Effect of lncRNA ZEB1-AS1 on proliferation, invasion and apoptosis of glioma U87 cells

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Abstract. This study aimed to investigate the effect of LncRNA ZEB1-AS1 on the proliferation, invasion and apoptosis of human glioma U87 cells. U87 glioma cells were divided into three groups. The Si group was transfected with LncRNA ZEB1-AS1 specific SiRNA. The NC group was transfected with non-specific scramble siRNA, and untransfected glioma cells were used as the blank group. After 48 h of transfection, the proliferation of U87 cells was detected by MTT assay, apoptosis of U87 cells was detected by flow cytometry, and Transwell invasion assay was used to detect cell invasion. The expression of LncZEB1-AS1 in Si group was significantly lower than that in the NC and blank groups (P<0.01). There was no statistical difference in the OD 490 between the three groups at 24 h (P>0.05). At 48 h, the Si group was significantly lower than the NC group and the blank group (P<0.01). After 48 h, the three groups showed a gradually increasing trend, but at all the time points, the Si group was always lower than the NC and blank groups (P<0.01). The OD values of the blank and NC groups were significantly higher than the same group at the previous time point (P<0.01). The OD values of Si group at 48 and 96 h were significantly higher than those at the previous time point (P <0.05). Although there was an upward trend between 72 and 48 h, the difference was not significant (P>0.05). Flow cytometry detected apoptosis in each group and found that the apoptosis rate in the Si group was significantly higher than that in the NC and blank groups (P<0.01). Inhibition of LncRNA ZEB1-AS1 can inhibit the proliferation and invasion of glioma U87 cells and promote apoptosis. LncRNA ZEB1-AS1 is expected to become a new target for the treatment of glioma.

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Introduction

Gliomas are the tumors with the highest morbidity and mortality in adult central nervous system tumors (1). With the improvement of the medical science level, the treatment methods for brain tumors (surgical resection, chemotherapy, and radiotherapy) have greatly improved, but the prognosis of glioma patients is still poor. At 5% is the lowest five-year survival rate of all cancer patients, the median survival time is approximately 14 months, even after receiving the maximum safety resection plus adjuvant chemotherapy, the median survival time can only be increased to approximately 15 months (2,3). Due to abundant blood supply, gliomas appear to be infiltrating growth, and it is difficult to excise the gliomas completely. It is difficult to avoid postoperative recurrence. Chemotherapy and radiotherapy are not highly specific for glioma cells and have great toxic and side effects (4). Therefore, the search for new molecular biomarkers and the development of safer and more effective molecular targeted therapies are important tasks in the treatment of gliomas.

lncRNA is an RNA with a base number of not less than 200 bp and does not encode protein, and regulates the expression of genes at several levels such as chromatin modification, transcription, and post-transcriptional modification (5). It has been found that many lncRNAs are abnormally expressed during carcinogenesis. These lncRNAs may be useful therapeutic targets for malignant tumors and molecular markers for the diagnosis of prognosis (6). lncRNA ZEB1-AS1 is a newly discovered lncRNA, and several studies have shown that lncRNA ZEB1-AS1 plays an important role in the occurrence and development of many types of tumors, including liver cancers (7), colorectal cancer (8), esophageal squamous cell carcinoma (9), and lung cancer (10). Li *et al* (11) studied gastric cancer and found that ZEB1-AS1 exerts carcinogenic effects by promoting the migration, invasion and EMT process of gastric cancer cells. The expression of ZEB1-AS1 is positively correlated with the prognosis of gastric cancer. Wang *et al* (9) investigated the expression of lncRNA ZEB1-AS1 in esophageal squamous cell carcinoma and found that the expression of lncRNA ZEB1-AS1 was increased in cancer tissues and was positively correlated with tumor grade, depth of invasion, and lymph node metastasis. However, the clinical significance, biological function, and mechanism of action of lncRNA ZEB1-AS1 in gliomas remain to be determined.

Table I. qPCR primer sequences.

Gene	Upstream sequence	Downstream sequence
GAPDH	5'-CCCATCACCATCTTCCAGGAG-3'	5'-GTTGTCATGGATGACCTTGGC-3'
ZEB1-AS1	5'-AACCTTGTTGCTAGGGACCG-3'	5'-AGTCACTTCCCATCCCGGTT-3'

Lv *et al* (12) found that ZEB1-AS1 is highly expressed in glioma tissues. Therefore, the purpose of this study was to investigate the effects of knockdown of lncRNA ZEB1-AS1 on proliferation, invasion, and apoptosis of glioma U87 cells in order to treat gliomas and to provide new therapeutic targets.

Materials and methods

Materials and reagents. U87MG glioblastoma cells of unknown origin (Shanghai Zeye Biological Technology Co., Ltd., catalogue no.: AC319); lncRNA ZEB1-AS1-specific SiRNA and non-specific scramble siRNA sequence design (Shandong Weizheng Biotech Co., Ltd., Shandong, China); fetal bovine serum, DMEM medium [Wuhan Puno (Sales Life Technology Co., Ltd.), Wuhan, China]; TransScript Green Two-Step RT-qPCR SuperMix kit (Beijing Quantum Biotechnology Co., Ltd., Beijing, China); Lipofectamine® 3000 Transfection Reagent, TRIzol Reagent, Thermo Scientific Revert Aid First Strand c DNA Synthesis kit (Thermo Fisher Scientific (China) Co., Ltd., Shanghai, China; qPCR Primer Sequences (Shanghai Sangon Bioengineering Co., Ltd., Shanghai, China); CCK8 kit (Shanghai Shengsheng Biotechnology Co., Ltd. Shanghai, China); and Transwell Cell (Beijing Yiming Fuxing Biotechnology Co., Ltd. Beijing, China) were used in the study.

The study was approved by the Ethics Committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

Plasmid transfection. The U87MG glioblastoma cell line was cultured in DMEM medium containing 20% fetal bovine serum at 37˚C in a 5% CO_2 incubator. When the cell confluence reached 80%, the cells were seeded in a 6-well plate at a concentration of $1x10^5$/per well. When the cell confluence reached 60%, Si RNA transfected with lncRNA ZEB1-AS1 was transfected into Si group, NC group was transfected with non-specific scramble siRNA, blank group was not transfected, and equal amount of complete medium was added. The instructions for Lipofectamine 3000 transfection reagents were strictly followed. After incubation in a 5% CO_2 incubator at 37˚C, assays were performed when cell confluency reached 80%.

RT-qPCR detection of lncZEB1-AS1 expression. TRIzol and chloroform reagents were used to extract total RNA from each group. The concentration and purity of RNA were measured by UV spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The ratio of A260/A280 between 1.8 and 2.0 was considered as qualified. The reverse transcription reaction system was prepared strictly in accordance with the instructions, incubated at 42˚C for 15 min, and reverse transcribed with 1 μg of total RNA at 85˚C for 5 sec. The qPCR reaction was strictly performed in accordance with the TransScript Green Two-Step RT-qPCR SuperMix kit instructions. The amplification was performed using a two-step method, pre-denaturing at 94˚C for 30 sec, 94˚C for 5 sec, and 60˚C for 30 sec for 40 cycles. Using GAPDH as an internal reference, the sequences of each primer are shown in Table I.

MTT test cell proliferation in each group. When the cells grew to logarithmic phase, the cells were digested and the cell suspension was prepared with RPMI-1640 containing 10% FBS. The cell density was adjusted to $2x10^4$ ml and seeded in 96-well plates at $3x10^3$/200 μl/well. The cell proliferation was measured by MTT assay every 24 h until 120 h. Five tests were performed. Of 5 mg/ml MTT solution 10 μl was added to each well for 4 h. Formanzan solution was added at 150 μl/well. The wells were mixed for 10 min at room temperature and vortexed, the absorbance (OD) was recorded at a wavelength of 490 nm using a microplate reader, and the assay was repeated three times per well.

Transwell invasion experiment. After 12 h of culture in serum-free RPMI-1640 medium, cell suspension was prepared and counted to adjust the cell density to $1x10^5$/ml. Then, 100 μl cell suspension was added to the Transwell upper chamber of 24-well plate, and 600 μl DMEM medium with 20% fetal bovine serum was added to the lower chamber, followed by incubation at 37˚C, 5% CO_2 for 24 h. The mixture was washed 3 times with PBS and the non-migrating cells of the upper chamber on the polycarbonate membrane were gently wiped off with a cotton swab. Then, 4% paraformaldehyde was fixed on a polycarbonate membrane for 10 min, followed by crystal violet staining for 10 min, prior to slowly washing 3 times with PBS and counting under a microscope at a magnification of x400. Five fields were randomly selected for counting and average value was recorded.

