Original Article

Function and mechanism of long non-coding RNA KIAA0495 in glioma

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Abstract: Objective: To investigate the expression of long non-coding RNA KIAA0495 in human glioma tissues, and the effect of U251 glioma cells on the biological function as well as its mechanism. Methods: We selected 100 cases of glioma specimens and 50 cases of normal brain tissue specimens, then used PCR to detect the expression levels of genes in cells, and used Pearson correlation analysis to detect the expression correlation of KIAA0495 and CTNNBIP1. Results: In glioma, the expression level of KIAA0495 was inversely proportional to the degree of malignant gliomas, HGG (high grade glioma) expression was significantly lower than that of the LGG (Lower grade glioma); correspondingly, the expression of CTNNBIP1 was inversely proportional to the degree of malignant gliomas and the expression level of HGG was lower than that of the LGG. Thus, the analysis of its relevance told us a positive correlation between them. The up-regulation of KIAA0495 expression inhibited cell proliferation, migration, cycle, but promoted cell apoptosis. On the basis of the over-expression of KIAA0495, we interfered the expression of CTNNBIP1, then found that compared with over-expressed KIAA0495 group, the proliferation, migration and cell cycle inhibition were all weakened. And the promoting effect of cell apoptosis was inhibited to some degree. Experiment of subcutaneously transplanted tumor (in vivo) in nude mice confirmed that the down regulation of CTNNBIP1 expression could weaken the inhibitory effect of KIAA0495 on tumor growth. Conclusion: In glioma cells, increasing lncRNA KIAA0495 can slow down the growth of glioma cells, and this inhibition can be achieved by regulating the expression of CTNNBIP1 protein. Thus, this result means that lncRNA KIAA0495 could become a new therapeutic target for glioma.

Keywords: Long non-coding RNA, glioma, U251 cells, biological behavior

Introduction

Recently, an increasing number of reports have confirmed that IncRNA plays an important role in many biological processes, including tumors. Glioma is a brain tumor that is harmful to human health and is not easy to be cured. And recently, some scholars have found a new IncRNA, KIAA0495 (TP73-AS1). Its expression down regulated in the plasma cells of multiple myeloma, and the reduction of this expression was correlated with promoter methylation. However, in primary myeloma cells and recurrence or progression of myeloma cells that have been confirmed, the methylation level of KIAA0495 promoter has not changed. Therefore, it was believed that methylation of KIAA0495 was not important to the pathogenesis and progression of myeloma [1]. Later, some scholars found that in esophageal squamous cell tumors, KIAA0495 had an oncogenes function, and the down regulated expression of KIAA0495 inhibited the expression of BDH2, the result of which inhibited cell proliferation and promotes apoptosis through CASPASE-3-dependent apoptosis pathway [2]. But some studies have reported that KIAA0495 plays the role of a tumor suppressor gene in glioma [3]. However, what is the function of KIAA0495 in glioma and how to exercise its function as a tumor suppressor gene have not been reported yet. In this study, by using PCR to detect the expression of KIAA0495 in glioma, using the lentivirus transfection technique and the establishment of model of hypodermatic xenografts of human brain glioma in nude mice, we observed the effects and related mechanism of KIAA0495 on the proliferation, migration, cell cycle and apoptosis of U251 cells, as well as explore the possibility of KIAA0495 as a new therapeutic target for glioma.
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Material and methods

Experimental specimen

100 cases of glioma tissue and 50 cases of normal brain tissue (Shanghai RuiSai Biotechnology Co., Ltd), pathological grade: grade I (22 cases), grade II (27 cases), grade III (26 cases), grade IV (25 cases); lower-grade glioma were 49 cases and high-grade glioma were 51 cases.

Experimental animal, cell strain and main reagent

BALB/c-nu/nu nude mice were purchased from Shanghai silaike experiment Co., Ltd, which was 4-6 weeks old, of either sex, 18-21 g, and under the condition of SPF level feeding. Human glioma U251 cells (American Type Culture Collection, ATCC), was frozen by the laboratory. Transwell chambers (Corning Costar Company); qRT-PCR kit SYBR Green (Roche company, USA); Annexin V FITC/PI (BD Biosciences company, USA); RNaseA (Boehringer Mannheim company, USA); CCK-8 and PI (Sigma company, USA); lentivirus vector carrying over-expressed KIAA0495 (Shanghai Ji Ma Pharmaceutical Co., Ltd).

Real time fluorescent quantitative PCR detection

Total RNA of cell samples or tissue samples were extracted by TRIzol method. In order to confirm quality of total RNA, the total RNA of each sample should be detected by ND1000 NanoDrop, and the integrity of RNA should be assessed by agarose gel electrophores in standard. Real time fluorescent quantitative PCR was in accordance with the SYBR Green operating instructions to prepare the reaction system, each sample was provided with 3 repeated holes. The primer sequences were as follow: hKIAA0495-F1: GCCTTGGTCCTTTCTATCTTTG, hKIAA0495-R1: GGTTCTCAGGT TTCTTGGTTTC, hCTNNBIP1-F1: CCGTTAGGCGGGATT TATG, hCTNNBIP1-R1: TGGGGCTTTTATGT GGGTT. Using β-actin as reference, the expressions of KIAA0495 and CTNNBIP1 were calculated by the method of 2-ΔCT.

Cell culture and the establishment of stable transfection cell strains

U251 cells should be cultured in DMEM medium (10% FBS) and should be put in incubator with conventional culture of 37°C, 5% CO₂, then, the cells were subcultured every other day, and selected in exponential phase to infect. The recombinant lentivirus vector carrying meaningless sequence was used as the control group (NC group); the lentiviral vector carrying KIAA0495 gene sequence was used as the over-expressed KIAA0495 group (KIAA0495 group). According to MOI=30, add up the virus fluid and transform them into normal culture solution after transfection for 24 h, after that, select by utilizing the puromycin (PM) for two weeks for further experiment. The CTNNBIP1 blocker was added to the over-expression of KIAA0495 group as the over-expression KIAA-0495 and interference of CTNNBIP1 group (K-OE+C-KD group). Finally, after the cells were cultured in 48 h, the total RNA was collected and extracted.

Detecting the proliferation of U251 cells by CCK8

Select the cells in the exponential phase and make them into single cell suspension. And the cells were inoculated in 96-well plate (5*10⁴/ml), 200 μL per hole, 6 holes in each group. Replace the culture medium every other day, and use CCK-8 method every 24 hours to measure absorbance value of each group at the wavelength of 450 nm for 5 days.

Detecting the migration of U251 cell by transwell method

Cells should be inoculated to transwell chambers with 24 holes, according to 5*10⁴/hole in each group, and was set 3 repeated holes in each group. The volume of upper chamber was 100 μL without serum DMEM medium, and the volume of lower chamber was 500 μL, which contained DMEM medium with 10% fetal bovine serum. After being put into 37°C incubator for 48 hours, cells were taken out from the upper chamber, and were placed into the 0.1% crystal violet staining solution for 15 min, then were scrubbed with PBS for 3 times, after that, the cells were wiped off without migration on upper layer with the cotton swab. Later, we observed the random selection of visual field under the optical microscope, and photographed them. Then we dehydrated them with 33% acetic acid, the crystal violet could completely eluted them, and finally, we put the eluant in microplat reader and measured the OD value in 570 nm.
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Figure 1. Comparison of expression amount of KIAA0495 in glioma with different pathological grades. **P<0.05.

Detecting the cell cycle of U251 cells by flow cytometry

Each group of U251 cells were inoculated in 6-well plate with the density of 5*10⁵/mL, then 48 h after making corresponding treatment, they were washed by PBS. After that, we digested and collected them in the flow tube using trypsin without EDTA, and placed them at -20°C for an hour after suspending again in 75% alcohol, then washed them again by the PBS. At last, flow cytometer was used to detect the cell cycle of U251 cells after suspending again in HBSS solution with 50 g/ml PI.

Detecting the apoptosis of U251 cell by flow cytometry

Each group of U251 cells were inoculated in 6-well plate with the density of 5*10⁵/mL. 24 hours later, after digesting and collecting them in the flow tube by using trypsin without EDTA, we centrifugated them for 6 minutes in 10000 r/min, and removed the supernatant, then washed them for 3 times with precooling PBS, next added 3 μL Annexin V-FITC and 3 μL PI into them and mix them gently, after that added 300 μL buffer solution into it, then reacted for 15 minutes without light in room temperature. At last, flow cytometer was used to detect cell apoptosis, and the apoptosis should be expressed in percentage.

