Long-Chain Non-Coding RNA Targeting miR Signal Axis Regulates the Mechanism of Apoptosis and Invasion and Migration of Glioma U251 Cells

Hailin Zhao[1#], Shiwei Li[2#], Lulu Wang[3], Dongdong Luo[1], Su Hu[1], Dan Li[1], Biao Peng[1*]

[1]Neurosurgery, Affiliated Cancer Hospital & Institute of Guangzhou Medical University, Guangzhou, 510090, China
[2]Intensive Care Unit, Affiliated Cancer Hospital &Institute of Guangzhou Medical University, Guangzhou, 510090, China
[3]Operation Room, Affiliated Cancer Hospital & Institute of Guangzhou Medical University, Guangzhou, 510090, China
#Hailin Zhao and Shiwei Li are co-first authors

ARTICLE INFO

ABSTRACT

The occurrence of glioma is gradually promoted by various factors, and it has gone through multiple stages of development, involving abnormal expression of multiple genes. One of the important reasons for the development of gliomas is the interaction of genetic factors and the environment. Non-coding transcripts can also form this high-level structure, and the formation of binding sites for interactions between lncRNA and proteins, DNA, and other RNA molecules may be related to their structural diversity. Due to the importance of glioma-related research and the potential effectiveness of lncRNA, this paper focuses on the mechanism of long-chain non-coding RNA targeting the Mir signal axis to regulate apoptosis, invasion and migration of glioma U251 cells. In this paper, human glioma cell line U251 was used as experimental material for simulation analysis. The results showed that after miR simulation, the pass rate of U251 stem cells through the filter was 17.3%, which was significantly less than 85.4% of group C; compared with 77.6% of the negative control group, the cell penetration rate of the miR inhibitor group was significantly improved. 92.5%. The miR expression level can affect the invasion ability of U251 stem cells, and can negatively regulate the expression of fzd4 to inhibit the invasion and metastasis of glioma U251 cells.

Introduction

The occurrence of tumors is mainly due to abnormal changes in genetic networks due to changes in genetic information. The main clinical manifestations of gliomas are increased intracranial pressure and focal neurological dysfunction. The origin of glioma is generally thought to occur in the neuroectoderm of gliocytes or their progenitor cells, accounting for more than half of intracranial tumors, most of which are malignant gliomas.

Current treatment strategies for gliomas rarely achieve long-term tumor control (1-2). Surulescu proposed a multi-scale model of the interaction of cells with the underlying tissue network and the growth of proliferation gliomas. Surulescu assumes that the cancer cell invasion path is nerve fibers, and diffusion tensor imaging can provide relevant information to open up the way for patient-specific modeling of glioma invasion. Surulescu conducted a numerical simulation based on DTI data to evaluate the performance of the modeling method (3-4). He explored the expression of NEAT1 in gliomas and its relationship with clinicopathological characteristics and prognosis. His research showed that NEAT1, as a potential prognostic predictor, is highly expressed in tumor tissues and participates in the canceration and progression of gliomas (5-6). Zheng explored the role of circ-TTBK2 and miR-217 in glioma cells by establishing a stable knockout of circ-TTBK2 or a high expression model of miR-217 glioma cell lines. Zheng's experiments show that circ-TTBK2 gene knockout combined with high expression of miR-217 leads to tumor regression in vivo, so circ-TTBK2 has important research value in the occurrence and development of gliomas (7-8). Xing used microarray transcriptome profiling to explore the differential expression of genes in gliomas. After gene chip analysis, Xing further verified the gene expression profiles of different grades of gliomas through bioinformatics analysis and co-expression network

construction. Xing's research shows that the occurrence of tumors is related to the expression of CAPG protein in gliomas. CAPG may be a biomarker for the pathological grade of gliomas (9-10). Yu has developed a multifunctional fusion protein for targeting and transporting therapeutic elements of glioma cells. Yu expressed multifunctional fusion protein in E. coli and purified by Ni-NTA resin affinity chromatography. Yu's research shows that GHPc+mT is a multifunctional protein that induces apoptosis, targets, and inhibits the proliferation of glioma cells, and is expected to develop into an effective drug delivery system for glioma therapeutic proteins (11-12).

