Si wei xiao liu yin group and temozolomide group: F = 5.426, $P = 0.035 < 0.05$; Si wei xiao liu yin group and the combination group: F = 11.339, $P = 0.005 < 0.05$; temozolomide group and combination group: F = 5.600, $P = 0.033 < 0.05$) (Tables 1 and 2, Fig. 3).

Table 1 Real-time-PCR(Ct) results comparison ($\overline{\chi} \pm s$).

Groups	miRNA-21	miRNA-221	Internal reference 18s
Saline group	21.35 ± 1.34	18.54 ± 1.01	12.89 ± 0.45
Si wei xiao liu yin group	21.82 ± 1.28	18.72 ± 1.25	12.18 ± 0.54
TMZ group	23.04 ± 1.26	19.65 ± 0.93	11.38 ± 0.45
Combination group	27.93 ± 1.24	24.91 ± 1.82	13.95 ± 0.55

Table 2 Comparison of real-time-PCR ($2^{-\triangle\triangle Ct}$) results comparison ($\overline{\chi} \pm s$).

Groups	miRNA-21	miRNA-221
Saline group	1.00 ± 0.17	1.00 ± 0.20
Si wei xiao liu yin group	0.44 ± 0.06	0.54 ± 0.05
TMZ group	0.12 ± 0.02	0.16 ± 0.02
Combination group	0.02 ± 0.01	0.02 ± 0.01

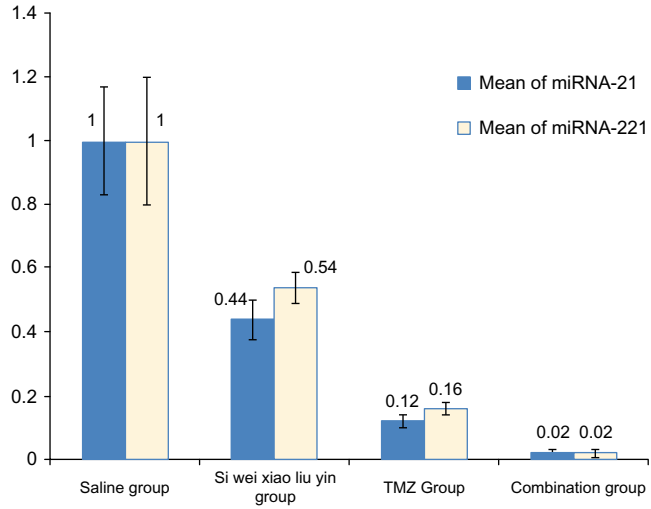

Fig. 3 $2^{-\triangle\triangle Ct}$ histogram of miRNA-21, 221.

4. Discussion

miRNAs are a class of small noncoding RNAs that regulate gene expression at posttranscriptional levels and have functions that regulate many physiological and pathological processes, including apoptosis, proliferation,

and differentiation (Van Wynsberghe, Chan, Slack, et al., 2011). An experimental study on the expression of miRNA-221/222 in human glial cells and tissues confirmed that the expression of miRN221/222 in glioma tissues and cells are significantly higher than that in normal brain tissues and normal cells. Highly malignant glioma tissues express significantly higher miRNA-221 and miRNA-222 than low-grade glioma tissues, its expression was positively correlated with the survival rate of glioma cells, and was closely related to the grading and prognosis of glioma (Xue, Wang, Yue, et al., 2017). miRNA-21 is an oncogene that has been reported to be overexpressed in a variety of tumors over the past few years (Bonci, 2010; Cheng & Zhang, 2010; Li, Liang, Xu, & Zou, 2012). A meta-analysis of miRNA-21 indicates that extracellular miRNA-21 is highly expressed in the cerebrospinal fluid of glioma patients and may be a potential biomarker for the diagnosis of brain cancer, its mechanism is related to the involvement of TGF-β/Smad3 signaling in mediating the release of miR-21 in glioma cells (Qu, Lin, Pang, et al., 2016). Yang and others found that (Yang, Yang, Tong, et al., 2017) down-regulation of miRNA-221 expression in SHG-44 cells can inhibit cell proliferation, migration and temozolomide tolerance, and both tumor tissue and exosome levels miRNA-221 expression is increased with the glioma grade. The experiment confirmed that the DNM3 gene is the target of miRNA-221, RELA induces the expression of miRNA-221, and RELA/miRNA-221 can be used as a target for the diagnosis and treatment of glioma.

The study found that, under the intervention of Si wei xiao liu yin combined with TMZ, the expression of miRNA-21 and 221 in the tumor tissue of tumor-bearing mice was inhibited, and the tumor volume was also significantly smaller than that of the control group. It was confirmed that Si wei xiao liu yin combined with TMZ had a certain synergistic effect on inhibiting glioma. Its antitumor mechanism may be related to the inhibition of miRNA-21 and miRNA-221 expression. However, the mechanism of down-regulation of miRNA-21 and miRNA-221 by Chinese medicine remains to be further studied.

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