Experiment of hypodermatic xenografts of human brain glioma in nude mice

The nude mice were divided into 3 groups randomly, with 8 rats in each group. U251 cells with over expression of KIAA0495, and over expression of KIAA0495+interference CTNNBIP1 as well as those infected by empty lentiviral vector were inoculated in each group respectively. We collected cells in exponential phase. After digesting, centrifugating and counting, we used the DMEM culture medium without serum to wash cells once and made them into cell suspension, with the concentration of 5*10⁶ cells/100 μL. 100 μL inoculated cells were subcutaneous injected after sterilized skin by 75% alcohol in nude mice, after that we observed the tumor growth every day, and used sliding caliper to measure the length and the short diameter every fifth day. Finally, the volumes of tumor were accounted.

Statistical methods

The SPSS17.0 software was used for statistical analysis, the measurement data was expressed by mean ± standard deviation. Quantitative data between two groups was detected by t-test; one-way ANOVA was applied when there were three groups, and enumeration data was examined by Chi-square test or Fisher exact test. Pearson correlation analysis method was used to detect the correlation between two objects. When P<0.05, the difference between the two groups is statistically significant.

Results

The expressions of KIAA0495 in glioma

In order to detect the expression of KIAA0495 in glioma, qPCR was chosen to detect the expression amount of KIAA0495. The result showed: clinical pathological grade (I-II) belonged to LGG (lower grade glioma), and the grade (III-IV) belonged to HGG (high grade glioma). Among different pathological grades in the glioma, the expression level of KIAA0495 had diversities, the higher pathology degree was, the lower expression level was. The difference between the two groups was statistically significant (P<0.05), as the Figure 1 shows.

The effects on the cell proliferation, migration, cycle and apoptosis of U251 by over-expression of KIAA0495

In order to study whether the expression of KIAA0495 was up-regulated after lentivirus-mediated over-expression of KIAA0495 in U251 cells or not, the expression of KIAA0495
in the two groups was detected by qPCR 48 h after transfection. Results found that it could significantly increase the expression amount of KIAA0495 in U251 cells, see from Figure 2A.
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Compared with the control group, over-expression of KIAA0495 can inhibit cell proliferation (Figure 2B), cell cycle (Figure 2C), migration (Figure 2E) and promote the cell apoptosis (Figure 2D) in U251 cells. The difference between the two groups was statistically significant (P<0.05).

The expression of CTNNBIP1 protein in glioma and its effects on the biological behavior of U251 cells

Q-PCR results showed that up-regulation of the expression of KIAA0495 in U251 cells promoted the expression of CTNNBIP1 (Figure 3A). Compared with low-grade glioma, the expression of CTNNBIP1 in high-grade glioma was down-regulated (Figure 3B), and the correlation analysis of expression level between CTNNBIP1 and KIAA0495 in the high-grade glioma showed they were positive (Figure 3C).

In order to verify whether KIAA0495 works by the effect of CTNNBIP1, and inhibit expression of CTNNBIP1 on the basis of over-expressed KIAA0495 (Figure 4A) or not. We found that compared with KIAA0495 group, degree of inhibition of proliferation (Figure 4B), cycle (Figure 4C) and migration (Figure 4E) in U251 cells have been weakened in K-OE+C-KD group, and the promoting effect of apoptosis also has been weakened (Figure 4D).

Observation and measurement on the growth of subcutaneously transplanted tumor in nude mice

In order to verify whether KIAA0495 affected the growth of glioma cells by CTNNBIP1 or not, NC lentiviral infected cells was regarded as control group, subcutaneous injection of 5×10^6 U251 cells with stable and over-expressed KIAA0495 and over-expression of KIAA0495+ interference of CTNNBIP1 in other two groups were made respectively. After 14 days, the size of the tumor began to be measured. After 40 days, solid tumor of glioma was taken out. The result showed that compared with control group, the tumor volume of subcutaneous injection of U251 cells with stable and over-expressed KIAA0495 decreased significantly. But, on the basis of interfered expression of CTNNBIP1, the inhibition degree of KIAA0495 which has on the growth of tumor volume reduced in some degree. See Figure 5 for details.