Most tumors are caused by the interaction of somatic and germline mutations with a range of environmental factors (13). Most of these mutations occur in genomic structures that lack protein-coding capabilities, but these structures contain other types of genes that function as RNA molecules. These genes are non-coding RNAs (14-15). Li detected the expression of SNHG20 in 107 colorectal cancer tissues. The detection showed that SNHG20 gene knockdown suppressed the proliferation, invasion and migration of colorectal cancer cells and the progression of the cell cycle. In addition, SNHG20 regulates cell growth by regulating a series of cell cycle-related genes. The disorder of SNHG20 is involved in the progression of colorectal cancer, which may become a potential therapeutic target for colorectal cancer patients (16-17). Han studied 83 patients with colorectal cancer and explored that LNCRNAH19 can recruit eIF4A3 and promote the proliferation of CRC cells(18-19). Shang aims to explore the regulatory effect of adriamycin on the malignant proliferation of gastric cancer cells and chemotherapy sensitivity and its clinical significance. Shang uses real-time quantitative PCR to detect the expression of UCA1. Shang believes that lncRNA UCA1 is a related gene that regulates the malignant proliferation of gastric cancer cells and doxorubicin resistance. UCA1 is expected to provide new targets and strategies for the treatment of gastric cancer (20-21). Chen conducted a genome-wide analysis of lncRNA expression to determine new targets, which laid the foundation for further study of colorectal cancer liver metastasis(22-23). Zhao found that long-chain non-coding RNA linc0092 is a node driver of cancer-associated fibroblast-mediated metastasis. The metastasis-promoting properties of CAF in vivo and in vivo are related to the high expression of chemokine CXCL14. Zhao's research indicates that CXCL14 positive CAF is essential for the progression of cancer-associated fibroblast-mediated metastasis (24-25).

The increasing expression of LncRNAs in malignant tumors provides a theoretical basis for LncRNAs as a potential therapeutic target for malignant tumors. In this article, we first predicted the potential binding sites between HOTAIR and miR, miR and FGF1 through bioinformatics, and then detected the three genes in the same group of normal and glioma tissues and cell lines. Source expression, and further study the three regulatory effects of HOTAIR and miR, FGF1.

Materials and Methods
Research Object and Methods

The glioma cell line U251 was purchased from a culture collection center and was stored in a refrigerator at -80℃ by our laboratory. The Transwell cell is shaped like a hat, with multiple tiny holes at the bottom, through which cells can pass. Place it in a cell culture plate and divide it into two different zones. The bottom of area A and area B are separated by a certain distance. A film is formed on the bottom of area A to seed cells in area A, and the culture fluid containing fetal bovine serum is added to area B. The culture fluid in area B also contains fetal bovine serum, which can attract the cells in area A to shuttle down, and observe the number of cells passing through the bottom of the upper chamber under the microscope to evaluate the cell's invasion ability. After culturing the cells, attach the cells to the wall, line the cell culture dish with a micropipette tip, change the medium and discard the cells, the time is recorded as "0h", and then put them into the cell incubator to continue culturing for 24 hours. Take out the cell culture dish, observe the migration distance of the cells around the scratch to the scratch area under the microscope, and then evaluate the migration ability of the cells.

Isolation and Identification of Stem Cells

Place glioma U251 cells in a 37°C, 5% CO_2 incubator, use CD133/1 beads to magnetically label

