Original Article
SnoRNA SNORD76 is downregulated in glioblastoma and inhibits cell proliferation through cyclinD1 and p21

Huguang Li[1*], Junhui Xiong[2*], Weiming Wang[1]

Departments of [1]Neurology, [2]Rheumatism, The People's Hospital of Lishui City (The Six Affiliated Hospital of Wenzhou Medical University), Lishui 323000, Zhejiang Province, PR China. *Equal contributors.

Abstract: Increasing evidence has showed that small nucleolar RNAs (snoRNAs) are activated and involved in carcinogenesis. The aim of this study was to verify the expression of snoRNA SNORD76 in human glioma. 40 surgically resected glioma tissues and 6 non-tumoral white matters, as well as glioblastoma multiforme (GBM) cell lines were collected and expression of SNORD76 was determined by quantitative real-time PCR (qRT-PCR). Then, we analyzed the potential relationship between SNORD76 expression and the clinical outcomes of glioma patients. We confirmed that SNORD76 expression was associated with the WHO classification and was remarkably decreased in GBM (WHO grade IV) tissues. Meanwhile, SNORD76 was also downregulated in U87 cells, comparing with that in other cell lines. In addition, survival analysis showed a positive and significant correlation of SNORD76 expression with poor overall survival of glioma patients. Functional and mechanism study showed the tumor suppressing roles of SNORD76 by targeting cyclinD1 and p21. The present study adds to a growing body of evidence that SNORD76 expression was decreased in GBM, and it might be a potential prognostic biomarker and therapeutic target for malignant GBM.

Keywords: Small nucleolar RNA, SNORD76, glioblastoma, cell proliferation

Introduction

Glioblastoma multiforme (GBM) is the most common and lethal intracranial tumor with a median survival ranging from 12 to 15 months [1-3]. It exhibits a relentless malignant progression characterized by widespread invasion throughout the brain. The disease development leads to deterioration in neurocognitive function, decreased functional independence, and a progressive decrease in health-related life quality [4, 5]. Despite the slight improvement achieved in surgery, chemotherapy and radiotherapy over the past few decades, the prognosis of GBM patients is still poor and needs an urgent elevation [6]. Hence, understanding GBM cell biology and identifying more effective and predictive biomarkers are of great clinical significance to develop novel therapeutic strategies.

The human genome project reveals that at least 90% of the human genome is actively transcribed to RNA, but less than 2% of RNA encodes proteins [7, 8]. Non-coding RNAs (ncRNAs) are a class of RNAs that do not code for proteins and constitute a major part of the eukaryote transcriptome. They actively function in many aspects of biological processes and include microRNAs (miRNAs), long non-coding RNAs (lncRNAs), tRNAs, small nuclear RNAs (snoRNAs), and small interfering RNAs (siRNAs) [9, 10]. Recently, ncRNAs have been reported to behave as rising stars in cancer genetics, especially for miRNAs and lncRNAs [11-14]. However, there are still few reports characterizing the features or functions of snoRNAs in carcinogenesis [15]. SnoRNAs constitute one of the major groups of functionally trans-acting ncRNAs currently known in mammalian cells [16]. They are abundant, evolutionarily ancient, and possess impressively diverse functions. According to the structural basis, snoRNAs could be classified into two groups, C/D box snoRNAs (SNORDs) which guide 2'-O-methylation, and H/ACA box snoRNAs (SNORAs) which guide pseudouridylation [16-18]. Recent findings indicate that snRNAs are also involved in the control of cell fate and tumorigenesis [19, 20].

Table 1. The clinical features of the glioma tissues used in this study

Feature	WHO grade		
	I-II (7)	III (15)	IV (18)
Age (Year)	30±5.6	45±7.2	64±10.8
Gender			
Male	5	8	12
Female	2	7	6
Predominant side			
Left	3	6	9
Right	3	8	7
Middle	1	1	2
Predominant location			
Frontal lobe	4	10	8
Temporal lobe	0	3	7
Parietal lobe	1	1	2
Saddle area	0	1	1
Cerebellum	2	0	0

A C/D box snoRNA U76 (SNORD76) is a snRNA excised from the third intron of *growth arrest-specific transcript 5* gene (*GAS5*). It was first mentioned in a paper that identified aberrant expression of snoRNA signatures associated with early stage of non-small lung cancer (NSCLC), in which it was increased in plasma of NSCLC cancer patients [21]. Recently, Chen et al proved that SNORD76 was inversely associated with Hox Transcript Antisense Intergenic RNA (HOTAIR) expression, and its overexpression could inhibit proliferation of glioma cells [22]. Although they had referred to SNORD76 as a potential tumor suppressor in GBM [22], the direct evidence is still lacking. For instance, the expression pattern of SNORD76 in GBM tissues and cell lines was unclear. Also, its prognosis value remains to be determined. In the present study, we profiled the expression of SNORD76 in glioma tissues and GBM cell lines, and investigate the functions and possible mechanisms in GBM cells.

Material and methods

Cell culture

Human astrocytes were purchased from Gibco (Invitrogen). U87, U251 and U343 GBM cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The GBM cells were maintained in DMEM supplemented with 10% FBS (Hyclone) in a 5% CO_2 atmosphere in a 37°C humidified chamber.

Clinical samples

Freshly resected tissues were immediately frozen in liquid nitrogen for subsequent total RNA extraction. All grades of glioma tissues with clinical data were collected from The people's Hospital of Lishui City. The study was approved by the hospital institutional review board. Also, the written informed consent was obtained from all patients. The information of surgical patients was provided in **Table 1**.

RNA extraction and qRT-PCR analysis

Total RNA was extracted using TRIzol (Invitrogen). Quantitative real-time PCR (qRT-PCR) assays were performed to measure the expression levels of SNORD76. QRT-PCR was performed using the SYBR Green PCR Master Mix (TOYOBO) according to the manufacturer's protocols. U6B was used for normalization of the SNORD76 expression. QRT-PCR data were analyzed by the comparative Ct method.

MTT assay

Cell growth rate was measured by MTT assay. After transfection with control siRNA or two independent SNORD76 siRNAs, cells were plated in 96-well plates. At 24 h, 48 h and 72 h, 20 μl of 5 mg/ml 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT) was added into each well. After incubation for 4 h at 37°C, the medium was aspirated and 200 μl DMSO was added per well. Absorbance was measured in a plate reader at 490 nm and the cell proliferation rate was calculated.

Crystal violet staining

U343 cells were transfected with control siRNA or two independent SNORD76 siRNAs. 48 h later, cells were fixed in 4% paraformaldehyde for 30 min. After washing, the cells were stained with 0.5% crystal violet solution for another 30 min. The plates were aspirated, washed, allowed to air dry and scanned with high resolution for photographs.

Western blot

Protein expression was determined by western blot, the cell lysate was run on 11% SDS-PAGE gels and then transferred onto nitrocellulose membranes. After blocking with 5% skimmed milk, blots were incubated with cyclinD1, p21
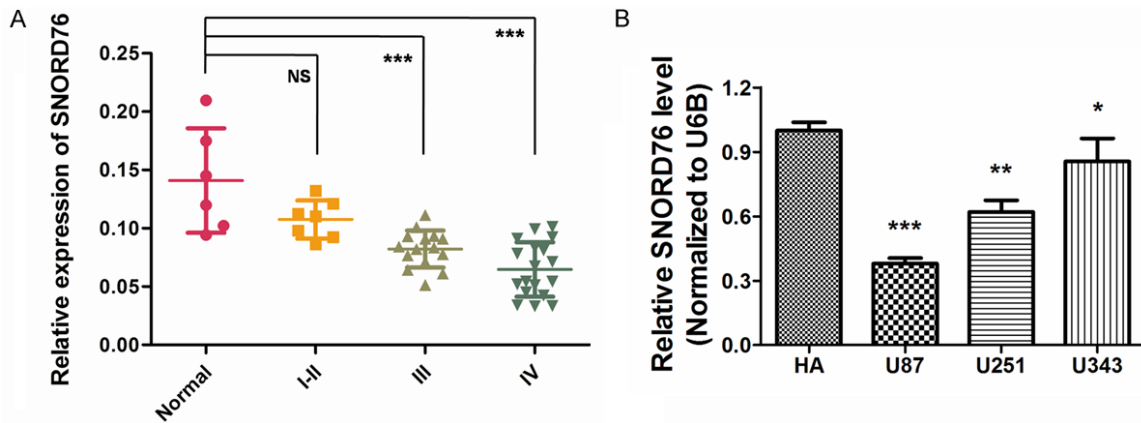
Figure 1. SNORD76 expression was downregulated in surgically resected glioma (WHO grade III and IV) tissues and GBM cell lines. A. Compared with non-tumor brain tissue (Normal), glioma tissues of WHO grade III (n=15) and grade IV (n=18) displayed significantly lower SNORD76 expression as determined by qPCR. ***$P<0.001$ (One-Way ANOVA); ns, no significant difference ($P>0.05$) relative to the other group. B. Expression of SNORD76 in 3 GBM cell lines, U87, U251, and U343, and normal human astrocytes was measured by qPCR *$P<0.05$.
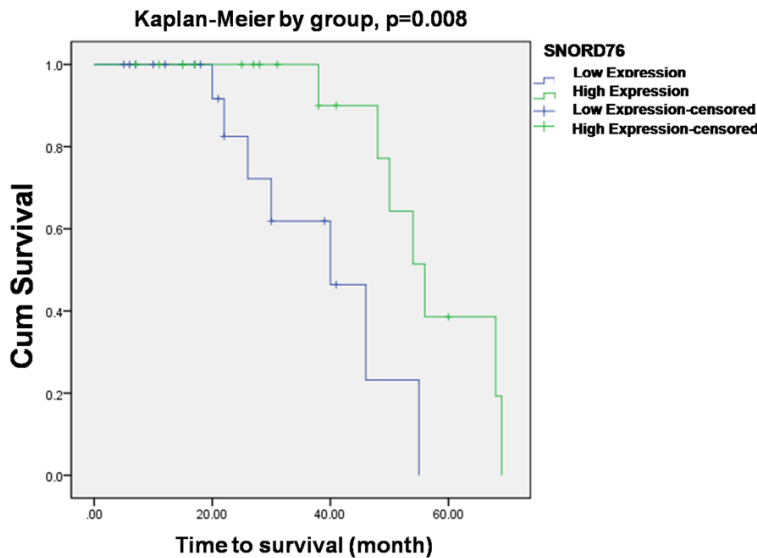

Figure 2. Kaplan-Meier post-operative survival curve for patterns of glioma patients and SNORD76 expression. The figure shows that patients of lower snoRNA SNORD76 expression tended to have worse overall survival (log rank test, $P=0.008$).

or β-actin antibodies (Cell Signaling Technology). β-actin was used as a loading control. A total of 50 μg of cell lysate was loaded in each lane for western blot analysis.

Statistical analysis

All statistical analysis was performed using SPSS 18.0 software (IBM). The statistical significance between groups was determined using One-Way ANOVA or the Student's *t* test.

The overall survival data was evaluated using the Kaplan-Meier method and compared using log-rank test.

Results

Expression of SNORD76 in human glioma tissues

One of the main aims of this study was to investigate whether SNORD76 exhibited abnormal expression in glioma tissues compared with non-tumor normal brain tissues, as well as in GBM cell lines compared with respected non-tumoral astrocytes. To answer this question, we collected 40 surgically resected glioma tissues of WHO grade I-II (n=7), grade III (n=15), grade IV (n=18), and non-tumor brain tissues (Normal, n=6) from clinic. RNA was purified and qRT-PCR was applied to detect the expression of SNORD76. U6B was used as the normalization control. Our results indicated that, SNORD76 was significantly decreased in grade III and IV (GBM), when compared with that in normal non-tumoral tissues. However, there was no significant difference between tumors of glioma grade I-II and normal non-tumoral tissues (**Figure 1A**).
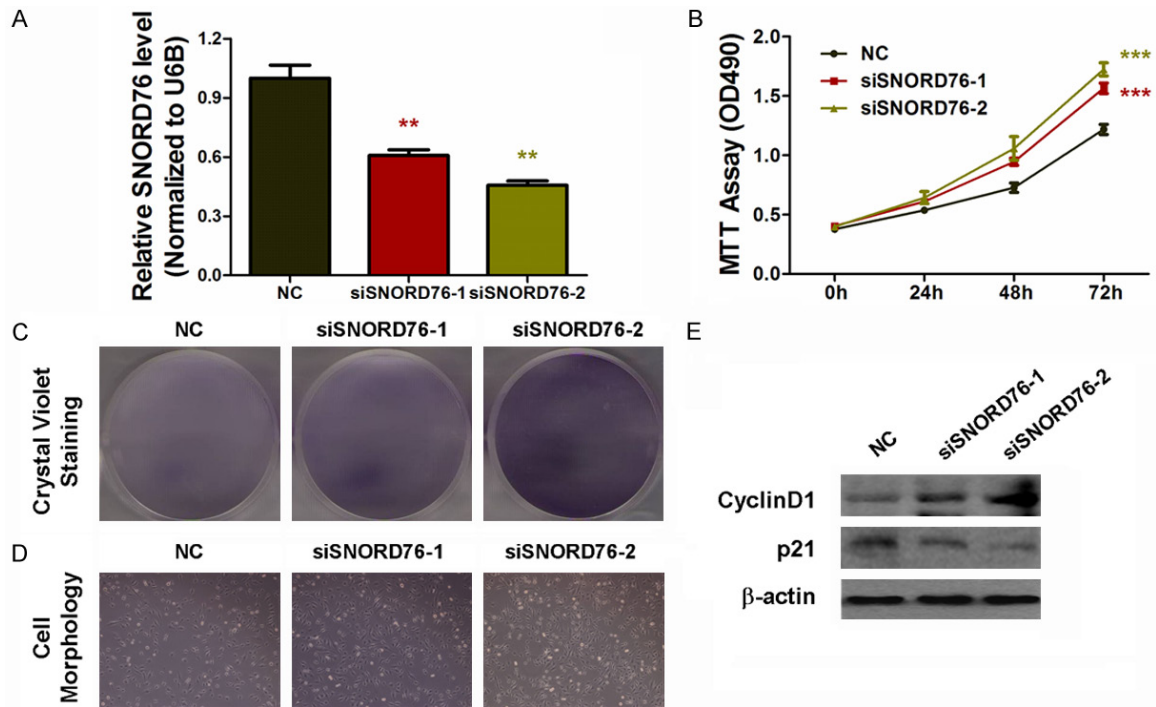
Figure 3. Knockdown of SNORD76 promoted cell proliferation through cyclinD1 and p21. A. U343 cells were transfected with control siRNA or two SNORD72 siRNAs. 48 h later, cells were harvested for qRT-PCR test. B, C. MTT assay over a 4-day period and crystal violate staining assay were performed to access cell growth rates. Data are expressed as the Mean ± SD of the experiments performed in triplicate. D. Knockdown of SNORD76 influenced cell density by morphological image U343. E. U343 cells were transfected with control siRNA or two SNORD76 siRNAs. 24 h later, cells were harvested for WB test (**: *P*<0.01, ***: *P*<0.001).

Expression of SNORD76 in GBM cell lines

To further examine SNORD76 expression in gliomas, RNA was firstly isolated from known GBM cell lines, including U87, U251, U343 cell lines, as well as the normal human astrocytes. The results from the qRT-PCR assay revealed that, SNORD76 was downregulated in these selected GBM cell lines compared with the control cells. Specifically, a most significant decrease of SNORD76 expression was observed in the U87 cell line (**Figure 1B**). From this aspect of results, we could conclude a downregulation of SNORD76 expression in GBM cell lines. The above results collectively illustrated that, SNORD76 was indeed downregulated in glioma tissues, especially decreased in GBM (grade IV) tissues and cell lines. Hence, we might regard SNORD76 as a potential usable biomarker for GBM diagnosis.

Relationship of SNORD76 expression with overall survival of glioma patients

To access the relationship of SNORD76 expression with overall survival of glioma patients,

Kaplan-Meier analysis was applied to disclosure this question and examine the prognostic value. The expression of snRNA SNORD76 in tumor tissues were categorized as low or high levels in relation to the mean value. The output survival curve revealed that, patients with lower SNORD76 expression tended to have worse (shorter) overall survival (log rank test, *P*=0.008, **Figure 2**). Hence, from this aspect, our results concluded that SNORD76 might be used as a prognosis predictor for glioma patients.

Knockdown of SNORD76 promotes cell proliferation and regulates cyclinD1 and p21

To explore the functions of SNORD76 in GBM cells, we performed loss-of-function experiments using siRNA transfection of U343 cells (with relative high endogenous SNORD76 expression. **Figure 3A** showed the knockdown effective of two independent SNORD76 siRNAs in U343 cells. The MTT assay and crystal violet staining assay demonstrated that cell proliferation rate was significantly enhanced in SNORD76 knockdown groups in comparison

with the control siRNA groups (NC) (**Figure 3B** and **3C**). Moreover, morphological views of the cells by microscope demonstrate that the density of the cells changed when expression of SNORD76 was inhibited by siRNAs (**Figure 3D**). Next, to investigate the underlying mechanism, key factors regulating cell proliferation were detected by western blot. The results showed that cyclinD1 was significantly elevated and p21 was significantly impaired in SNORD76 knockdown groups, which were consistent with phenotype (**Figure 3E**). Our finding suggested that the tumor suppression activity of SNORD76 might act through cyclinD1 and p21 as least partially.

Discussion

GBM is the most common and biologically aggressive malignant glioma in human. Its current poor prognosis is crucially linked to tumor invasion. In the past few years, efforts have been made in understanding the molecular pathogenesis of malignant gliomas. However, little light has been shed on the non-coding genome or transcriptome in cancer, which is extremely obvious in the snRNA field. Identification of novel tumor associated snRNAs is important for understanding their roles in tumorigenesis, and they might be crucial for development of novel therapeutic targets. Until recently, snRNAs have been reported to have functions in carcinogenesis in various human cancers. Nevertheless, there are almost none previous reports characterizing snRNA expression and their roles in gliomas. In this study, we focused on the snRNA SNORD76.

Although Chen and his colleagues have discovered a tumor suppressing function of SNORD76 in GBM, by suppressing GBM cell proliferation [22], they did not examine the expression and clinical significance of SNORD76 in glioma tissues. In this manuscript, we investigated SNORD76 expression in 46 cases of glioma tissues (including 6 non-tumoral brain tissues) and GBM cell lines. The results all came out a decrease of SNORD76 expression in GBM tissues and cell lines. Furthermore, the overall survival analysis showed that lower SNORD76 expression correlated with poorer overall survival of glioma patients. Together with the above evidence, we proposed that snRNA SNORD76 could be regarded as a novel diagnosis and prognosis biomarker for human glioma,

especially for high grade GBM patients. Nevertheless, we realized that our data was a bit weak when talking about its expression in GBM cell lines. Although all three selected cell lines exhibited consistent downregulation of SNORD76, we thought it was better to take more cell lines into this comparison. Also, hybridization in situ assay would be the best choice for characterizing SNORD76 expression in human glioma tissues.

In the long run, more attention should be paid on the other biological function and the more precise underlined mechanisms of SNORD76 in the pathology of GBM. More *in vitro* and *in vivo* evidence are still lacking for characterizing the function of SNORD76. Meanwhile, we should also ask a question why it was decreased in GBM. Is this linked with epigenetic modulation or transcription repression? It is worth noting that more precise mechanisms are involved in this regulation.

In conclusion, our results proved that snRNA SNORD76 expression was indeed downregulated in GBM tissues and cell lines. And its downregulation was associated with poor prognosis. Cellular function assay demonstrated that knockdown of SNORD76 enhanced cell proliferation rate and regulated key moleculars in cycle cell. These results indicated that snRNA SNORD76 might be a novel prognostic indicator in GBM and may be a potential target for gene therapy. We also recognized that more work need to be done to fully interpret its role in GBM.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Weiming Wang, Department of Neurology, The People's Hospital of Lishui City (The Six Affiliated Hospital of Wenzhou Medical University), No. 15 Dazhong Road, Liandu District, Lishui, Zhejiang Province, China. Tel: +86 (0578) 2780150; E-mail: wangwm_neuro@163.com

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