Discussion

Glioma is the most common primary brain tumor in the brain in clinic, accounting for about 45% of the central nervous system tumors, and it has high degree of malignancy, high recurrence as well as poor prognosis [4, 5]. Glioma is similar to other malignant tumors, and its occurrence and development are effected by the two major mechanisms-epigenetics and genetics. This is the pathological process of polygene involvement and multistage variation accumulation [6-8]. Previous studies are usually based on the molecular mechanisms, genetic levels and pathways to study the prevention and treatment of glioma, but the mechanism of the occurrence and development of glioma remains unclear at present. Although surgical resection, chemotherapy and radiotherapy have gotten some progress in the treatment of glioma, the prognosis of patients is still poor. Therefore, how to effectively inhibit the malignant progression of brain glioma is one of the key factors for the treatment of glioma.
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Non-coding RNA includes the representative short RNA such as miRNA, siRNA, and long non-coding RNA. Long non-coding RNA is a kind of RNA whose transcription of the length is more than 200nt, and it is not encode protein. Compared with the short non-coding RNA, a lot of functions of long non-coding RNA are

Figure 4. Effects of interfer CTNNB1 to over-expression of KIAA0495 on the proliferation, migration, cell cycle and apoptosis in U251 cells, *P<0.05, **P<0.05, ***P<0.05.

Figure 5. Comparison of the growth of tumor in nude mice (in vivo) after inoculating cells for 14 days. ***P<0.05.
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not clear [9, 10]. However, with the development of genomic technology, more and more evidences are showing that long non-coding RNA has effects on cell function in many areas by regulating the expression of target genes, or by modifying the transcription products [11, 12]. Compared with normal tissue, long non-coding RNA with abnormal expression in tumor tissue is likely to affect the oncogenes and tumor suppressor gene, which can promote or inhibit the formation of tumors [13, 14]. The role of lncRNA in glioma is still at exploratory stage, but it has made some progress. Studies had shown that in the glioma, high expression of long non-coding RNA HOTAIR showed increasing trend with elevating tumor grade. For patients with brain glioma, high expression of HOTAIR suggested that patients had poor prognosis [15, 16]. Further studies showed that HOTAIR affected on the target gene PCR2 and ultimately promoted tumor growth. Another study also reported that in U251 and U87 over-expressed lncRNA-MEG3 could significantly inhibit the proliferation of brain glioma cells, and promoted apoptosis of tumor cells [17, 18]. MEG3 could regulate cell proliferation by P53, MDM2 and GDF15 genes' interaction, and it also showed that IncRNA was closely related to the occurrence and development of glioma [19, 20]. In addition, a series of differentially expressed IncRNA was found in glioblastoma IncRNA and mRNA expression profile chip, it analyzed and predicted two IncRNA (ASLNC20819 and ASLNC22381) through bioinformatics, and they participated in the malignant progression of glioma through the target gene IGF-1 [21, 22]. Thus, studying the function of IncRNA in glioma cells has great significance on revealing the exact molecular mechanisms of the occurrence and development of glioma, and designing reasonable targeted drugs and estimating prognosis, as well as further improving the level of the treatment of glioma.

TP73-AS1 was a recently discovered and abnormal expressed IncRNA in brain glioma. In this study, firstly, q-PCR was used to detect the expression of TP73-AS1 in brain glioma tissues and U251 glioma cells. Results found that the expression of TP73-AS1 in grade III, IV glioma tissues was significantly lower than that of grade I, II, which meant TP73-AS1 may be one of the predictors for the degree of malignant in tumor. And it confirmed that TP73-AS1 in the glioma may play a role as a tumor suppressor gene. Then, we transfected lentiviral vector carrying over-expressed TP73-AS1 in U251 cells, and the results showed that the expression amount of TP73-AS1 significantly increased after transfection. Meanwhile, the over-expressed TP73-AS1 could significantly inhibit the proliferation, migration, cell cycle and apoptosis of U251 glioma cell. Visibly, TP73-AS1 in U251 glioma cells also played a role of tumor suppressor gene. Subsequent animal and cell experiment results showed that TP73-AS1 regulated biological behavior of U251 cells through regulating expression of CTNNBIP1 protein, which suggested that the inhibited effect of TP73-AS1 was achieved by regulating the WNT pathway of CTNNBIP1 and inhibiting the level of protein.

To sum up, KIAA0495 is also known as TP73-AS1, and belongs to long non-coding RNA. In glioma, it is inversely proportional to the degree of malignancy, and the increase of expression amount can inhibit the development of glioma. Its inhibited effect is positively correlated with CTNNBIP1, which control the growth of glioma by regulating CTNNBIP1.

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Disclosure of conflict of interest

None.

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