cells in a logarithmic growth phase, and resuspend in PBS containing 0.5% fetal bovine serum and 2mmol/LEDTA The mouse anti-human CD133 antibody was PE-labeled and added to it at a dilution ratio of 1:11. After incubation at 4°C in the dark, the cells were washed twice with buffer. After resuspending the buffer solution and the anti-PE magnetic beads at a ratio of 1:5, mix and incubate again in the dark at 4 ℃ for 11 min, then add the buffer again to wash, and rotate at 1000r/min Centrifuge for 10 min, discard the supernatant, and resuspend the cells in the buffer. The column was washed with buffer, and then the cell suspension was passed through the column. Wash the column with a pump to pressurize 1mL of buffer solution, collect and separate the washing solution to obtain positive cells. The expression rate of CD133 in the sorted cells was detected by flow cytometry. Human basal fiber growth factor, recombinant epidermal growth factor and human leukocyte inhibitory factor were added to stem cell maintenance solution DMEM/F12 medium at a standard of 20ng/ml to culture the cells, and 2% B27 was used to maintain the stemness of stem cells. Further, use CD133 antibody and GFAP antibody staining verification. The experiment was divided into miR mimetics group, miR inhibitor group, control group and negative control group.

Statistical Method

The experiment was repeated at least three times, and the experimental data were expressed as mean±standard deviation. IBM SPSS statistics19 statistical software was used for data analysis. When comparing the two groups, a t-test was used; when comparing the multiple groups, ANOVA was used. When $p < 0.05$, the difference was statistically significant. The formula of mean and standard deviation is as follows:

$$\mu = A_n = \frac{a_1 + a_2 + a_3 + ... + a_n}{n} \tag{1}$$

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2} \tag{2}$$

Results and Discussion

Analysis of the Effect of miR Expression Level on the Invasion Ability of U251 Glioma Stem Cells

After 24 hours, the invasiveness of U251 cells was detected by the Transwell method. The results showed

that the ratio of U251 stem cells crossing the cell membrane after miR mock transfection was 17.3%, which was significantly lower than 85.4% in group C. Compared with 77.6% in group D, the cell penetration rate of the miR inhibitor group was 92.5%. It can be seen that the expression level of miR significantly affects the invasiveness of U251 stem cells. U251 stem cells were transfected with miR analogs or inhibitors respectively, and Western blot analysis was performed 48 hours later. The results are shown in Figure 1.
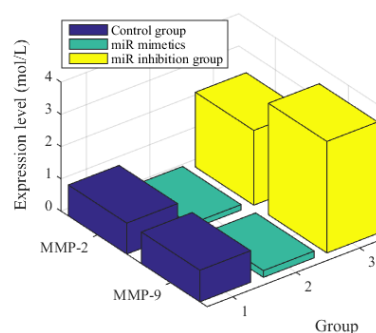

Figure 1. Expression of MMP-2 and MMP-9 in U251 cells of each group

The expression results of U251 cells in each group showed that the expression levels of MM 9 and matrix metalloproteinase(MM) 2 of U251 stem cells were significantly reduced after miR mimicry intervention. The expression levels of MM 2 and MM 9 in U251 stem cells intervened by miR inhibitors were significantly increased. The expression of MM 9 and MM 2, two invasion-related proteins of U251 stem cells, was obviously affected by the level of miR expression.

Analysis of the Expression Level of FZD4 Protein in U251 Cells after Transfection

The expression level of fzd4 protein in U251 cells after transfection is shown in Figure 2.
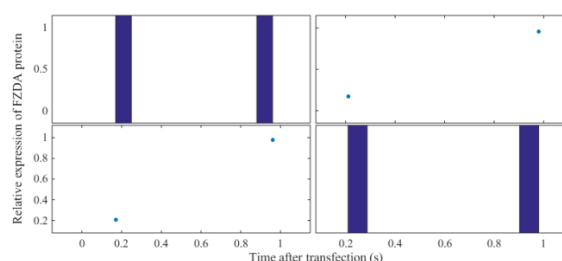

Figure 2. The expression level of FZD4 protein in U251 cells after transfection

The results showed that the expression level of FZD4 protein in the simulated group was significantly lower than that in group C, indicating that miR can inhibit the expression of FZD4 protein. FZD4 participates in the signal transduction process of various malignant tumor cells, plays a similar role to tumor genes in tumor cells, and participates in regulating important physiological and pathological activities of the body. In order to increase the expression of mir-136 in U251 cells, it was tested whether the expression of FZD4 downstream of U251 cells was affected. The results showed that the up-regulation of miR can significantly inhibit the expression of FZD4 mRNA and FZD4 protein. Therefore, miR can negatively regulate the expression of FZD4, thereby inhibiting the proliferation, invasion and metastasis of glioma U251 cells.