Flow cytometry detection of apoptosis. After 48 h of transfection, the cells were first trypsinized, and collected. Following digestion with trypsin, the cells were re-precipitated (4˚C) in 0.01 mol/l PBS, counted to adjust the cell density to $1x10^5$/ml, and 150 μl of Annexin V-FITC was added to the cells to resuspend the cells, which were then transferred to a flow test tube. Annexin V-FIT (5 μl) and 15 μl of propidium iodide was added to each tube and gently mixed, and reacted at room temperature for 15 min in the dark. The samples were detected by flow cytometry. The process was completed within 1 h, with each sample repeated 3 times. Apoptosis rate was expressed as a percentage and data analysis was performed using flow software Flowjo 7.5.

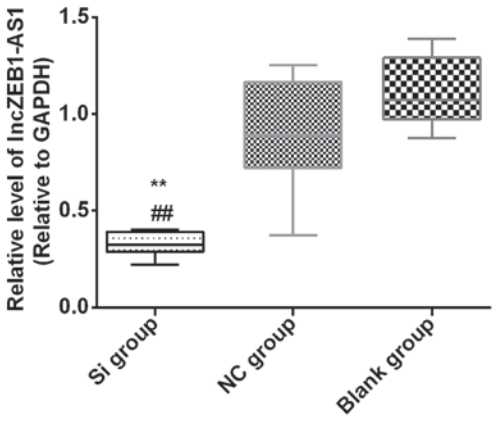

Figure 1. Expression of lncZEB1-AS1 in each group by RT-qPCR. The expression of lncZEB1-AS1 was detected by RT-qPCR 48 h after transfection. The expression of lncZEB1-AS1 in Si group was significantly lower than that in the NC and blank groups (P<0.01). There was no statistical difference between the NC and blank groups (P>0.05). **P<0.01 compared with NC group. ##P<0.01 compared with blank group.

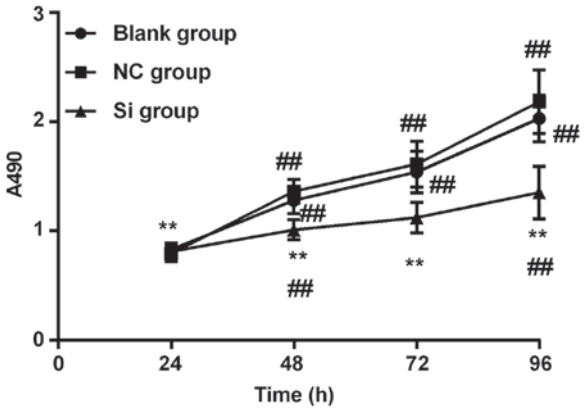

Figure 2. MTT test for cell proliferation in each group: the three groups were found at the same time point, there was no statistically significant difference in OD490 in three groups at 24 h (P>0.05), at 48 h, Si group was significantly lower than the NC and blank groups (P<0.00), there was no difference between the NC and blank groups (P>0.05). The three groups showed a gradual upward trend from 48 h, but Si group was always lower than the NC group and blank group at each time point (P<0.01). There was no significant difference between the NC and blank groups (P<0.05). The variance analysis of repeated measurement at the same group at different time points showed that the OD values of blank group at 72, 96, 120 h significantly increased compared with the previous time point (P<0.01), OD values of NC group at 72, 96, 120 h were significantly higher compared with the previous time point (P<0.01). The OD values of Si group at 48 and 96 h were significantly higher than those at the previous time point (P<0.01). Although there was an upward trend between 72 and 48 h, the difference was not statistically significant (P>0.05). At the same time point, **P<0.01 compared with NC group and blank group. In the same group, ##P<0.01 compared with the previous time point.

Statistical analysis. The statistical analysis was performed using the SPSS 19.0 software system (IBM, SPSS, Chicago, IL, USA). Data were expressed as the mean ± standard deviation (mean ± SD). The mean of multiple groups was compared using a single factor variance analysis and analysis of repeated measures. Multiple comparison was performed using LSD test. P<0.05 was considered to indicate a statistically significant difference.

Results

RT-qPCR detection of lncZEB1-AS1 expression. The expression of lncZEB1-AS1 was detected by RT-qPCR 48 h after transfection. The expression of lncZEB1-AS1 in the Si group (0.33±0.02) was significantly lower than that in the NC group (0.91±0.10) and blank group (1.11±0.06) (P<0.01). There was no statistical difference between the NC and blank groups (P>0.05) (Fig. 1).

