Int J Clin Exp Pathol 2016;9(4):4601-4614
www.ijcep.com /ISSN:1936-2625/IJCEP0018801

Original Article

Let-7g regulates p16ink4a/p19arf and inhibits proliferation of glioma cells: roles of HMGA2

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Received October 29, 2015; Accepted March 5, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: We carried out this study for the purpose of unveiling the association among let-7g, HMGA2 and p16ink4a/p19arf pathway in glioma. This study incorporated 78 glioma tissues which were collected from glioma patients and 10 normal brain tissues. Both real-time quantitative RT-PCR (qRT-PCR) and in situ hybridization were implemented for the detection of let-7g expression level in tissues and cells. Moreover, immunohistochemistry and western blot assay were used to measure HMGA2, p16ink4a and p19arf. We also transfected different groups of vectors into NGA cells and these vectors include let-7g inhibitors, lenti-HMGA2 and let-7g inhibitors + HMGA2 siRNA. U251 cells also were transfected with different groups of vectors including let-7g mimics, HMGA2 siRNA and let-7g mimics + lenti-HMGA2. Cell proliferation and apoptosis status were assessed by standard assays. Glioma xenograft models were constructed on mice in order to unfold effects of let-7g and HMGA2 on glioma tissues *in vivo*. Let-7g expression was significantly down-regulated in both glioma tissues and cells, while HMGA2 was significantly up-regulated compared to the reference group of normal brain cells (all $P < 0.05$). As demonstrated by transfected NGA cells, both let-7g inhibitors and lenti-HMGA2 significantly increased HMGA2 expression, decreased both ink4a and arf expressions and up-regulated the proliferation rate of glioma cells, while HMGA2 siRNA antagonized the effect of let-7g inhibitors on glioma cells (all $P < 0.05$). Transfected U251 cells suggested that let-7g mimics and HMGA2 siRNA significantly decreased HMGA2 expression, increased both ink4a and arf expressions, down-regulated the proliferation rate of glioma cells and up-regulated the apoptosis rate of glioma cells, whereas lenti-HMGA2 antagonized the effect of let-7g mimics on glioma cells (all $P < 0.05$). As suggested by glioma xenograft models, let-7g mimics and HMGA2 siRNA effectively inhibited the growth of glioma tumors. Let-7g may have significant impact on the proliferation and apoptosis of glioma cells via mediating p16ink4a/p19arf by targeting HMGA2.

Keywords: Let-7g, HMGA2, p16ink4a, p19arf, glioma, proliferation, apoptosis, xenograft model

Introduction

Glioma is a kind of malignant carcinoma that mainly occurs in the brain and it accounts for 81% cases of intracranial tumors [1]. As suggested by the histologic type of glioma and the *International Classification of Diseases-Oncology* version 3 (ICD-O-3), glioma cases can be divided into three groups: astrocytic type, oligodendrocytic type and a hybrid of these two types [2]. Compared with other types of tumors, the incidence of glioma is relatively low ranging from 4.67 to 5.73 per hundred million populations. However, this disease is associated with a large number of deaths and it results in an unexpectedly high mortality [3]. Moreover, glioblastoma is the most malignant one with a five-year survival rate of less than 5% [1]. Studies have reported that genetic and epidemiologic risk factors have significant influence on the development of glioma and single nucleotide polymorphism of seven genes including TERT, EGFR, RTEL1, TP53, CDKN2B, PHLDB1 and CCDC26 are associated with this disease [4]. Potential epidemiologic risk factors for glioma include allergy, ionizing radiation, non-ionizing radiation and exposure to chemicals [4]. So far, magnetic resonance imaging and computerized tomography have been introduced into clinical practice in order to assist in glioma diagnosis [5]. Surgery, immunotherapy, chemotherapy and radiotherapy are major approaches for managing patients with glioma and they appeared to have limited effectiveness due to

Table 1. Primer sequences of GAPDH and let-7g for implemention of RT-PCR

Gene		Primer sequence
GAPDH	Sense	5'-TGGTATCGTGGAAGGACTCAT-3'
	Antisense	5'-GTGGGTGTCGCTGTTGAAGTC-3'
Let-7g	Sens	5'-UGAGGUAGUAGGUUGUAUAGUU-3'
	Antisense	5'-CUAUACAACCUACUACCUCAUU-3'

GAPDH: glyceraldehyde phosphate dehydrogenase, RT-PCR: real time-polymerase chain reaction.

various reasons [6]. As a result, discovery of alternative therapies that are able to manage glioma in an effective and safe manner has been prioritized into the agenda. As suggested by recent studies, miRNAs influence the proliferation of glioma cells and this may provide additional information for understanding the formation of glioma.

MiRNAs are a class of non-coding RNAs with a length ranging from 18 to 24 bp and they play a role in tumorgenesis which affects tumor development and prognosis [7, 8]. MiRNAs were aberrantly expressed in some carcinomas and therefore using miRNAs as diagnosis biomarkers and therapeutic targets may be feasible [9]. It is reported that let-7 regulates human gliogenesis through NOTCH signaling pathway [10]. Let-7 is a family of miRNAs which are highly conserved in animal species and they are involved in tumorgenesis via different signaling pathways [11]. For example, let-7a suppresses the expression of K-Ras and inhibits the migration of glioma cells [12]. Moreover, let-7g has been reported to inhibit the proliferation and migration of hepatocellular carcinoma by targeting HMGA2 [13].

HMGA2 belongs to the high-mobility group A family and it is highly expressed in embryogenesis but they were rarely detected in adult tissues [14]. Besides, HMGA2 contributes to stemness maintenance which can trigger neoplasm formation when HMGA2 is overexpressed in adult tissues [15]. Studies have reported that HMGA2 plays a pivotal role in EMT maintenance and promotes cell proliferation in pancreatic cancer [16] Furthermore, HMGA2 is suppressed by let-7 in order to prevent neoplastic transformation [17]. Therefore, we hypothesized that let-7g is able to suppress glioma by targeting HMGA2.

It has been reported that HMGA2 could negatively regulate p16ink4a and p19arf for stimulating the self-renewal process of neural stem cells [15] and let-7g can inhibit hepatocellular tumor proliferation by up-regulating the expression of p16ink4a [18]. Both p16ink4a and p19arf are encoded by *p16INK4a* gene in the MTS1 locus [19] and they are considered as predictors of malignant carcinoma including non-small cell lung carcinoma, ewing sarcoma and colorectal carcinoma [20-22]. Both p16ink4a and p19arf are able to slowdown cell cycle progression and induce cell senescence by targeting Rb and P53, respectively [23, 24], suggesting that p16ink4a and p19arf could suppress cell proliferation and tumor formation. Hence, we suspected that let-7g targets HMGA2 and suppresses glioma formation via p16ink4a/p19arf signaling pathway.

Recently, a study provided evidence that let-7 targets HMGA2 through TGF-β/Smad3 pathway in glioma carcinoma [25]. However, let-7 also is likely to target HMGA2 through p16ink4a/p19arf signaling pathway for suppressing glioma carcinoma proliferation. Our experiments *in vitro* and *in vivo* revealed that let-7 inhibited HMGA2, suppressed glioma cell proliferation and enhanced glioma cell apoptosis. Hence, let-7 is a potential biomarker for both diagnosing and managing patients with glioma carcinoma.

Materials and methods

Ethics statement

Collection of human tissues was agreed by the Institutional Ethics Committee according to the Helsinki Declaration. Informed consent was obtained from patients prior to subject inclusion. All rat experiments were complied with the Guidance for Care and Usage of Laboratory Animals, and were adopted by National Cancer Institute Animal Care and Use Committee.

Clinical samples and cell lines

Glioma biopsy specimens (n = 78) were collected from the First Hospital of Jilin University between May 2013 and Oct 2014. Glioma patients were pathologically confirmed and they did not receive any radiotherapy or chemotherapy before surgical operation. As suggested by the World Health Organization (WHO) [26], glioma patients were classified as: low-grade group (9 grade I cases and 15 grade II cases) and high-grade group (32 grade III cases

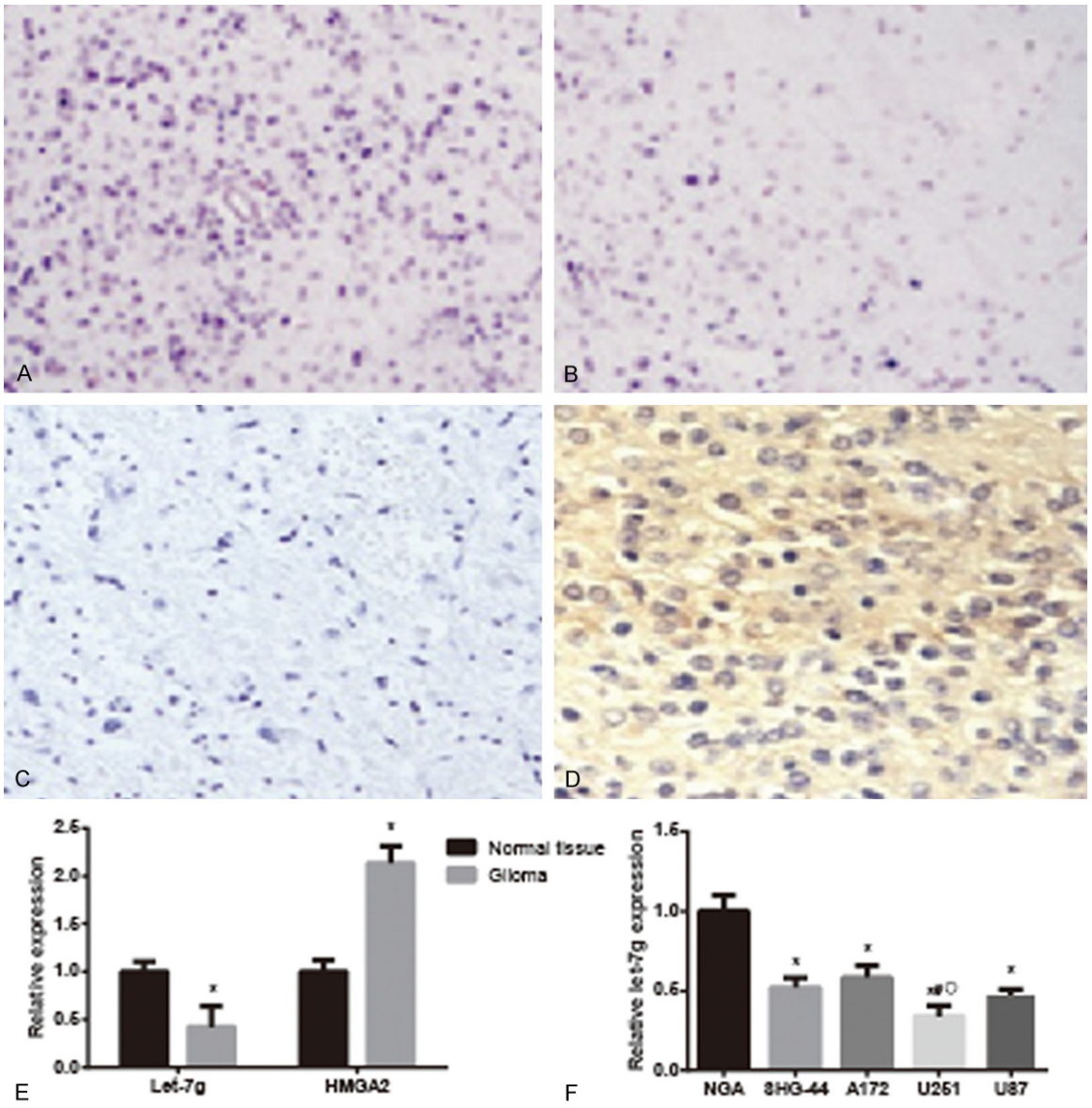

Figure 1. Let-7g and HMGA2 expressions in glioma tissues and cell lines. (A, B) In situ hybridization of let-7g in normal tissues (A) and glioma tissues (B). (C, D) Immunohistochemical staining of HMGA2 in normal tissues (C) and glioma tissues (D). (E) Relative let-7g expressions in glioma tissues and normal tissues. (F) decreased let-7g expression in glioma cell lines compared with normal cell lines.

and 22 grade IV cases). A total of 50 males and 28 females were incorporated with an average age of 48.2±14.6 years. The control group included 10 normal cerebral tissue samples (male:female = 7:3; average age: 47.8±13.1 years old) which were obtained from brain trauma patients who received intracranial decompression operation. All clinical characteristics of patients were presented in **Table 1**. Specimens collected in operation were treated with liquid nitrogen and conserved under -70°C.

One normal human brain astroglia cell line 1800 ATCC (NGA) and four human glioma cell lines (SHG-44, A172, U251 and U87) were purchased from the Institute of American Type Culture Collection (ATCC, American). Cells were cultured in Dulbecco modified Eagle medium (Gibco, Carlsbad, CA) with 10% fetal bovine serum (Gibco, Carlsbad, CA) at 37°C in an incubator with 5% CO_2. Cell passage was carried out when cell attachment rate of monolayer cells reached the level of 80%.

Table 2. The correlation between let-7a and clinicopathological characteristics of glioma patients

Characteristic	Number	The expression of let-7a	*P* value
Age			0.705
< 50	50	0.41±0.23	
≥ 50	28	0.43±0.21	
Gender			0.567
Male	50	0.41±0.21	
Female	28	0.44±0.24	
Glioma histopathology			0.935
Astrocytic tumors	58	0.42±0.22	
Oligodendrogial tumors	8	0.39±0.19	
Oligoastrocytic tumors	12	0.42±0.23	
WHO grade			< 0.0001
Low-grade (Grade I-II)	24	0.73±0.18	
High-grade (Grade III-IV)	54	0.28±0.11	

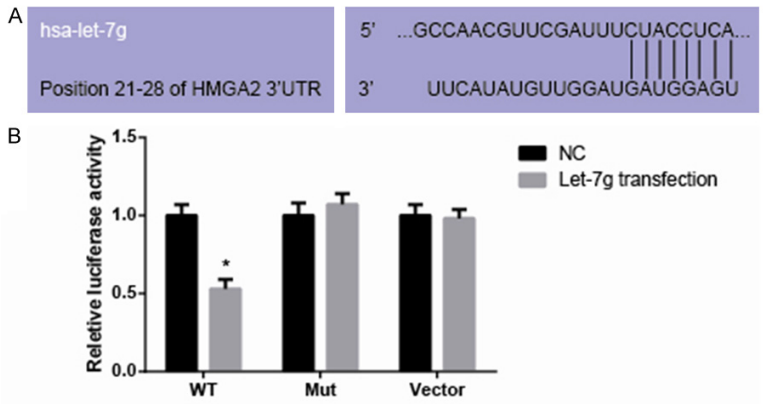

Figure 2. Binding site prediction and results of luciferase report assay between let-7g and HMGA2. A. Putative target sites predicted by online database. B. Relative luciferase activity resulted from the binding of HMGA2 3'UTR reporter and let-7g in NGA cells 48 h after transfection. *$P < 0.05$ versus NC group. WT: wide type of HMGA2-3'UTR; Mut: HMGA2 mut-3'UTR; NC: negative control.

Immunohistochemistry and in situ hybridization

Immunohistochemical analysis of HMGA2 in glioma tissues was performed with the two-step EnVision method as described previously [27]. Rabbit polyclonal anti-HMGA2 antibody (Zhongshan Biology Company, Beijing) was diluted for 100 times. In situ hybridization of let-7g for formalin-fixed and paraffin-embedded tissue samples were performed with a miRCURY LNA™ let-7g detection probe, via ISH optimization kit (Exiqon, Vedbaek). Scramble-miR probes were used as negative controls.

Luciferase activity assay

The 3'untranslated region (UTR) of *HMGA2* containing let-7g binding sites was amplified through polymerase chain reaction (PCR) and was cloned into the downstream of the psiCHECK-2 luciferase vector (Promega, USA), which was named as wt 3'UTR. The binding site was mutated using GeneTailor Site-Directed Mutagenesis System (Invitrogen, USA) and the resultant mutant 3'UTR was cloned into the same vector which was named as mu 3'UTR.

NGA cells maintained in 48-well plates were co-transfected with different groups of vectors: one group was transfected with the combination of 200 ng pGL3-control luciferase reporter, 10 ng pRL-TK vector and let-7g vector, while let-7g was replaced by the negative control vector. Transfected cells were analyzed with the Dual-Luciferase Reporter Assay System (Promega) 48 hours after their transfection.

Lenti-virus transduction and transfection

Four groups of fragments, separately containing let-7g mimics, let-7g inhibitors, HMGA2 siRNA and Lenti-HMGA2 were cloned into the pCDH vector. Then the pCDH vector was transfected into cells with other packaging plasmids using Lipofectamine LTX kit (Invitrogen, CA) and viral particles therein were collected 48 hours after transfection.

NGA cells were infected with five groups of recombinant lentivirus and 8 ug/ml polybrene: control group (cells without any transfection), NC group (cells transfected with negative control), let-7g inhibitors group (cells transfected with let-7g inhibitors), lenti-HMGA2 group (cells transfected with Lenti-HMGA2) and let-7g inhib-

Table 3. Relative luciferase activity in wt HMGA2, Mut HMGA2 and vector groups

Relative luciferase activity	Wt	Mut	Vector
NC	1.00±0.07	1.00±0.08	1.00±0.07
Transfection group	0.53±0.06*	1.07±0.07	0.98±0.06

Wt: wild type, Mut: mutation, NC: negative control. *P < 0.05 versus NC group.

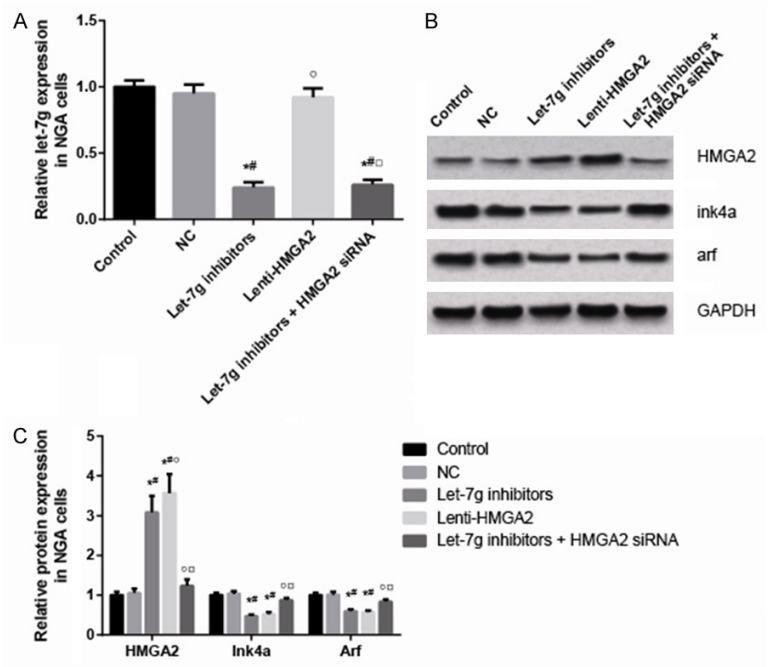

Figure 3. Expressions of let-7g, HMGA2, ink4a and arf in NGA cells after transfection. A. Quantitative data of mRNA level of let-7g in cells with different controls (i.e. NC, let-7g inhibitors, Lenti-HMGA2 and let-7g inhibitors + HMGA2 siRNA). B. Western blot analysis of HMGA2, ink4a and arf with GAPDH as the internal control in cells. C. Quantitative protein level of HMGA2, ink4a and arf in cells. Data were presented as mean ± SD for three independent experiments. *P < 0.05 versus control group; #P < 0.05 versus NC group; ○P < 0.05 versus let-7g inhibitors group; □P < 0.05 versus lenti-HMGA2 group.

itors + HMGA2 siRNA group (cells co-transfected with both HMGA2 siRNA and let-7g inhibitors). Besides that, U251 cells were transfected and divided into five groups: control group (cells without any transfection), NC group (cells transfected with negative control), let-7g mimics group (cells transfected with let-7g mimics), HMGA2 siRNA group (cells transfected with HMGA2 siRNA) and let-7g mimics + lenti-HMGA2 group (cells co-transfected with both lenti-HMGA2 and let-7g mimics).

RNA isolation and RT-PCR

Total RNA were extracted from tissues or cells with the aid of TRIzol reagent (Invitrogen). The ReverTra Ace qPCR RT Kit (Toyobo, Japan) was used to reversely transcribe total RNA into cDNA and real time-PCR (RT-PCR) was performed using THUNDERBIRD SYBR® qPCR Mix (Toyobo, Japan) at the instrument of CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relevant primers were enlisted in **Table 1**. Target gene expression levels were normalized to that of the control gene (GAPDH) and were calculated by the method of $2^{-\Delta\Delta CT}$.

Western blot

Tissues and cells were harvested and lysed using radio immunoprecipitation assay (RIPA) buffer. Total protein was separated and calculated in line with the Bradford method [28]. Then total protein was denatured in boiled water and transferred onto Polyvinylidene Fluoride (PVDF) membranes once sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was completed. Membranes were blocked in Tris Buffered Saline Tween (TBST) with 5% skim milk for 1 h and were then treated with primary antibodies against HMGA, p16ink4a and p19arf (1:800 dilution, Zhongshan Biology Company, Beijing) at 4°C overnight. Then membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-goat, 1:2000 dilution, Zhongshan Biology Company, Beijing) after they were completely washed. Samples in which reduced glyceraldehydes-phosphate dehydrogenase (GAPDH) was set as the endogenous control, were ultimately processed with enhanced chemiluminescence and quantified by Lab Works4.5 software (Mitov Software).

Cell proliferation and apoptosis assay

MTT assay was conducted to observe cell proliferation status. A total of 3×10^3 cells were cul-

Table 4. Expressions of let-7g, HMGA2, ink4a and arf in transfected NGA cells

Group	Control	NC	Let-7g inhibitors	Lenti-HMGA2	Let-7g inhibitors + HMGA2 siRNA
Let-7g	1.00±0.05	0.95±0.07	0.24±0.04*,#	0.92±0.07○	0.26±0.04*,#,□
HMGA2	1.00±0.08	1.05±0.11	3.08±0.42*,#	3.57±0.48*,#,○	1.23±0.17○,□
Ink4a	1.00±0.06	1.03±0.07	0.47±0.04*,#	0.52±0.05*,#	0.87±0.06○,□
Arf	1.00±0.06	1.01±0.07	0.59±0.05*,#	0.57±0.05*,#	0.83±0.06○,□

NC: negative control. *P < 0.05 versus control group; #P < 0.05 versus NC group; ○P < 0.05 versus let-7g inhibitors group; □P < 0.05 versus lenti-HMGA2 group.

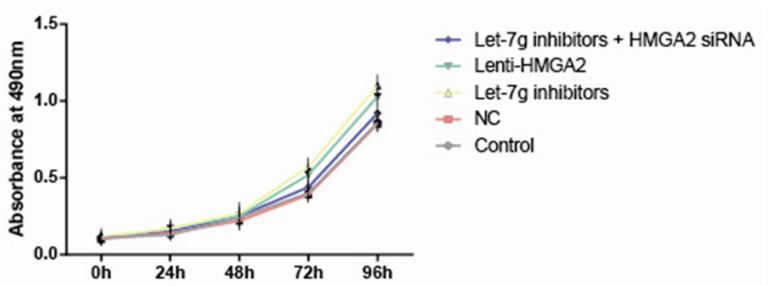

Figure 4. Effects of let-7g inhibitors, Lenti-HMGA2 and HMGA2 siRNA on proliferation of NGA cells estimated by MTT assay. Data were presented as mean ± SD for three independent experiments.

tured in 96 well plates and incubated for four different periods of time (24 h, 48 h, 72 h, 96 h). Then cultured cells were stained with 0.5 mg/ml MTT for 4 hours. Cell supernatant was discarded and 200-ul dimethylsulfoxide was added to cultured cells for dissolving precipitate. Samples were measured at 490 nm by an ELISA reader. Cell apoptosis rate in samples was assessed by flow cytometry once cells were stained with Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences). Cell necrosis was represented by Annexin V+ and PI+, cell apoptosis was represented by V+ and PI-whereas normal cells were represented by Annexin V- and PI-.

Animal model

Twenty male BALB/c nude mice (Institute of Zoology, Chinese Academy of Sciences, Beijing, China) with an average age of 4 weeks and weight of 16-18 g were obtained for building tumor growth models. Briefly, U251 cells were subcutaneously injected into the neck of mice (1×10^6 cells each mice). After 7 days when tumor volumes were quantified, tumor sites in the four groups of mice (5 mice per group) were transfected with four types of lenti-virus expressing vectors (control, let-7g mimics, HMGA2 siRNA, let-7g mimics + lenti-HMGA2) for 16 days through direct injection (2×10^7 units each time, twice a week). Tumor volume of mice in each group was calculated every 4 days using the following formula: volume = $(A\times B^2)/2$, where A is the biggest diameter of tumor and B is the smallest diameter of tumor.

Statistical analysis

All statistical analyses were performed with SPSS 18.0 software (Chicago, Illinois, USA). Data were presented in the form of mean ± standard deviation (SD). Two-tailed student's *t*-test or one-way analysis of variance (ANOVA) was used to analyze between-group or among-group comparisons. Counted data was compared and analyzed by the chi-squared test. P < 0.05 provided sufficient evidence for statistical significance.

Results

Glioma tissues or cells exhibited down-regulated let-7g and up-regulated HMGA2

Compared with normal tissue samples obtained from brain trauma patients, glioma tissues exhibited significantly lower let-7g expression and significantly higher HMGA2 expression (all P < 0.05, **Figure 1E**). We also analyzed the association between let-7g expression and clinicopathological characteristics of glioma patients, including age, gender, histopathology type and WHO grade. As shown in **Table 2**, patients with WHO III-IV stage had relatively lower let-7g expression compared with patients with WHO I-II stage (P < 0.05), while no significant relationship was found between let-7g expression and age, gender or histopathology type of patients (all P > 0.05). In addition, both immunohistochemistry and in situ hybridization further confirmed that glioma tissues exhibited

Table 5. Effects of let-7g and HMGA2 on proliferation of NGA cells

Group		Control	NC	Let-7g inhibitors	Lenti-HMGA2	Let-7g inhibitors + HMGA2 siRNA
Relative cell proliferation	0 h	0.11±0.04	0.10±0.04	0.12±0.05	0.10±0.04	0.11±0.04
	24 h	0.13±0.04	0.14±0.04	0.17±0.06	0.17±0.05	0.15±0.04
	48 h	0.24±0.06	0.22±0.06	0.27±0.07	0.25±0.06	0.25±0.06
	72 h	0.40±0.05	0.39±0.05	0.57±0.06*,#	0.52±0.06*,#	0.44±0.05○
	96 h	0.86±0.06	0.85±0.05	1.10±0.07*,#	1.03±0.05*,#	0.92±0.06○,□

*P < 0.05 versus control group; #P < 0.05 versus NC group; ○P < 0.05 versus let-7g inhibitors group; □P < 0.05 versus lenti-HMGA2 group.

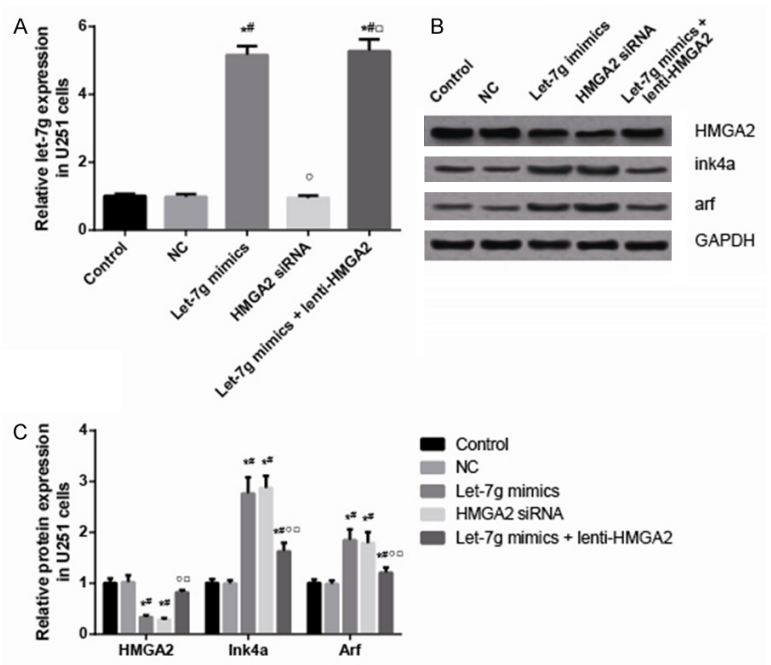

Figure 5. Expressions of let-7g, HMGA2, ink4a and arf in U251 cells after transfection. A. Quantitative data of mRNA level of let-7g in cells with different controls (i.e. NC, let-7g mimics, HMGA2 siRNA and let-7g mimics + lenti-HMGA2). B. Western blot analysis of HMGA2, ink4a and arf with GAPDH as the internal control in cells. C. Quantitative protein level of HMGA2, ink4a and arf in cells. Data were presented as mean ± SD for three independent experiments. *P < 0.05 versus control group; #P < 0.05 versus NC group; ○P < 0.05 versus let-7g mimics group; □P < 0.05 versus HMGA2 siRNA group.

lower let-7g levels but higher HMGA2 levels than normal tissues (**Figure 1A-D**).

Four types of glioma cell lines (SHG-44, A172, U251 and U87) were included in our research and they all exhibited significantly lower let-7g expression compared with normal brain astroglia cell line (NGA) (P < 0.05, **Figure 1F**). Among four glioma cell lines with different cell differentiation status, U251 cells had further lower let-7g expression compared with SHG-44 and A172 (P < 0.05).

HGMA2 expression was suppressed by the binding of let-7g with 3'UTR of HGMA2

As suggested by online database (www.microrna.org), one highly conserved let-7g binding site in the 3'-UTR of HMGA2 was predicted (**Figure 2A**). Moreover, luciferase activity assay revealed that binding of let-7g with normal 3'-UTR of HMGA2 in NGA cells could significantly decrease relative luciferase activity (P < 0.05), whereas there was no remarkable difference in the relative luciferase activity between the miR-31 vector and NC group which were contained in both of the blank and mut group (**Figure 2B**; **Table 3**).

Effects of let-7g inhibitors and lenti-HMGA2 on NGA cells

No significant differences in let-7g, HMGA2, ink4a or arf expression were identified between the control and NC group (P > 0.05, **Figure 3**; **Table 4**). Transfection of let-7g inhibitors remarkably up-regulated HMGA2 expression as well as down-regulated let-7g, ink4a and arf expressions compared with the control and NC group (P < 0.05). By contrast, over-expression of HMGA2 had no significant effects on let-7g expression (P > 0.05), while lenti-HMGA2 group exhibited significantly higher HMGA2 expression and lower ink4a and arf expressions compared with the control and NC group (P < 0.05). Besides

Table 6. Expressions of let-7g, HMGA2, ink4a and arf in transfected U251 cells

Group	Control	NC	Let-7g mimics	HMGA2 siRNA	Let-7g mimics + lenti-HMGA2
Let-7g	1.00±0.07	0.97±0.09	5.15±0.27*,#	0.95±0.07○	5.26±0.36*,#,□
HMGA2	1.00±0.09	1.02±0.14	0.33±0.04*,#	0.28±0.04*,#	0.82±0.04○,□
Ink4a	1.00±0.08	0.99±0.07	2.76±0.32*,#	2.87±0.24*,#	1.62±0.18*,#,○,□
Arf	1.00±0.07	0.98±0.08	1.84±0.22*,#	1.79±0.21*,#	1.20±0.11○,□

*P < 0.05 versus control group; #P < 0.05 versus NC group; ○P < 0.05 versus let-7g mimics group; □P < 0.05 versus HMGA2 siRNA group.

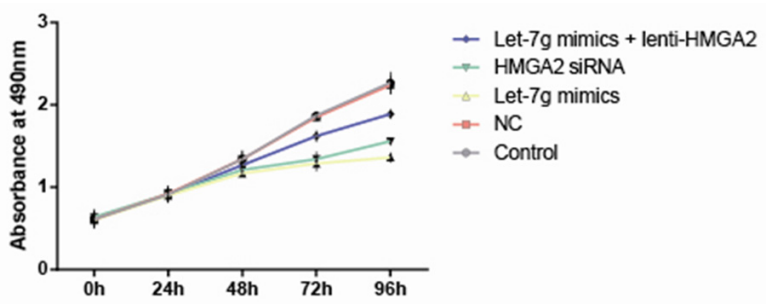

Figure 6. Effects of let-7g mimics, HMGA2 siRNA and lenti-HMGA2 on proliferation of U251 cells assessed by MTT assay. Data were presented as mean ± SD for three independent experiments.

that, HMGA2 siRNA antagonized the effect of let-7g inhibitors on HMGA2, ink4a and arf expressions, suggesting that no significant difference in the expression of HMGA2, ink4a or arf was observed between the let-7g inhibitors + HMGA2 siRNA and the control/NC group(P > 0.05, **Figure 3**; **Table 4**).

Proliferation status of NGA cells was indicated by MTT assay (**Figure 4**; **Table 5**) in which the number of cells was represented by the OD value. No obvious difference in cell proliferation status was identified among the control, NC or let-7g inhibitors + HMGA2 siRNA group at each time point (P > 0.05). However, both the let-7g inhibitors and lenti-HMGA2 group exhibited significantly higher relative proliferation rate after 72 hours compared with the control and NC group (P < 0.05). Apart from that, the let-7g inhibitors + HMGA2 siRNA group had lower proliferation rate than that of the let-7g inhibitors group at 72 h and it exhibited lower proliferation rate in comparison to the let-7g inhibitors and lenti-HMGA2 group at 96 hours (P < 0.05).

Effects of let-7g mimics and HMGA2 siRNA on U251 cells

There was no significant difference in let-7g, HMGA2, ink4a or arf expression between the control and NC group (P > 0.05, **Figure 5**; **Table 6**). Transfection of let-7g mimics significantly decreased HMGA2 expression and increased let-7g, ink4a and arf expressions compared with the control and NC group (P < 0.05). Unlike let-7g, HMGA2 siRNA suppressed HMGA 2 expression and elevated both ink4a and arf expressions. Furthermore, over-expression of HMGA2 antagonized the effect of let-7g mimics on HMGA2, ink4a and arf expressions, suggesting that no significant difference in HMGA2 or arf expression was observed between the let-7g inhibitors + HMGA2 siRNA and control/NC group (P > 0.05), while ink4a expression in the let-7g inhibitors + HMGA2 siRNA group was higher than those of the control and NC group but lower than those of the let-7g mimics and HMGA2 siRNA group (P < 0.05, **Figure 5**; **Table 6**).

Furthermore, no obvious difference in the proliferation status of U251 cells was identified between the control and NC group at each time point (P > 0.05). Let-7g mimics group had significantly lower proliferation rate than the control and NC group after 48 hours (P < 0.05). Transfection of HMGA2 siRNA remarkably down-regulated cell proliferation after 72 hours in comparison to those of the control and NC group, while the HMGA2 siRNA group exhibited significantly higher proliferation rate than the let-7g mimics group at 96 hours. Besides, proliferation rate of the let-7g mimics + lenti-HMGA2 group was significantly lower than those of the control and NC group but higher than the let-7g mimics and HMGA2 siRNA group after 72-hour transfection (P < 0.05, **Figure 6**; **Table 7**).

In addition, apoptosis rates of U251 cells were evaluated using of the annexin V-FITC/PI staining approach. No obvious difference in the apoptosis rate was identified between the con-

Table 7. Effects of let-7g and HMGA2 on proliferation and apoptosis of U251 cells

Group		Control	NC	Let-7g mimics	HMGA2 siRNA	Let-7g mimics + lenti-HMGA2
Relative cell proliferation	0 h	0.61±0.10	0.61±0.10	0.60±0.10	0.64±0.09	0.62±0.08
	24 h	0.91±0.10	0.92±0.10	0.90±0.08	0.92±0.08	0.91±0.09
	48 h	1.34±0.10	1.34±0.10	1.17±0.04*,#	1.21±0.05	1.27±0.07
	72 h	1.87±0.05	1.85±0.05	1.29±0.09*,#	1.34±0.09*,#	1.62±0.07*,#,○,□
	96 h	2.27±0.13	2.24±0.11	1.36±0.06*,#	1.56±0.04*,#,○	1.89±0.06*,#,○,□
Apoptosis rate		1±0.04	3.45±0.26	3.44±0.27	26.92±3.12*,#	24.88±2.47*,#

*P < 0.05 versus control group; #P < 0.05 versus NC group; ○P < 0.05 versus let-7g mimics group; □P < 0.05 versus HMGA2 siRNA group.

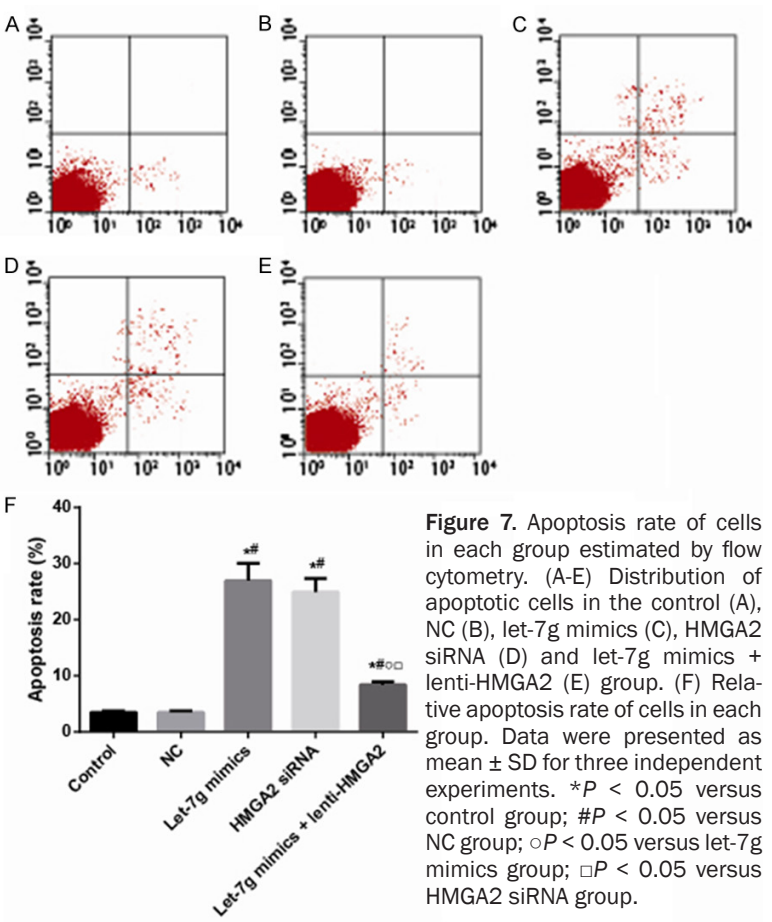

Figure 7. Apoptosis rate of cells in each group estimated by flow cytometry. (A-E) Distribution of apoptotic cells in the control (A), NC (B), let-7g mimics (C), HMGA2 siRNA (D) and let-7g mimics + lenti-HMGA2 (E) group. (F) Relative apoptosis rate of cells in each group. Data were presented as mean ± SD for three independent experiments. *P < 0.05 versus control group; #P < 0.05 versus NC group; ○P < 0.05 versus let-7g mimics group; □P < 0.05 versus HMGA2 siRNA group.

trol and NC group (P > 0.05). Transfection of let-7g mimics and HMGA2 siRNA had similar effects on the apoptosis status of U251 cells and they both significantly increased the apoptosis rate of U251 cells compared with the control and NC group (P < 0.05). On top of that, the let-7g mimics + lenti-HMGA2 group exhibited significantly higher apoptosis rate than those of the control and NC group but lower apoptosis rate compared to the let-7g mimics and HMGA2 siRNA group (P < 0.05, **Figure 7**; **Table 7**).

Results of animal model

A human glioma model was constructed on mice by injecting U2521 cells into mice in order to achieve tumor transplantation so that the effects of let-7g mimics and HMGA2 siRNA on glioma tumors can be confirmed *in vivo*. Significant differences in tumor size and volume were identified since day 8 of the operation. The let-7g mimics and HMGA siRNA group exhibited significantly smaller tumor volumes than those of the control and let-7g mimics + lenti-HMGA2 group on day 8 (P < 0.05). The control group had remarkably larger tumor volumes compared with the other three groups on day 12, while the let-7g mimics and HMGA2 siRNA group exhibited much smaller tumor volumes in comparison to the let-7g mimics + lenti-HMGA2 group (P < 0.05, **Figure 8**; **Table 8**).

Discussion

Identification of both miRNAs and their relevant targeted proteins is essential for unveiling the influence of miRNAs on tumorgenesis [25]. It has been widely acknowledged that dysregulated miRNAs are closely linked to the growth,

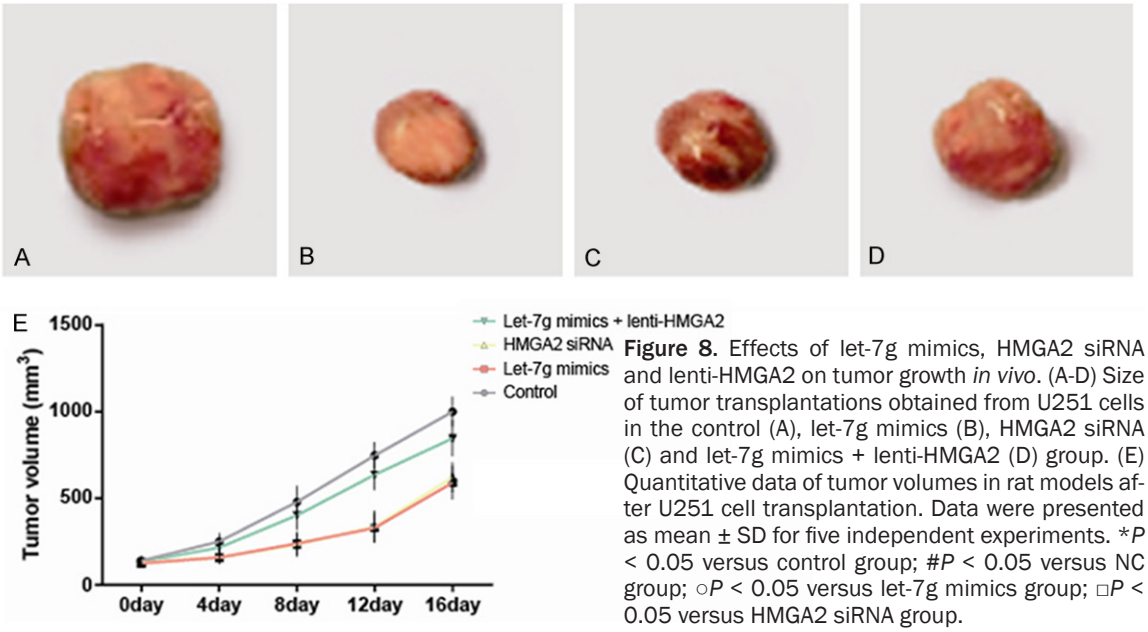

Figure 8. Effects of let-7g mimics, HMGA2 siRNA and lenti-HMGA2 on tumor growth *in vivo*. (A-D) Size of tumor transplantations obtained from U251 cells in the control (A), let-7g mimics (B), HMGA2 siRNA (C) and let-7g mimics + lenti-HMGA2 (D) group. (E) Quantitative data of tumor volumes in rat models after U251 cell transplantation. Data were presented as mean ± SD for five independent experiments. **P* < 0.05 versus control group; #*P* < 0.05 versus NC group; ○*P* < 0.05 versus let-7g mimics group; □*P* < 0.05 versus HMGA2 siRNA group.

Table 8. Tumor volumes of mice model in each group

Group/(mm^3)	Control	Let-7g mimics	HMGA2 siRNA	Let-7g mimics + lenti-HMGA2
0 d	138.23±23.23	122.74±18.92	127.57±23.57	131.41±22.12
4 d	249.59±47.06	156.54±23.57	157.54±39.05	214.47±33.65
8 d	478.17±93.18	238.28±67.72*	230.33±69.95*	401.23±82.01#,○
12 d	746.68±77.83	328.70±82.77*	334.82±93.31*	635.34±88.73*,#,○
16 d	999.70±85.57	589.53±96.67*	620.43±85.31*	845.86±104.58*,#,○

**P* < 0.05 versus control group; #*P* < 0.05 versus let-7g mimics group; ○*P* < 0.05 versus HMGA2 siRNA group.

apoptosis and metastasis of tumor cells due to the aberrant expression of miRNAs [29-31]. Let-7, as an original member of the miRNA family, has been indicated to be a putative tumor suppressor [32, 33]. As suggested by resent studies, the expression of let-7 appeared to be down-regulated in lung, breast, head and neck, pancreatic, colon and ovarian carcinomas [34-39] and let-7 expression levels substantially varied with cancer progression [40]. As the most frequently observed brain tumors, gliomas are also reported to be involved with regulation pathways which are dominated by let-7 [12, 41]. This study enabled us to discover that let-7g expression was significantly decreased in glioma tissues and cell lines, suggesting that let-7g was associated with glioma progression. These results are in line with previous reports from Lee *et al.* [41] and Amaral *et al.* [42]. We extended our experiments to other types of glioma cell lines and discovered that different characteristics of glioma cell lines would influence let-7g expression. In addition, our study demonstrated that over-expressed let-7g can suppress the proliferation of U251 cell lines whereas inhibited let-7g expression exacerbated the proliferation of NGA. These findings suggested that let-7g has a negative regulatory impact on tumor genesis and glioma progression.

High-mobility group A2 protein is an architectural transcription factor which is universally expressed during embryogenesis and other various tumor progression stages [43-45]. Previous studies observed that HMGA2 was involved in pituitary tumorigenesis [46-48], while others suggested that HMGA2 was negatively correlated with the expression of let-7 miRNAs family [17, 40, 49]. Madison *et al.* illustrated that inhibition of let-7 induced the development of intestinal cancer mainly through targeting HMGA2 [50]. Furthermore, Keane *et al.* established a pathway of MYCN/LIN28B/Let-7/

HMGA2 in suppressing post-natal proliferation [51]. However, little evidence has been revealed with respect to the correlation between let-7g/HMGA2 pathway and tumorgenesis of glioma. In the current study, we noted that HMGA2 exhibited significantly higher expression in glioma tissues compared with normal tissue samples. Besides that, both suppression of let-7g and over-expression of HMGA2 were related to stimulated proliferation of glioma cells, while high level of let-7g and repression of HMGA2 had the opposite effect on the proliferation of glioma cells and this trend was confirmed by the mice model. Bianchini *et al.* illustrated that inhibition of let-7 maybe not a primary stimulator of up-regulated HMGA2, but rather a subordinate participant in the down-regulation of HMGA2 [52]. Our results further indicated that the enhancement of let-7g significantly down-regulated the expression of HMGA2, while the inhibition of let-7g had the opposite impact on HMGA2 expression. Several reports indicated that human HMGA2 gene is a common target of chromosomal rearrangements which are able to induce C terminus loss of both the protein and the 3'-UTR of the mRNA [17, 40, 49, 53]. This conclusion has been confirmed in our study by luciferase activity assay which bound let-7g with normal 3'-UTR of HMGA2 in NGA cells. As a result, we suspected that let-7g targeted HMGA2 and regulated glioma cell metastasis by binding with the 3'-UTR of HMGA2.

Recently, both p16ink4a and p14arf gene have been widely studied as tumor suppressors [54-56]. For instance, Vanbrocklin *et al.* suggested that p16ink4a and p14arf played vital roles in the development of glioma cells [57]. Moreover, Nishino *et al.* has illustrated that fetal and young adult stem cells which contain suppressed HMGA2 exhibited up-regulated p16Ink4a and p19Arf expressions [15]. However, the effect of let-7g/HMGA2 on p16ink4a/p14arf in human glioma tumorigenesis has not been completely disclosed. In the current study, let-7g down-regulated HMGA2, stimulated ink4a/arf expression and then inhibited the proliferation of glioma cells. Furthermore, the antagonism between let-7g inhibitors and HMGA2 siRNA, let-7g mimics and lenti-HMGA2 were both effective in regulating ink4a/arf expression, suggesting that ink4a/arf is located in the downstream of HMGA2. Vanbrocklin *et al.* illustrated that ink4a/arf regulated the recurrence of tumors through RAS pathway which was not incorporated in our study [57]. Intriguingly, Markowski *et al.* reported that p14/arf is able to repress the expression of HMGA2 and therefore further studies may be carried out to confirm these conclusions [45]. All of these outcomes revealed that let-7g increased ink4a/arf expression through targeting HMGA2 in glioma and this may provide evidence for unveiling the function of let-7g in glioma.

In conclusion, suppressed let-7g expression and enhanced HMGA2 expression were observed in glioma cells and tissues. Furthermore, let-7g negatively regulated ink4a/arf genes by targeting HMGA2 in suppressing the progression of glioma. However, relatively small sample size and the lack of explanation for the mechanism of ink4a/arf in glioma cells are major limitations of this study. Therefore, future studies are encouraged in order to verify the feasibility of using let-7g/HGMA2/ink4a/arf pathway for designing alternative targeted therapies in managing glioma.

Acknowledgements

This work was funded by National Natural Science Foundation of China (grant Nos. 81201188 and 81472344) and Research Fund for the Scientific and Technological development plan project in Jilin Province (grant No. 20150414031GH).

Disclosure of conflict of interest

None.

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