MTT Cell Proliferation Experiment to Detect the Effect of miR on U251 Proliferation

The proliferation of U251 cells detected by MTT within three days after transfection is shown in Table 1. The MTT cell proliferation experiment showed that there was no difference between the groups. The MTT cell proliferation experiment The effect of miR on U251 cell proliferation is shown in Figure 3.

Table 1. MTT test U251 cell proliferation within four days after cell transfection(OD value)

	miR mimic group	miR inhibitor group	Control group	Negative control group
D0	0.128	0.113	0.137	0.146
	0.129	0.132	0.139	0.141
	0.139	0.165	0.124	0.142
	0.134	0.129	0.113	0.102
D1	0.234	0.209	0.204	0.211
	0.214	0.167	0.178	0.228
	0.134	0.145	0.246	0.206
	0.141	0.154	0.165	0.224
D2	0.367	0.295	0.318	0.323
	0.341	0.304	0.353	0.324
	0.307	0.401	0.321	0.386
	0.378	0.357	0.317	0.325
D3	0.426	0.402	0.422	0.445
	0.471	0.413	0.454	0.453
	0.463	0.384	0.408	0.413
	0.474	0.438	0.406	0.468

In the glioma cell line U251, miR directly inhibits the expression of CD44 and TGFBR2 by directly targeting the 3'UTR of CD44 mRNA and TGFBR2 mRNA, and exerts the effect of inhibiting the migration ability of U251 cells. CD44 is a transmembrane glycoprotein receptor that is expressed on most vertebrate cell membranes. After inhibiting the expression of CD44 by RNAi, the migration ability of U251 cells was significantly inhibited; and overexpression of CD44 can restore the inhibitory effect of miR on U251 cells to a certain extent, which shows that miR plays a role in inhibiting the migration of U251 cells. To a certain extent, it is achieved by down-regulating the expression of CD44.
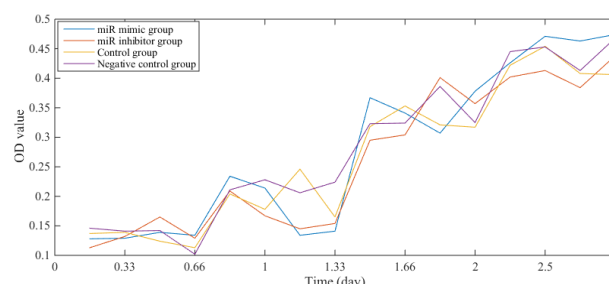

Figure 3. MTT cell proliferation experiment the effect of miR on U251 cell proliferation

Analysis of the Effect of Knocking down CRNDE on miR Expression and EMT Protein Expression

Inhibition of Mir expression reverses the effect of crnde knockdown on EMT-related protein expression, as shown in Figure 4.

Compared with Mir mimic group, the expression level of N-cadherin and vimentin decreased and E-cadherin increased in the Mir inhibitor group; the expression level of N-cadherin and vimentin increased and E-cadherin decreased in the control group; the protein expression levels of E-cadherin, N-cadherin and vimentin in the negative control group had no significant difference. Compared with the Mir mimic group, the expression of N-cadherin and vimentin in the control group increased, while the expression of E-cadherin decreased. It is suggested that Si crnde can affect the expression of EMT-related proteins by promoting the expression of Mir, which can be reversed by Mir inhibitor.
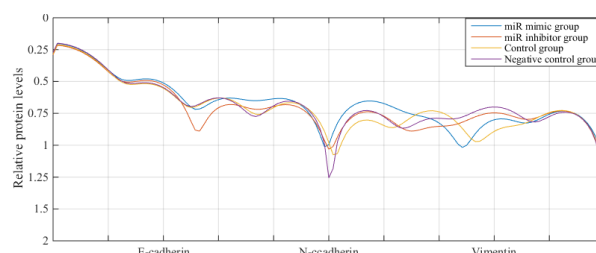

Figure 4. Inhibition of miR expression reverses the effect of knockdown of CRNDE on EMT-related protein expression