MTT detection of cell proliferation in each group. The comparison between the three groups at the same time point showed that there was no significant difference in OD490 among the three groups at 24 h (P>0.05). At 48 h, the Si group was significantly lower than the NC group and the blank group (P<0.05), there was no difference between the NC group and the blank group (P>0.05). From 48 h, the three groups showed a gradual upward trend, but at all time points, the Si group was lower than the NC group and the blank group (P<0.01). There was no statistical significance between the NC group and the blank group (P<0.05); The variance analysis of repeated measures at the same group at different time points showed that the OD values of the blank group at 72, 96, and 120 h were significantly higher than those at the previous time point (P<0.01). The OD values of the NC groups at 72, 96 and 120 h were significantly higher than those at the previous time point (P<0.01). The OD values of Si group at 48 and 96 h were significantly higher than those at the previous time point (P<0.01). Although there was an upward trend between 72 and 48 h, the difference was not statistically significant (P>0.05) (Fig. 2).

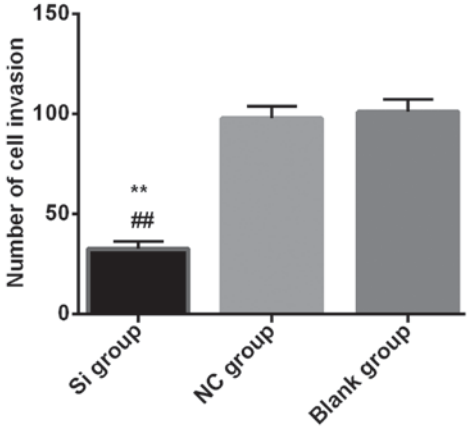

Figure 3. Transwell invasion assay to detect the invasive ability of each group: Transwell invasion assay to detect the invasive ability of each group found that the number of invading cells in the Si group (32.51±3.71) was significantly lower than the NC group (97.82±5.92) and blank group (101.09±6.22) (P<0.01). There was no statistical difference in the number of invasions between the NC group and the blank group (P>0.05). **P<0.01 compared with NC group. ##P<0.01 compared with blank group

Transwell invasion experiment. Transwell invasion assay to detect the invasive ability of each group found that the number of invading cells in the Si group (32.51±3.71) was significantly lower than that in the NC group (97.82±5.92) and the blank group (101.09±6.22) (P<0.01), There was no statistical difference in the number of invasive cells between NC group and blank group (P>0.05) (Fig. 3).

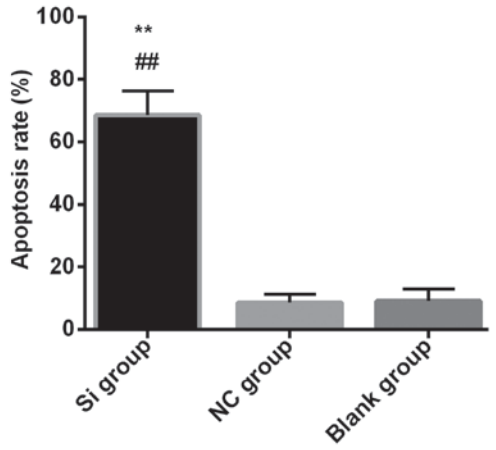

Figure 4. Flow cytometry detection of apoptosis: Flow cytometry was used to detect apoptosis in each group. The apoptosis rate in Si group was significantly higher than NC group and blank group (P<0.01). There was no statistical significance between the NC and blank groups (P>0.05). **P<0.01 compared with NC group. ##P<0.01 compared with blank group.

Flow cytometry detection of apoptosis. Flow cytometry detected apoptosis in each group the apoptosis rate in the Si group 68.54±7.71% was significantly higher than that in the NC group 8.51±2.71% and in the blank group 9.16±3.71% (P<0.01). There was no statistical significance between NC group and blank group (P>0.05) (Fig. 4).

Discussion

Gliomas are the most common type of malignant brain tumors, accounting for approximately 46%. According to the time of onset, they are divided into adult and child types. The prognosis is extremely poor, which jeopardizes the patient's physical health and social economic development, leading to great economic and psychological burden to the family (13,14). lncRNA ZEB1-AS1 is upregulated in a variety of tumors and is closely related to clinical stage, prognosis and lymph node metastasis, but the relationship between the mechanism of action of glioma cells and clinical features has not yet been clarified (15). This study explored the effect of lncRNA ZEB1-AS1 on the proliferation, invasion and apoptosis of U87MG glioblastoma cells and the role of lncRNA ZEB1-AS1 in glioblastoma cells in order to provide molecular-targeted therapy and theoretical basis for gliomas.

The expression of lncZEB1-AS1 was detected by RT-qPCR and it was found that the expression of lncZEB1-AS1 in Si group was significantly lower than that in NC group and blank group after transfection, indicating that ZEB1-AS1 SiRNA was successfully transfected into Si group; other subsequent results in cells were comparable. MTT detection of cell proliferation in each group found that at 24 h, OD490 of the three groups did not show statistical difference, from 48 h, the three groups showed a gradual upward trend, but at each time point Si group was lower than the NC and blank groups, there was no statistical significance between the NC and blank groups, indicating that transfection of ZEB1-AS1 SiRNA inhibited the proliferation of U87MG cells. In other words, lncZEB1-AS1 promote the proliferation of glioblastoma cells and Transwell invasion assay detected the invasion ability of cells in each group. The number of invading cells in the Si group was significantly lower than that in the NC and blank groups. There was no statistical difference in the number of invading cells between the two groups, indicating that the transfection of ZEB1-AS1 SiRNA inhibited the invasion of U87MG cells, i.e., lncZEB1-AS1 could promote the invasion of brain glioma cells. Apoptosis was detected by flow cytometry and it was found that the apoptosis rate of Si group was significantly higher than that of the NC and blank groups, indicating that ZEB1-AS1 SiRNA promoted apoptosis of U87MG cells after transfection. Lnc ZEB1-AS1 can inhibit apoptosis of glioma cells. Lv *et al* (12) found that lncZEB1-AS1 is highly expressed in glioma tissues and is closely related to the clinical stage and prognosis of glioma. lncZEB1-AS1 is an independent prognostic factor for glioma patients. lncRNA ZEB1-AS1 inhibits proliferation and invasion and promotes apoptosis in U87MG glioblastoma cells. The possible reason is that lncRNA ZEB1-AS1 is derived from the promoter region of ZEB1, and lncRNA ZEB1-AS1 can induce H3K4me3 modification of ZEB1 promoter region and activate ZEB1 transcription. Thus, ZEB1 exerts the role of oncogenes by regulating its downstream targets (16,17). The miR-101/ZEB1 axis can promote the hypoxia-induced epithelial-mesenchymal transition in malignant gliomas (18). ZEB1-AS1 can also regulate the proliferation and migration of osteosarcoma cells through miR-200 (19).

The present findings showed that lncRNA ZEB1-AS1 can promote the proliferation and invasion of U87MG glioblastoma cells and inhibit apoptosis, indicating that lncRNA ZEB1-AS1 plays an oncogenic role, and if there is an additional grouping of normal brain glial cells, with RT-qPCR detection of lncZEB1-AS1 expression, lncRNA ZEB1-AS1 level in the normal brain glial cells was significantly lower than glioma U87 cells, we can further verify this conclusion. In this study, only one type of glioma cell was selected for testing. It does not fully represent the actual progress of glioma. It should be tested in other glioma cell lines. Moreover, the cell test is *in vitro*, it does not represent the true situation in the body, so it should also be used to further verify the glioma cells in the rat subcutaneous tumorigenesis test (20).

In summary, inhibition of lncRNA ZEB1-AS1 can inhibit proliferation and invasion of U87MG glioblastoma cells and promote apoptosis. lncRNA ZEB1-AS1 is expected to become a new target for the treatment of glioma.

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Availability of data and materials

