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MiR-301a is interrelated with poor prognosis and contributes to temozolomide resistance in human glioma

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Objective: To study the role of microRNA-301a (miR-301a) in glioma. *Methods:* Real-time quantitative RT-PCR (qRT-PCR) was carried out to detect the miR-301a expression in tissues. CCK-8 assay and cell viability assay were used to determine cell proliferation and temozolomide (TMZ) resistance. The association between mir-301a and BTG1 was confirmed by luciferase reporter assay as well as Western blot. *Results*: The miR-301a expression was raised in glioma significantly. Obvious interrelation was detected between high miR-301a expression level with high WHO grade. The 5-year overall survival (OS) rate was dramatically reduced in patients with high miR-301a expression. Besides, miR-301a was a prognostic variable for OS rate. Moreover, miR-301a overexpression promoted glioblastoma cell proliferation and TMZ resistance through regulating BTG1. *Conclusions:* High miR-301a expression in glioma is participated in glioblastoma cell proliferation as well as TMZ resistance, which may help for the prognosis and treatment for glioma.

1. Introduction

Glioblastoma multiforme is a kind of gliomas with most malignant behavior (Liang et al. 2017). Despite great progress, glioma survival remains a major challenge, especially for high-grade glioma (Houshyari et al. 2015). At present, Karnofsky performance status score (KPS) and WHO grade are the most recognized prognostic indicators for glioma. However, because of the heterogeneity among patients, these factors are not able to provide an accurate estimate of prognosis in glioma patients.

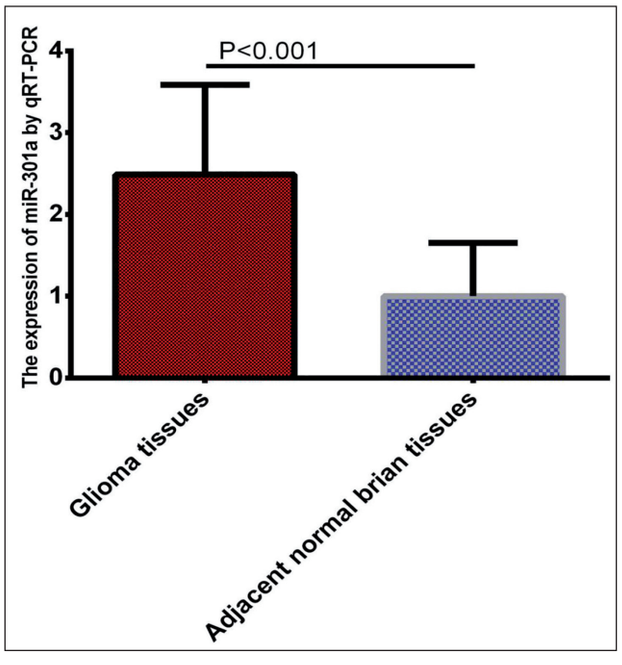

Fig. 1: Relative miR-301a expression in glioma tissues and the normal.

MicroRNAs (miRNAs) play role in regulating gene expression *via* binding the target mRNAs (Luo et al. 2015; Ameres and Zamore 2013). Recently, miRNAs have shown the possibility of being used as diagnostic and prognostic biomarkers for cancers (Challagundla et al. 2014; Lei et al. 2020; Li et al. 2019). Interestingly, miR-301a has proven its role in several types of cancers (Fang et al. 2015; Ma et al. 2014; Shi et al. 2016; Xia et al. 2015; Xu et al. 2013; Zhou et al. 2012). Notably, miR-301a level is significantly increased in glioma. Furthermore, it was found that miR-301a exerts its role in promoting glioma cell invasion through the Wnt/miR-301a/SEPT7 pathway (Yue et al. 2016). However, the significance in clinic and biological roles of miR-301a in glioma remain unknown. Thus, we tried to study the significance and biological functions of miR-301a in glioma.

2. Investigations and results

2.1. MiR-301a was upregulated in glioma

Obviously, MiR-301a expression was increased in human glioma tissues in contrast to the adjacent normal (P<0.001, Fig. 1). Patients with glioma were divided into the low-expression group (n=50) and the high-expression group (n=50) in the light of the median miR-301a expression.

2.2. Correlation between miR-301a and characteristics of glioma patients

Significant associations were detected between high miR-301a expression level and high WHO grade (P<0.001), as well as low KPS score (P=0.001; shown in Table 1). However, no obvious relationship existed between miR-301a and age, gender, or tumor size (P>0.05, respectively; Table 2).

2.3. miR-301a is related to poor prognosis in glioma patients

The 5-year OS rate was obviously reduced in the high-expression group than the low-expression group (P=0.034; shown in Fig. 2). Multivariate analysis indicated that miR-301a was a prognostic variable for OS rate (HR=3.384, 95% CI: 1.383-10.021, P=0.021, Table 2).

Table 1: Correlations between miR-301a expression and clinicopathological variables of glioma patients

Variables	Case(n)	miR-301a expression level		*P* value
		High(n=50)	Low(n=50)	
Age				
≤50	43	18	25	0.225
>50	57	32	25	
Gender				
Male	61	29	32	0.682
Female	39	21	18	
KPS score				
>80	59	21	38	0.001
≤80	41	29	12	
Tumor size(cm)				
≤5	55	24	31	0.228
>5	45	26	19	
grade WHO				
I- II	47	11	36	<0.001
III-IV	53	39	14	

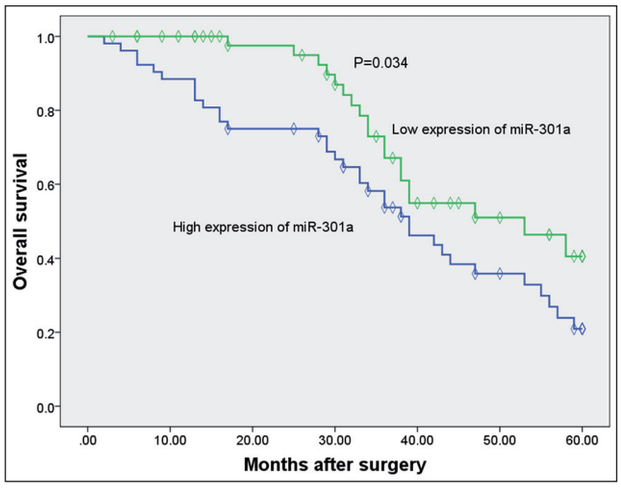

Fig. 2: Kaplan-Meier curves with the log-rank test for patients with glioma according to miR-301a expression level.

2.4. MiR-301a overexpression promotes glioblastoma cell proliferation

To clarify miR-301a's role in the glioblastoma cell proliferation, we established two glioblastoma cell lines, which stably expressed miR-301a. The miR-301a was raised significantly in miR-301a overexpression U251 and LN229 cells (Fig. 3A). Compared to the proliferation of control cells, CCK-8 assay indicated that the miR-301a overexpression U251 and LN229 cells was much higher (Fig. 3B). Moreover, miR-301a overexpression significantly upregulated the levels of proliferation markers Ki67 and PCNA in U251 and LN229 cells (Fig. 3C).

2.5. Overexpression of miR-301a enhances TMZ resistance of glioblastoma cells

We next attempted to clarify miR-301a's role in TMZ resistance of glioblastoma cells. Cell viability assay revealed that miR-301a

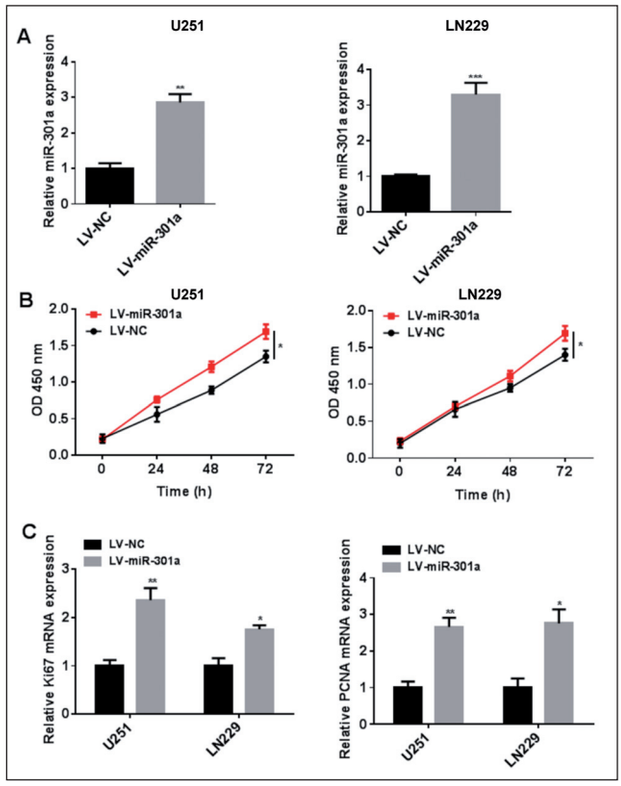

Fig. 3: MiR-301a enhances glioblastoma cell proliferation. (A) The miR-301a levels in U251 and LN229 cells (B) The proliferation rate of U251 and LN229 cells. (C) The Ki67 and PCNA mRNA levels in U251 and LN229 cells. (*P<0.05, **P<0.01, ***P<0.001)

overexpression U251 and LN229 cells showed increased TMZ resistance compared with control cells (Fig. 4A). Moreover, miR-301a overexpression significantly decreased the apoptosis rate of U251 and LN229 cells treated with TMZ (Fig. 4B).

2.6. MiR-301a directly targets BTG1

Among the targets predicted by TargetScan, BTG1, a negative regulator of glioma, was noted. There was a predicted binding site between miR-301a and BTG1 3'UTR (Fig. 5A). MiR-301a

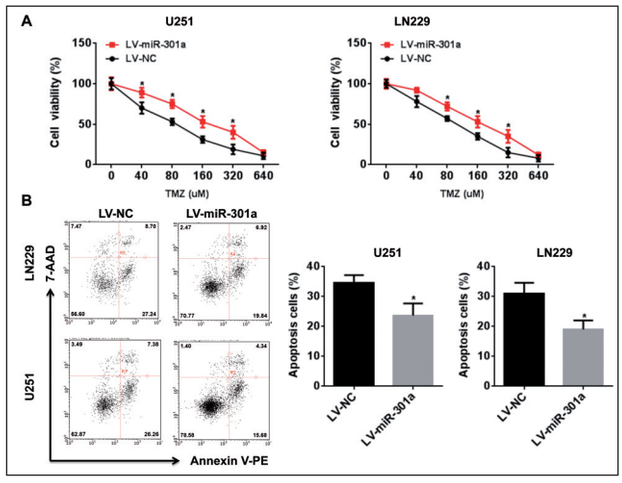

Fig. 4: MiR-301a strengthens glioblastoma cells to TMZ resistance. (A) The cell viability of U251 and LN229 cells was analyzed by CCK-8 assay after with TMZ (0-1000 μM) treatment. (B) The cell apoptosis of U251 and LN229 cells was analyzed by flow cytometry after with TMZ (80 μM) treatment. (*P<0.05)

mimic attenuated the luciferase activity of luciferase vector with WT BTG1 3'UTR significantly, but had no influence on luciferase vector with Mut BTG1 3'UTR (Fig. 5B, 5C). Moreover, miR-301a overexpression substantially restrained the BTG1 protein expressions in U251 and LN229 cells (Fig. 5C).

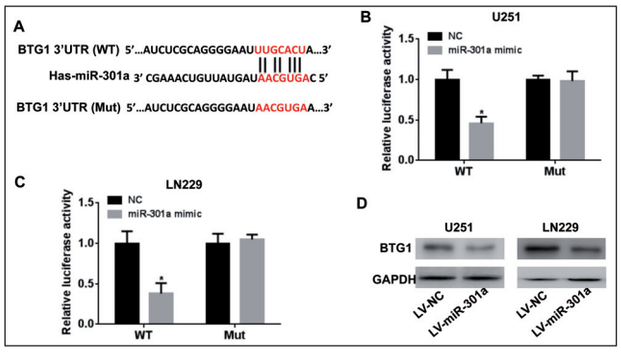

Fig. 5: BTG1 is a target of miR-301a. (A) Schematic miR-301a putative target sites in 3'UTRs of BTG1 and sequence of miR-301a-mutant. (B, C) The luciferase activity of U251 (B) and LN229 (C) cells after co-treated with luciferase reporter vectors containing the wide type (WT) or mutant (Mut) BTG1 3'UTR and miR-301a mimic. (D) The BTG1 protein expression in U251 and LN229 cells. (*P<0.05)

2.7. *MiR-301a promotes glioblastoma cell proliferation and TMZ resistance via BTG1*

To explore whether BTG1 regulated glioblastoma cell proliferation and TMZ resistance induced by miR-301a, BTG1 overexpression vector was used. As shown in Fig. 6A, BTG1 overexpression significantly down-regulated the proliferation rate of U251 and LN229 cells induced by miR-301a. Moreover, the cell viability assay revealed that BTG1 overexpression abolished the role of miR-301a overexpression on TMZ resistance (Fig. 6B).

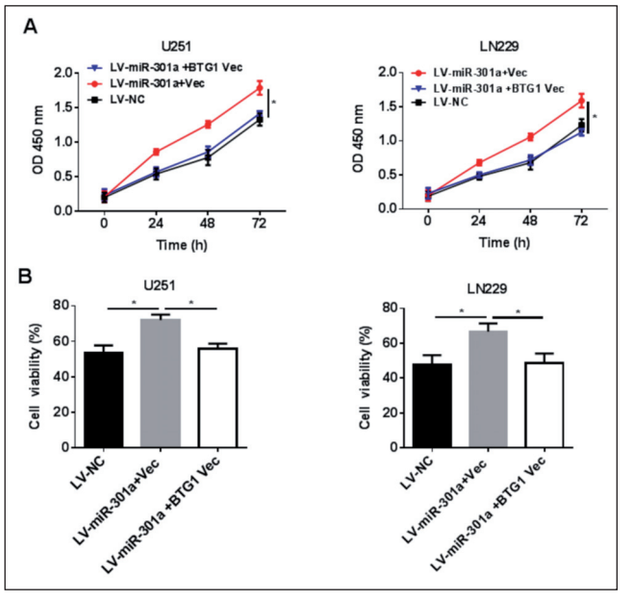

Fig. 6: BTG1 abolished the effect of miR-301a overexpression on cell proliferation and TMZ resistance. (A) The proliferation rate of U251 and LN229 cells. (B) The cell viability of U251 and LN229 cells was analyzed by CCK-8 assay after with TMZ (80 μM) treatment. (*P<0.05)

3. Discussion

Glioma, arising from the glials cell in the brain and spinal cord, is common in the nervous system (Mrugala 2013). Like other tumors, gliomas are also caused by the interaction between congenital genetic risk factors and environmental carcinogenic factors (Arcella et al. 2020; Wrensch et al. 2002). Recently, the development of molecular genetics has provided new insights for the classification and prediction of treatment response in gliomas (Ghotme et al. 2017; Wang et al. 2017).

More and more study results show that miRNAs are related to tumor prognosis, and can thus be considered as biomarkers (Corsini et al. 2012; Fabbri 2010; Sun et al. 2015). MiR-301a has proven to be increased in numerous cancers before. For instance, miR-301a was increased in gastric cancers, which was significantly associated with tumor TNM stage and prognosis (Xu et al. 2013). Besides, miR-301a was significantly raised in non-small-cell lung cancer (NSCLC), which was relevant to advanced TNM stage, poorly tumor differentiation, lymph node metastasis as well as poor prognosis (Shi et al. 2016). What's more, upregulated miR-301a expression was related to the progression of triple-negative breast cancer (TNBC), indicating that miR-301a might be a novel biomarker for TNBC prognosis (Yu et al. 2014). