European Review for Medical and Pharmacological Sciences 2017; 21: 723-729

Down-regulation of miR-124 target protein SCP-1 inhibits neuroglioma cell migration

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have been widely studied as a potential cancer agent, but its efficacy does not get improvement due to poor targeting. miRNAs have been reported to play multiple roles in the development of the tumor. miR-124 is expressed in various tumor. This study aimed to elucidate the expression of miR-124 in neuroglioma cells as well as its related mechanism.

MATERIALS AND METHODS: Expression of miR-124 in neuroglioma cells was assessed by Quantitative PCR (q-PCR). Astrocytes (RA cells) were used as control group. The relationship between miR-124 and SCP-1 was explored with bioinformatics tools. Luciferase reporter assay was performed to examine the expression of miR-124 target protein SCP-1. Gene interference technology was used to regulate expression of miR-124 and SCP-1 in neuroglioma cells, and q-PCR was performed to confirm gene interference effects. Migration of miR-124 and SCP-1 in neuroglioma cell was measured by wound healing assay and cell migration test.

RESULTS: Compared with control group, the expressions of miR-124 (p=0.0015) and SCP-1 (p=0.0042) were higher in neuroglioma cells. Luciferase reporter assay proved that SCP-1 was the target of miR-124. Wound healing assay and migration test showed down-regulation of SCP-1 inhibited neuroglioma cell migration. Down-regulation of miR-124 didn't influence neuroglioma cell migration movement.

CONCLUSIONS: miR-124 and SCP-1 in neuroglioma cell were highly expressed. MiR-124 impeded the progression of neuroglioma via down-regulating SCP-1.

Key Words:
miR-124, SCP-1, Neuroglioma.

Introduction

Immature astrocytoma and glioblastoma pertain to malignant glioma, and are both common brain tumor with high invasiveness[1,2]. Current therapeutic regimen for brain tumor includes surgery, radiotherapy and chemotherapy. Despite promising development in these treatments, their efficacy and prognosis of brain tumor patients were still unsatisfactory. Previous studies[3-6] showed that median survival time was only 12 to 16 months, and prognosis of malignant glioma has not been remarkably improved. As a kind of non-coding RNA, microRNAs (miRNAs) can mediate transcription of RNA and promotes or inhibits expression of tumor genes. Current studies[7-9] showed more than 1000 miRNAs were used as biomarkers for diagnosis, prognosis and efficacy in a wide range of diseases, including cancer and diabetes. Repairing and mediating function of microRNA were proved to play a crucial role in the pathogenesis of malignant glioma in recent years. Moreover, microRNA can regulate proliferation, apoptosis, tumor invasiveness and tumor angiogenesis[10,11]. It is suggested that both over expression or down-regulation of microRNA can influence tumor treatment. For instance, over-expression of microRNA was mostly reported in neuroglioma such as miR-10b, miR-21, miR-26a, and lower-expression of miR-124 was identified in glioma stem cells and malignant glioma[12-14]. In spite of its promising therapeutic potential, microRNA was not fully understood in the field of clinical applications mainly due to complicating mechanism. Synaptonemal complex proteins 1 (SCP-1) is located at chromosome IP12-13, and its encoding protein, with a molecular weight of 111KD, contains 946 amino acids. As a member of CAT, SCP-1 was reported to be expressed in tissues of intestinal cancer and liver cancer[15,16]. Our study was aimed to reveal the relationship between miR-124 and neuroglioma, seeking to explore whether miR-124 regulated SCP-1 to influence tumor progress.

Materials and Methods

Materials

U8 glioma cell line and normal astrocytes (RA cells) were bought from the Institute of Cell Biology, Chinese Academy of Sciences (China). MTT

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kit (Gibco, Rockville, MD, USA), fetal calf serum (FCS), RPMI-1640 culture (HyClone, South Logan, UT, USA) were from Thermo-Scientific (Waltham, MA, USA). Transwell was provided by BD, the reagents of common use all bought from Shanghai Sangon Biotech (Shanghai, China).