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

Authors' contributions

WZ drafted the manuscript. WZ and LX were mainly devoted to collecting and interpreting the data. WZ helped with flow cytometry detection. LX performed Transwell invasion experiment. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Agarwal S, Sane R, Oberoi R, Ohlfest JR and Elmquist WF: Delivery of molecularly targeted therapy to malignant glioma, a disease of the whole brain. Expert Rev Mol Med 13: e17, 2011.
2. Delgado-López PD and Corrales-García EM: Survival in glioblastoma: A review on the impact of treatment modalities. Clin Transl Oncol 18: 1062-1071, 2016.
3. Yan W, Zhang W and Jiang T: Oncogene addiction in gliomas: Implications for molecular targeted therapy. J Exp Clin Cancer Res 30: 58, 2011.
4. Jia L, Tian Y, Chen Y and Zhang G: The silencing of LncRNA-H19 decreases chemoresistance of human glioma cells to temozolomide by suppressing epithelial-mesenchymal transition via the Wnt/β-catenin pathway. Onco Targets Ther 11: 313-321, 2018.
5. Wang Q, Du X, Yang M, Xiao S, Cao J, Song J and Wang L: LncRNA ZEB1-AS1 contributes to STAT3 activation by associating with IL-11 in B-lymphoblastic leukemia. Biotechnol Lett 39: 1801-1810, 2017.
6. Lin J, Zhan Y, Liu Y, Chen Z, Liang J, Li W, He A, Zhou L, Mei H, Wang F, *et al*: Increased expression of ZEB1-AS1 correlates with higher histopathological grade and promotes tumorigenesis in bladder cancer. Oncotarget 8: 24202-24212, 2017.
7. Li T, Xie J, Shen C, Cheng D, Shi Y, Wu Z, Deng X, Chen H, Shen B, Peng C, *et al*: Upregulation of long noncoding RNA ZEB1-AS1 promotes tumor metastasis and predicts poor prognosis in hepatocellular carcinoma. Oncogene 35: 1575-1584, 2016.
8. Gong H, Wen H, Zhu X, Lian Y, Yang X, Qian Z and Zhu J: High expression of long non-coding RNA ZEB1-AS1 promotes colorectal cancer cell proliferation partially by suppressing p15 expression. Tumour Biol 39: 1010428317705336, 2017.
9. Wang YL, Bai Y, Yao WJ, Guo L and Wang ZM: Expression of long non-coding RNA ZEB1-AS1 in esophageal squamous cell carcinoma and its correlation with tumor progression and patient survival. Int J Clin Exp Pathol 8: 11871-11876, 2015.
10. Rong L, Zhao R and Lu J: Highly expressed long non-coding RNA FOXD2-AS1 promotes non-small cell lung cancer progression via Wnt/β-catenin signaling. Biochem Biophys Res Commun 484: 586-591, 2017.
11. Li Y, Wen X, Wang L, Sun X, Ma H, Fu Z and Li L: LncRNA ZEB1-AS1 predicts unfavorable prognosis in gastric cancer. Surg Oncol 26: 527-534, 2017.
12. Lv QL, Hu L, Chen SH, Sun B, Fu ML, Qin CZ, Qu Q, Wang GH, He CJ and Zhou HH: A long noncoding RNA ZEB1-AS1 promotes tumorigenesis and predicts poor prognosis in glioma. Int J Mol Sci 17: 17, 2016.
13. Fu J and Cui Y: Long noncoding RNA ZEB1-AS1 expression predicts progression and poor prognosis of colorectal cancer. Int J Biol Markers 32: e428-e433, 2017.
14. Bonavia R, Inda MM, Vandenberg S, Cheng SY, Nagane M, Hadwiger P, Tan P, Sah DW, Cavenee WK and Furnari FB: EGFRvIII promotes glioma angiogenesis and growth through the NF-κB, interleukin-8 pathway. Oncogene 31: 4054-4066, 2012.
15. Li J, Li Z, Leng K, Xu Y, Ji D, Huang L, Cui Y and Jiang X: ZEB1-AS1: A crucial cancer-related long non-coding RNA. Cell Prolif 51: e12423, 2018.
16. Su W, Xu M, Chen X, Chen N, Gong J, Nie L, Li L, Li X, Zhang M and Zhou Q: Long noncoding RNA ZEB1-AS1 epigenetically regulates the expressions of ZEB1 and downstream molecules in prostate cancer. Mol Cancer 16: 142, 2017.
17. Fang C, Zan J, Yue B, Liu C, He C and Yan D: Long non-coding ribonucleic acid zinc finger antisense 1 promotes the progression of colonic cancer by modulating ZEB1 expression. J Gastroenterol Hepatol 32: 1204-1211, 2017.
18. Zhang S, Wang W, Liu G, Xie S, Li Q, Li Y and Lin Z: Long non-coding RNA HOTTIP promotes hypoxia-induced epithelial-mesenchymal transition of malignant glioma by regulating the miR-101/ZEB1 axis. Biomed Pharmacother 95: 711-720, 2017.
19. Liu C, Pan C, Cai Y and Wang H: Interplay between long noncoding RNA ZEB1-AS1 and miR-200s regulates osteosarcoma cell proliferation and migration. J Cell Biochem 118: 2250-2260, 2017.
20. Lenting K, Verhaak R, Ter Laan M, Wesseling P and Leenders W: Glioma: Experimental models and reality. Acta Neuropathol 133: 263-282, 2017.