The human genome contains less than 2% of protein-coding genes, most of which will be transcribed into non-coding RNA and expressed in proteins. Long-chain non-coding RNA is an RNA with a transcription length greater than 200 nt. It does not encode the protein itself. The full-length cDNA sequence was used to analyze a large number of long-chain non-coding Rna in humans, mice and flies. The study found that some lncRNA plays a role in imprint control, cell differentiation, immune response, etc., and even affects the process of human disease and tumor formation. Although more and more lincRNAs have been found, their functions are still unknown. In general, lincRNAs contain coding genes with weak conservation like introns, and their conservation is weaker than untranslated regions of mRNA. The study of the whole-genome spectrum shows that compared with non-glioma tissue, many lincRNAs are abnormal in glioma, and the differential expression also appears to span the different malignant degrees of glioma (12, 26-29).

This article found that the expression of miR in glioma tissues and cells was significantly reduced, and decreased with the increase of the pathological grade of tumor tissue. Compared with group D, the migration ability of glioma U251 cells decreased after CRNDE gene knockout, but the migration ability of glioma U251 cells after CRNDE gene knockout and miR inhibition was not significantly different from that of the negative control group, thus avoiding the inhibitory effect of the CRNDE gene knock-out on the migration of glioma U251 cells was investigated. The results showed that CRNDE gene knockout can promote the expression of Mir and reduce the migration ability of U251 cells, and miR inhibitors can reverse this effect.

Acknowledgements
None.

Interest conflict
None.

Reference

1. Yue X, Lan F, Hu M. Downregulation of serum microRNA-205 as a potential diagnostic and prognostic biomarker for human glioma. J Neurosurg 2016; 124(1): 122-128.

2. Zhou Y, Peng Y, Liu M. MicroRNA-181b Inhibits Cellular Proliferation and Invasion of Glioma Cells via Targeting Sal-Like Protein 4. Oncol Res Featur Preclin Clin Cancer Therapy 2017; 25(6): 947-957.

3. Surulescu C, Knappitsch M, Engwer C. A multiscale model for glioma spread including cell-tissue interactions and proliferation. Math Biosci Eng 2016; 13(2): 443-460.

4. Bush NAO, Chang SM, Berger MS. Current and future strategies for treatment of glioma. Neurosurg Rev 2017; 40(1): 1-14.

5. He C, Jiang B, Ma J. Aberrant NEAT1 expression is associated with clinical outcome in high grade glioma patients. Apmis 2016; 124(3): 169-174.

6. Chai C, Song LJ, Yang B. Circulating miR-199a-3p in plasma and its potential diagnostic and prognostic value in glioma. Eur Rev Med Pharmacol Sci 2016; 20(23): 4885-4890.

7. Zheng J, Liu X, Xue Y. TTBK2 circular RNA promotes glioma malignancy by regulating miR-217/HNF1β/Derlin-1 pathway. J Hematol Oncol 2017; 10(1): 1-19.

8. Zhang R, Pang B, Xin T. Plasma miR-221/222 Family as Novel Descriptive and Prognostic Biomarkers for Glioma. Mol Neurobiol 2016; 53(3): 1452-1460.

9. Xing W, Zeng C. An integrated transcriptomic and computational analysis for biomarker identification in human glioma. Tumor Biol 2016; 37(6): 7185-7192.

10. Zhu YG, Zhao HG, Rao M. MicroRNA-365 inhibits proliferation, migration and invasion of glioma by targeting PIK3R3. Oncol Rep 2017; 37(4): 2185-2192.

11. Yu J, Guo M, Wang T. Inhibition of cell proliferation, migration and invasion by a glioma-targeted fusion protein combining the p53 C terminus and MDM2-binding domain. Cell Prolif 2016; 49(1): 79-89.

12. Wei J, Nduom EK, Kong LY. MiR-138 exerts anti-glioma efficacy by targeting immune checkpoints. Neuro Oncol 2016; 18(5): 639-648.

13. Kawasaki N, Miwa T, Hokari S. Long noncoding RNA NORAD regulates transforming growth factor -β signaling and epithelial-to-mesenchymal transition-like phenotype. Cancer Sci 2018; 109(7): 2211-2220.

14. Schmitz SU, Grote P, Herrmann BG. Mechanisms of long noncoding RNA function in development and disease. Cell Mol Life 2016; 73(13): 2491-2509.

15. Pan L, Liang W, Fu M. Exosomes-mediated transfer of long noncoding RNA ZFAS1 promotes gastric cancer progression. J Cancer Res Clin Oncol 2017; 143(6): 991-1004.

16. Li C, Zhou L, He J. Increased long noncoding RNA SNHG20 predicts poor prognosis in colorectal cancer. BMC Cancer 2016; 16(1): 1-9.

17. Li D, Liu X, Zhou J. Long noncoding RNA HULC modulates the phosphorylation of YB-1 through serving as a scaffold of extracellular signal-regulated kinase and YB-1 to enhance hepatocarcinogenesis. Hepatol 2017; 65(5): 1612-1627.

18. Han D, Gao X, Wang M. Long noncoding RNA H19 indicates a poor prognosis of colorectal cancer and promotes tumor growth by recruiting and binding to eIF4A3. Oncotarget 2016; 7(16): 22159-22173.

19. Malakar P, Shilo A, Mogilevsky A. Long Noncoding RNA MALAT1 Promotes Hepatocellular Carcinoma Development by SRSF1 Upregulation and mTOR Activation. Cancer Res 2017; 77(5): 1155-1167.

20. Shang C, Guo Y, Zhang J. Silence of long noncoding RNA UCA1 inhibits malignant proliferation and chemotherapy resistance to adriamycin in gastric cancer. Cancer Chemother Pharmacol 2016; 77(5): 1061-1067.

21. Cai X, Liu Y, Yang W. Long noncoding RNA MALAT1 as a potential therapeutic target in osteosarcoma. J Orthopaedic Res 2016; 34(6): 932-941.

22. Chen D, Sun Q, Cheng X. Genome-wide analysis of long noncoding RNA (lncRNA) expression in colorectal cancer tissues from patients with liver metastasis. Cancer Med 2016; 5(7): 1629-1639.

23. Kazemi E, Zargooshi J, Kaboudi M, Heidari P, Kahrizi D, Mahaki B, Mohammadian Y, Khazaei H, Ahmed K. A genome-wide association study to identify candidate genes for erectile dysfunction. Brief Bioinforma 2021;22(4):bbaa338. https://doi.org/10.1093/bib/bbaa338.

24. Zhao L, Ji G, Le X. Long Noncoding RNA LINC00092 Acts in Cancer-Associated Fibroblasts to Drive Glycolysis and Progression of Ovarian Cancer. Cancer Res 2017; 77(6): 1369-1382.

25. Liu B, Ye B, Yang L. Long noncoding RNA lncKdm2b is required for ILC3 maintenance by initiation of Zfp292 expression. Nat Immunol 2017; 18(5): 499-508.

26. Fathi A., Barak M, Damandan M, Amani F, Moradpour R, Khalilova I., Valizadeh M. Neonatal Screening for Glucose-6-phosphate dehydrogenase Deficiency in Ardabil Province, Iran, 2018-2019. Cell Mol Biomed Rep 2021; 1(1): 1-6.

27. Tourang M, Fang L, Zhong Y, Suthar R. Association between Human Endogenous Retrovirus K gene expression and breast cancer. Cell Mol Biomed Rep 2021; 1(1): 7-13.

28. Bilal I, Xie S, Elburki M, Aziziaram Z, Ahmed S, Jalal Balaky S. Cytotoxic effect of diferuloylmethane, a derivative of turmeric on different human glioblastoma cell lines. Cell Mol Biomed Rep 2021; 1(1): 14-22.

29. Aziziaram, Z., Bilal, I., Zhong, Y., Mahmod, A., Roshandel, M. Protective effects of curcumin against naproxen-induced mitochondrial dysfunction in rat kidney tissue. Cell Mol Biomed Rep 2021; 1(1): 23-32.