Glioma Cell Line miR-124 Over Expression and Low Expression

U87-RA cells of logarithmic phase were seeded in 6 well cell culture plate, to keep each well 3×10^5-8×10^5 cells. 5-μl lipo2000 were added after mixing with 100-μl serum-free cultures. After incubation for 5 min, 12 μl plasmid of over-expression and low-expression (RIBOBIO) were mixed with 100 μl serum-free culture, respectively. After 5 min, the liquid was mixed for 20 min, then added with 1800 μl serum-free culture for further RNA extraction 48 h later.

Q-PCR

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) in the absence of RNAase. The primers were shown in Table I. PCR were performed by one-step RT-PCR kit (TaKaRa, Otsu, Shiga, Japan), which reverse transcribed RNA into cDNA, and followed by PCR amplification, transferred 5 μl amplification products for agarose gel assay and photographed. Reaction condition: denaturation 45 s, 94°C, renaturation 45 s, 59°C, elongation 60 s, 72°C, 35 cycles.

Western Blot

The expression of SCP-1 in transfected glioma cell was determined as follows: 100-μl glioma cells were seeded into 96 well cell plates, and each group seeded for 4 plates. The protein was extracted and qualified in room temperature by Beyotime protein extraction kit (Beyotime, Shanghai, China) and BCA Thermo-Scientific (Waltham, MA, USA). All protein samples concentration were adjusted equally to the same volume, then added with 2× bromophenol blue. The same concentration of protein and 6-μl marker were added into gel well. Initial voltage 80 V for stacking gel running and the voltage of separating was up to 120 V.

Polyvinylidene fluoride (PVDF) was blocked with 5% skim milk for 1 h after trans-membrane at 15 V for 30 minutes. Polyvinylidene fluoride (PVDF) was incubated with primary antibody (1:1000) Abcam (Cambridge, UK) over night, then washed with tris buffered saline-tween (TBST) for three times. The secondary antibody (1:5000) Abcam (Cambridge, UK) was selected to treat for 1 h. Polyvinylidene fluoride (PVDF) membrane was imaged after rinsing 5 min by tris buffered saline-tween (TBST) for 3 times.

Cell Migration Assay

100 μl glioma cells were seeded into 96 well cell plates, each group seeded for 4 plates, siRNA and NC were transfected into 70% confluent cells with transfection concentration of 100 nmol and 50 nmol respectively. After 72 h transfection, digested cells (final cell concentration 10^4/mL) were transferred into transwell up-chamber, and then 5 % fetal bovine serum (FBS) was added into down-chamber, for 24 h incubation. Transwell chamber was taken out and stained with crystal violet after washing. The total number of wear membrane cells in 5 views was counted under an inverted microscope (Olympus, Tokyo, Japan).

Wound-Healing Assay

After transfection with miR-124 or not, logarithmic U87 cells were seeded into 6 well cell plate, 3×10^5-8×10^5 cells per well. Cells were scraped by 'shape of cross' while zero, photographed after rinsing with PBS, then the same view was imaged after 6 and 12 h and the motor ability was compared by the transfection of miRNA-124.

Dual-luciferase Reporter Gene Assay

Dual-luciferase reporter gene assay: the plasmids were transfected into glioma cells. Groups were as following: miR-124 mimics+Wt SCP-1, miR-124a mimics NC+ Wt SCP-1, miR-124 mimics+Mut SCP-1, miR-124 mimics NC+ Mut SCP-1. The luciferase activity was detected after transfection for 48 h by dual-luciferase reporter gene assay kit Promega (Madison, WI, USA): cells were rinsed with phosphate buffered saline (PBS), and then LAR was added after spalling cells. Fluorescence value was read by enzyme detector, then adding stopping solution, detecting the fluorescence value again, calculating the rela-

Table I. RT-PCR primer.

Gene	Primer (5′-3′)	bp
miR-124	For: TGGGTTCGGTGGTCAAGTC Rev: CGCTCTGGTAGTGCTGGGA	134
SCP-1	For: CCCAGGACTCAGACAAGATC Rev: CGCTTCAACACGTAGACCTG	241
GADPH	For: AGCCACATCGCTCAGACA Rev: TGGACTCCACGACGTACT	314

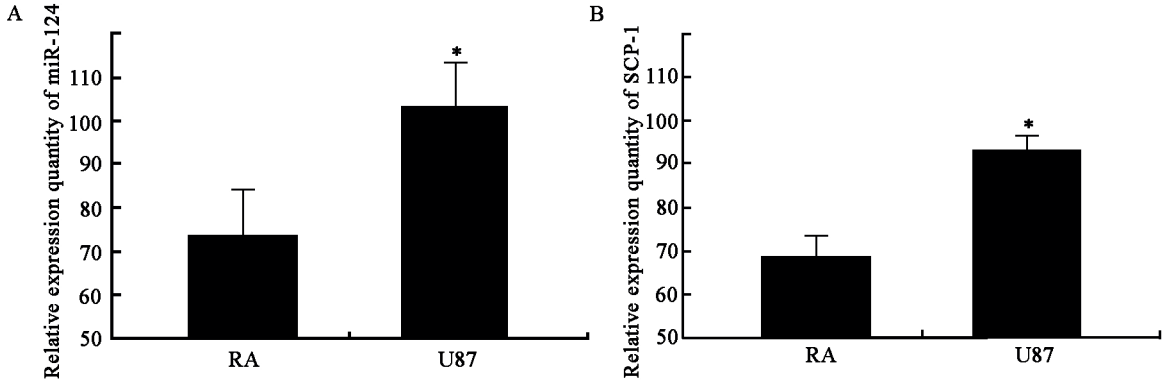

Figure 1. ***(A)*** Expression level of miR-124 in RA cell and neuroglioma cell ***(B)*** Expression level of SCP-1 in RA cell and neuroglioma cell.

tive fluorescence value. Fluorescence value design formula was as following: relative fluorescence value = firefly luciferin / ranilla luciferin.

Statistical Analysis

Results of Western blot were summarized and analyzed by Image-J, and shown as mean value ± standard error. *T*-test was performed by SPSS 13.0 software (SPSS Inc., Chicago, IL, USA), and p-value < 0.05 were considered to be statistically significant.

Results

Expression Level of miR-124 and SCP-1 in Neuroglioma cell

Compared with RA control group, levels of miR-124 in U87 neuroglioma cell group were significantly higher (Figure 1A, p=0.0015). Meanwhile, expression of SCP-1 in neuroglioma cell was higher than control group (Figure 1B, p=0.0042).

SCP-1 was the Target Protein of miR-124.

According to the analysis result of bioinformatics tools, Target Screen, SCP-1 gene sequence was the binding target of miR-124 (Figure 2A). Q-PCR result indicated a decrease of SCP-1 and miR-124 in both U87 and C6 cells, suggesting that glioma cell model with lower-expression SCP-1 and miR-124 was successfully established (Figure 2B and 2C). MiR-124 mimics, NC, wild type vector pGL3-SCP-1 3'UTR-Wt and mutant type vector pGL3-SCP-1 3'UTR-Mut were successfully transfected into U87 cells, respectively. Compared with other groups, fluorescence intensity was significantly weaker in co-transfection group of miR-124 mimics and wild type vector pGL3-SCP-1 3'UTR-Wt (Figure 2D, p<0.01). There was no difference in fluorescence intensity among other three groups. All of these results suggested SCP-1 was the target protein of miR-124.

Down-regulation of SCP-1 Attenuated Migration of Glioma Cell.

We found that both miR-124 and SCP-1 presented a high level of expression, so we hypothesized miR-124 or SCP-1 influenced cells motility, enhanced cells migration and exacerbate tumor progression. According to results of wound healing assay and cells migration test, down-regulation of SCP-1 was indicated to inhibited cells migration (Figure 3, A and C). Analysis data was shown in Figure 3B and Figure 3D.

miR-124 did not Influence Cells Motility and Migration

Down-regulation of miR-124 did not attenuate cells motility and migration according to results of wound healing assay and cells migration test (Figure 4A and 4C). Analysis data was shown in Figure 4B and Figure 4D.

Discussion

Development and progression of glioma are a multistep processes, which involve multi-factors. All these glioma factors result in oncogene activation and anti-oncogene inactivation[17]. Accumulative evidence proved miRNA played multiple roles in tumor progress, including oncogene activation and anti-oncogene inactiva-

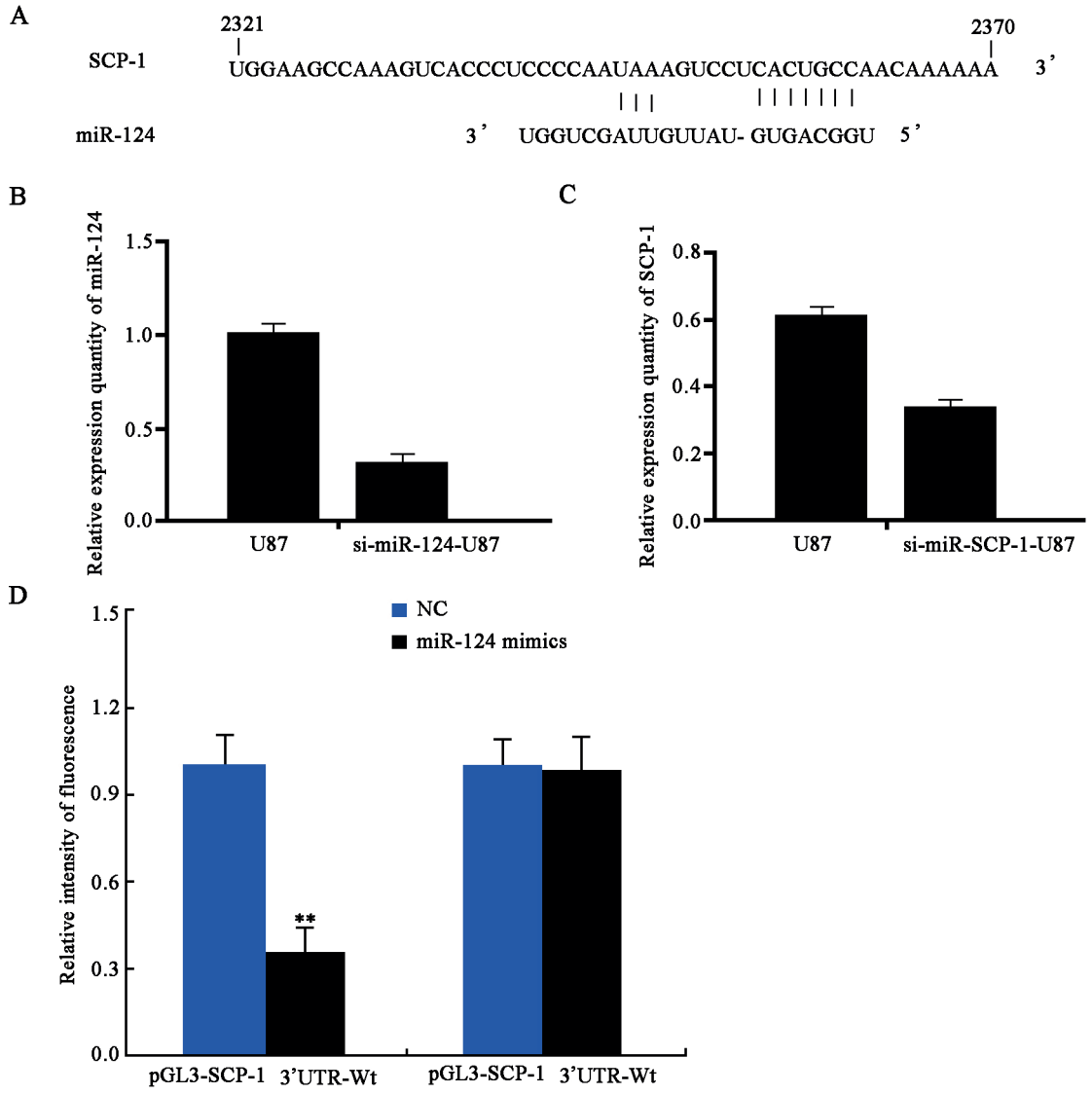

Figure 2. ***(A)*** Result of bioinformatics tools. ***(B)*** Expression of miR-124 was interfered. ***(C)*** Expression of SCP-1 was interfered. ***(D)*** Fluorescence intensity of four groups.

tion, and influenced proliferation, apoptosis, signaling and regulation of pathogenesis in tumor[18]. Glioma cell cancer is known for its high invasiveness, resulting in recurrence of cancer. Current therapy for glioma cell cancer is thus focused on surgery and post-surgery chemotherapy[19].

MiRNAs belong to endogenous non-coding small RNA of the evolutionarily conserved. MiRNA can bind with 3 -'UTR region of target gene, resulting in inhibition of transcription and translation of target gene. Although miR-124 has developed into a novel medicament in clinical treatment, a study found there were still questions to be answered. For example, how miRNA reached the location of tumor, and which mechanisms of miRNAs were involved in the modulation of tumor. As a member of CAT, SCP-1 was reported widely expressed in multiple tissues, including intestinal cancer and liver cancer. Our study proved SCP-1 was also expressed in glioma, suggesting that SCP-1 could influence the progress of glioma. We firstly proved SCP-1 was the target gene of miR-124 by bioinformatics examination, and following protein mediation tests confirmed this regulation relationship. After finding down-regulation of SCP-1 inhibited tumor mi-

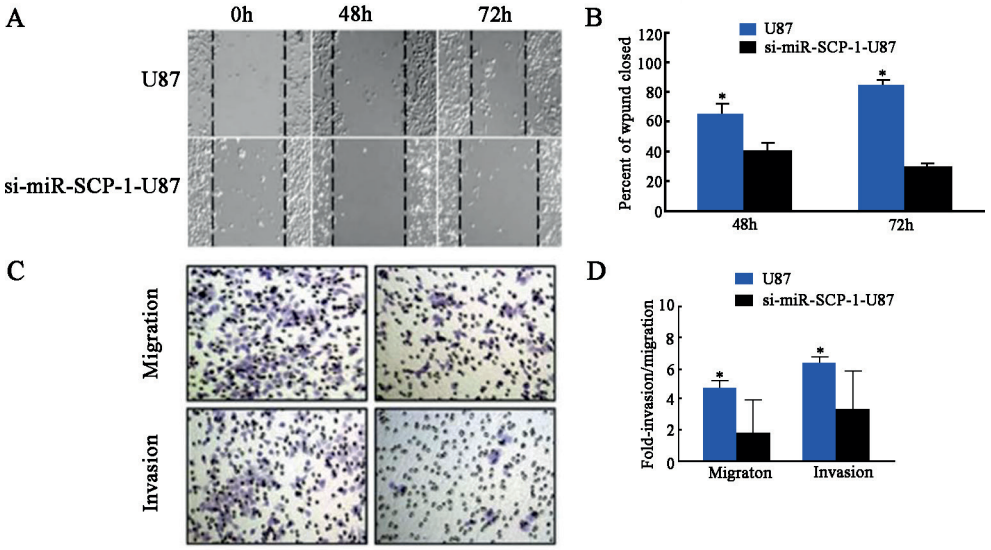

Figure 3. ***(A)*** Wound healing assay test of interfering SCP-1 group and control group. Interfering SCP-1 attenuated cells motility. ***(B)*** Analysis result for wound healing assay test in 48 h and 72 h. Compared with control group, interfering SCP-1 group had a significantly lower percentage of wound closed (48 h, p=0.0021. 72 h, p=0.0047). ***(C)*** Cells migration test of interfering SCP-1 group and control group. ***(D)*** Analysis result for cells migration test in 48 h and 72 h. Compared with control group, interfering SCP-1 group had a significantly lower fold of invasion/migration (48 h, p=0.0034. 72 h, p=0.0042).

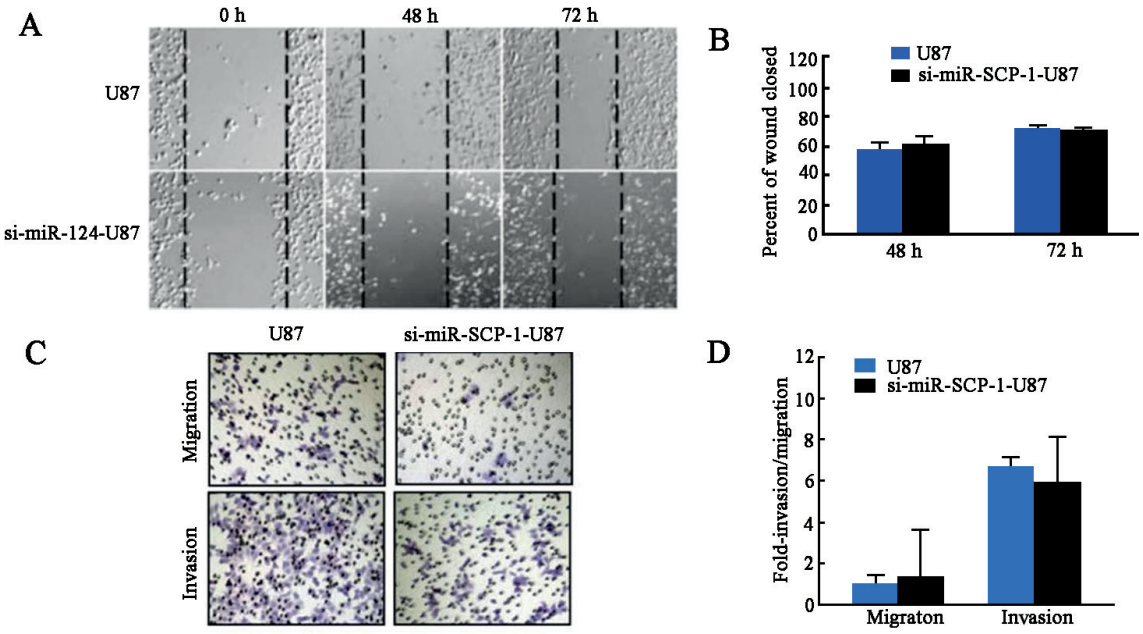

Figure 4. ***(A)*** Wound healing assay test of interfering miR-124 group and control group. Interfering miR-124 did not attenuate cells motility. ***(B)*** Analysis result for wound healing assay test in 48 h and 72 h. There was no significant difference in percentage of wound closed (48 h, p=0.087. 72 h, p=0.124). ***(C)*** Cells migration test of interfering miR-124 group and control group. ***(D)*** Analysis result for cells migration test in 48 h and 72 h. C There was no significant difference in fold of invasion/migration (48 h, p=0.068. 72 h, p=0.221).

gration, we further explored whether miR-124, upstream regulating factors of SCP-1, could directly influence tumor migration. Final test data showed down-regulation of miR-124 did not directly influence tumor migration; however, miR-124 could influence tumor migration

via regulating SCP-1. Therefore, SCP-1 is the direct regulating factor of tumor migration[20].

About how miRNA reached the location of tumor, researchers have multiple opinions. Some believed miR-124 was transported to lung cancer lesion via mesenchymal stem cell[21], while it was also considered miR-124 was transported to intestinal cancer lesion via downstream target protein[22]. Although our work did not identify how miR-124 was transported to glioma lesion, our results showed miR-124 influenced glioma cell migration via downstream SCP-1.

Conclusions

Our study firstly revealed the relationship between miR-124 and SCP-1, and proved that SCP-1 was involved in glioma progress. The limitation of our study is to identify the details of miR-124 and SCP-1.

Conflict of interest

The authors declare no conflicts of interest.

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