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MiR-324-5p inhibits proliferation of glioma by target regulation of GLI1

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Abstract. – OBJECTIVES: To study the effects of the miR-324-5p on the glioma cells proliferation via the targeted regulation of the glioma-associated oncogene 1.

METHODS: The luciferase reporter gene was used to test whether the glioma-associated oncogene 1 was the target of the miR-324-5p microRNA. The glioma-associated oncogene 1 expression was detected by Western blot. The proliferation and cell cycle were evaluated by MTT assay and flow cytometry.

RESULTS: The glioma-associated oncogene 1 is a target of the miR-324-5p. An over-expressed miR-324-5p could reduce the cell survival rate and increase the G1/G0 phase rate in the glioma cell lines.

CONCLUSIONS: The miR-324-5p can inhibit proliferation of the glioma cells via the targeted regulation of the glioma-associated oncogene 1.

Key Words:
Glioma-associated oncogene 1, miR-324-5p, Glioma cell.

Introduction

Micro RNAs (miRNAs) were discovered to be the endogenous non-coding small RNA molecules consisting of around 22 nucleotides. After transcription, the miRNAs regulate translation and expression of the mRNA target genes[1]. The micro RNA studies report that there is a close relationship between the micro RNA and the occurrence of tumors. The MiR-324-5p was confirmed to be active in cell proliferation, apoptosis and invasion in various cancers, such as lung and ovarian cancer and lymphoma. However, the related mechanism still needs to be clarified. This study explores the targeted regulation relationship of the miR-324-5p with the glioma-associated oncogene 1 (GLI1) in glioma cells, and investigates its function in cell proliferation.

Materials

Human glioma cell strains of U87 and LN229 were purchased from a cell bank at the Chinese Academy of Sciences. Transfection reagent Lipo2000 was purchased from Invitrogen (St. Louis, MO, USA). The MTT kit was purchased from Nanjing KeyGEN Company (Nanjing, China). The primary and secondary antibodies used in the Western blot were purchased from Santa Cruz Biotechnology Company (Santa Cruz, CA, USA). The PGL3 plasmid was purchased from Promega Company (Madison, WI, USA). Luciferase activities were detected by dual luciferase assay kit purchased from the Promega Company.

Methods

Cell culture andexperimental grouping

The U87 and LN229 cells were cultured in DMEM culture medium containing 10% fetal bovine serum (FBS) and placed in 5% CO_2 incubation chamber at 37°C. Experimental groups were divided into blank, control and miR-324-5p transfected groups.

Oligonucleotide transfected into glioma cells in vitro

The miRNA mimic was an analog of the endogenous miRNAs that was synthesized by chemical synthesis. The mimic could enhance the function of the endogenous miRNA. The miR-324-5p mimic was synthesized by Shang Hai GenePharma Company. The sequences of miR-324-5p mimic: 5′-UGUGGU-UACGGGAUCCCCUACGC-3′, nonsense sequences: 5′-UUCUCCGAACGUGUCACGUTT-3′. Glioma cells of each group with concentration of (2-5) × 10^5/ml were seeded in six-well plates and incubated at 37°C, in 5% CO_2 incubation for 24 hours. The Lipo2000 transfection

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reagent and miR-324-5p mimic in 20 μM concentration were mixed in a ratio of 1:1 by volume, incubated in serum-free medium for 20 minutes, and then added to the wells and cultured. After six to eight hours, the serum-free medium was replaced by DMEM of 10% FBS for cell culture.

Growth of Glioma Cell Analyzed by MTT Assay

Oligonucleotide was transfected into glioma cells in each group. After transfection, the glioma cells (1×10^4 cells per well) were seeded in 96-well plate, 100 μl culture solution was added in each well and the glioma cells were cultured at 37°C, in 5% CO_2 incubation for 12,24, 48 and 96 hours. A quantity of 50 μl 1 × MTT solution was added in each well at four hours before testing. At checkpoint, the culture solution was discarded. Blue-violet crystals dissolved in 150 μl dimethyl sulfoxide (DMSO) were added to each well. After 10 minutes, the absorbency of each well was measured by microplate reader.

Cycle of Glioma cell Detected by Flow Cytometry

The glioma cells from each group were cultured in six-well plate, trypsinized at 48 hours after transfection of the miR-324-5p mimic, and then collected by centrifuging for five minutes 1500 r/minute. The cells were washed with phosphate buffered saline (PBS), centrifuged and ruptured in 70% alcohol for one hour. The supernatant was discarded after centrifuging. The cells were washed with PBS, stained by a mixture of propidium iodine (PI) and RnaseA solution, and detected by flow cytometry.

Vector Construction of Luciferase Reporter Gene and Activity Detection

According to the GLI1 sequences and the predicted result of the Target Scan software (http://www.tar-getscan.org/), the luciferase reporter gene vector including GLI13' untranslated region (3'UTR) sequences of the human miR-324-5p binding sites was designed. Upstream primer (5′-GCTCTAGAAGAGTAGGGAATCTCATCCATCACAGATCG-3′ and 5′-ATTTGCGGCCGCCTGATGCAGTTCCTTTATTATCAGGAAACAGTGT-3′) of GLI1 3′UTR including miR-324-5p binding sites was amplified by PCR (1). The PCR products were digested and transferred into pGL3 plasmid to construct the recombinant pGL3-promoter-GLI13'UTR-wild type. As the template of this recombinant plasmid, complementary to the miR-324-5p binding site in GLI13'UTR sequence was taken as a deletion mutation to reconstruct the pGL3-promoter-GLI13'UTR-mutant type plasmid. The LN229 cells were seeded in 24-well plates, and the cell count for each well was 4×10^4. After 24 hours, the pRL-TK plasmid vector was used as a standard to calibrate the differences. The two types of recombinant plasmids, the miR-324-5p mimics and the nonsense sequence as the control group, were transfected using Lipo2000. After 48 hours, the cells were lysed and the fluorescence intensity was detected using the dual luciferase assay kit.

Protein Expression Levels Detected by Western Blot

The cells in each group were transfected after 48 hours, removed from the culture medium, washed with PBS, and centrifuged at the speed of 3000 r/min, for 15 minutes. Total proteins were extracted by radioimmunoprecipitation assay (RIPA) protein extraction. Protein samples were loaded in 12% SDS-PAGE gel for electrophoresis. Gels were transferred to the membrane for two hours at 4°C, run at a current of 125 mA and blocked for two hours. The membrane was incubated overnight in GLI1 primary antibody (1:1000) and washed with tris buffered saline and Tween-20 (TBST) three times, for five minutes each time. Then, the membrane was rinsed after incubation with the secondary antibody, for 1.5 hours, and imaging was performed with electronchemiluminescence (ECL) emitting solution.

Statistical Analysis

The SPSS 11.5 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the data. Measurement data were expressed as $\bar{x} \pm s$. Each experiment was repeated three times. $p < 0.05$ was considered statistically significant.

Results

GLI1 Expression Targeted Regulated by miR-324-5p

The Western blot assay showed that the GLI1 expression decreased significantly compared with the blank and control group after the miR-324-5p mimics were transfected in the glioma cell lines U87 and LN229. The miR-324-5p binding sites

were in the GLI1 mRNA 3'UTR region as predicted by Target-scan software. The binding sites were in a conservative sequence in many of the species including humans, mice, rats, dogs and chimpanzees. In order to verify that GLI1 was the target gene of the miR-324-5p, we reconstructed the wild-type and the mutant 3'UTR luciferase reporter vector of the GLI1. The assay of the luciferase reporter gene showed that after co-transfection of the miR-324-5p and the 3'UTR vector of the wild-type GLI1 in the glioma cells, the relative fluorescence intensity decreased by approximately 45.5% compared to the transfected no sense nucleotide sequence group ($p < 0.05$). However, after the co-transfection of the miR-324-5p and 3'UTR vector of the mutant GLI1 in the glioma cells, the relative fluorescence intensity had no significant difference with the group that transfected the no sense nucleotide sequence. This showed that the miR-324-5p could be combined with the GLI1 3'UTR region, thereby inhibiting its expression (Figure 1).

miR-324-5p Inhibited Glioma Cells Prolif Eration

The MTT assay analysis showed that the ratio of the U87 cells number of miR-324-5p mimics transfected groups to blank group were (95.71 ± 3.35)%, (12 hours after transfection), (91.83 ± 4.42)%, (24 hours after transfection), (75.81 ± 4.31)%, (48 hours after transfection), (63.81 ± 5.31)%, (72 hours after transfection) and (60.32 ± 4.18)%, (96 hours after transfection). However, the cell numbers in the control group showed no significant decline. In the other group, glioma cells line LN229, the results of MTT assay analysis were similar. The survival rates of the transfected groups were (94.71±3.56)%, (12 hours after transfection), (90.83±5.12)%, (24hours after transfection), (78.8±6.12)%, (48 hours after transfection), (63.83±5.12)%, (72 hours after transfection) and (61.33±6.34)%, (96 hours after transfection). The cell count in the control group showed no significant difference. The results showed that the miR-324-5p mimics the inhibited glioma cell proliferation (Figure 2).

miR-324-5p Inhibited Glioma Cell Cycle at G1/G0

Flow cytometric analysis showed that the G1/G0 phases of the U87cells were (60.43 ± 2.78)%, in the transfected group, (45.03 ± 2.04)% in the blank group and (47.51 ± 3.65)%, in the control group. The G1/G0 phases of the LN229 cells were (63.47 ± 2.17)% in the transfected group, (43.71 ± 2.72)%, in the blank group and (45.54 ± 3.04)%, in the control group. In both of these cells, the G1/G0 phas rate of transfected cells was more than the blank and the control group by 10% ($p < 0.05$). There was no significant difference between the blank and the control group.

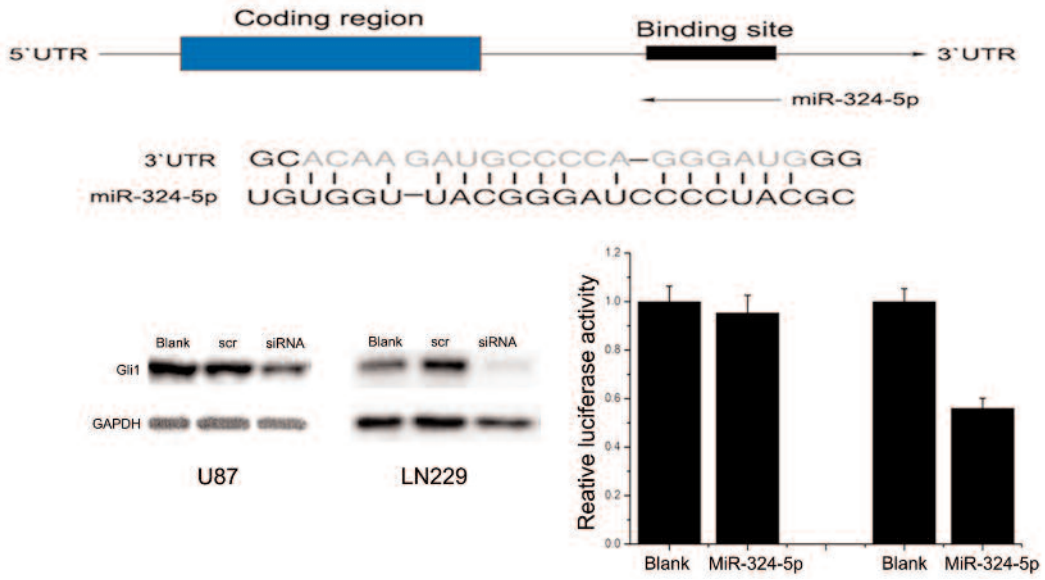

Figure 1. 4.2 miR-324-5p inhibited glioma cells proliferation.

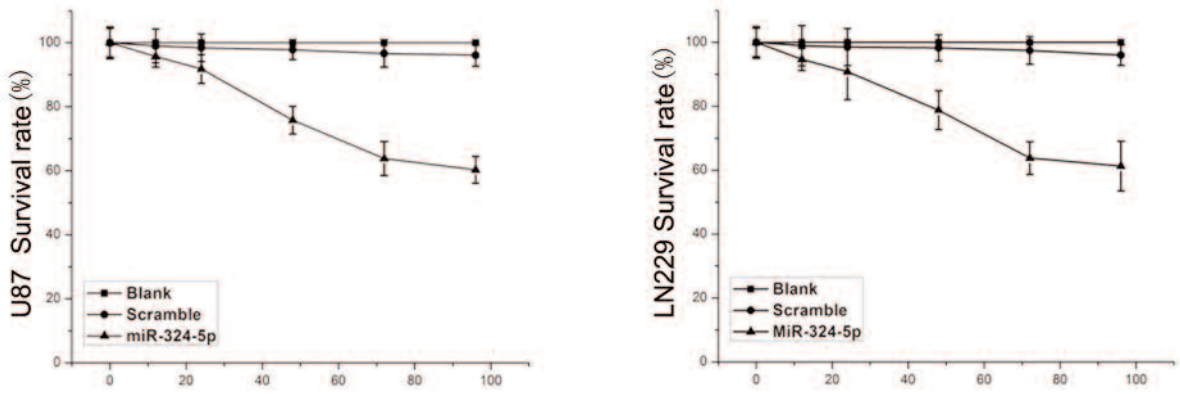

Figure 2. 3miR-324-5p inhibited glioma cell cycle at G1/G0.

Discussion

The micro RNAs were the endogenous non-coding single-stranded RNA molecules consisting of 18-25 nucleotides. The lin-4 small molecule RNA was first discovered by Lee et al[2], and was named the micro RNA. Following the base complementary principle, the micro RNA bound to the targeted mRNA3'-UTR untranslated region, caused the degradation of the targeted mRNA or blocked the translation involved in regulating the gene function of post-transcriptional level[3]. In recent years, many studies have shown that micro RNA is involved in the post-transcriptional regulation and plays an important role in both the biological development and tumorigenesis[4]. Abnormal expression of the micro RNA is closely related to the occurrence of human malignant tumors. Human glioblastoma samples were detected by Ciafre et al[5], using the gene chip technology. Compared with normal brain tissue, a number of abnormally expressed micro RNAs were found in these samples. An increase in expression of the micro RNAs were considered to play a role as a cancer gene; and some of the micro RNAs with downregulated expression played a role as tumor suppressor genes. Further studies about the functions showed that micro RNA involved in regulation of important biological processes of the glioma cells, such as cell differentiation, proliferation, apoptosis, invasion, metastasis, drug resistance and angiogenesis. It was found that over 50% of the micro RNAs positioned in the cancer-related genomic regions, including loss of heterozygosity, chromosomal amplified region and brittleness, etc. These micro RNAs played the roles of oncogenes or tumor suppressor genes, and were widely involved in tumor development, though a variety of molecular mechanisms[6-8]. Hedgehog (HH) signaling pathway involved in the occurrence and development of many types of tumors[9]. Abnormal active expression of HH/GLI1 signaling pathways were detected in the pathological specimens of the brain gliomas. With higher expression of the GLI1, proliferation index, histological grade and recurrence of tumors increasing in varying degrees. Meanwhile, it has an important impact in the biological behavior of the glioma cells, such as cell cycle and apoptosis, tolerance of chemotherapy or radiation, etc[10]. Further studies confirmed that the growth of the glioma cell is inhibited after the abnormal signaling pathway that is blocked *in vitro*. When this pathway is inhibited, the number of cancer stem cells and tumor formation is reduced. Therefore, abnormal activation of HH/GLI1 signaling pathways is one of the important molecular mechanisms of brain glioma[11].

The glioma-associated oncogene 1 is a nuclear transcription factor of the Hh signaling pathway in the GU family. The hedgehog signaling pathways included HH ligand proteins (sonic, Indian and desert hedgehogs), cross-membrane proteins (PTCH and SMO), and transcription factors (GU) and FU, SUFU, etc. There were three kinds of transcription factors (GU1, GU2, and GU3). Among these, GU1 was a transcriptional activator with strong activity[12]. It was discovered that miR-324-5p may directly act on the key gene of the HH/GLI1 signaling pathway in medulloblastoma[1]. Therefore, through the luciferase reporter gene, we discovered that the miR-324-5p could target and regu-

late the GLI1 in the glioma cells. The MiR-324-5p is transfected into the glioma cell lines, and it was discovered that the expression of the GLI1 decreased. An increase in the MiR-324-5p levels in the U87 and LN229 glioma cell lines could cause a decline in cell proliferation, block cell growth in the G1/G0 phase and induce apoptosis.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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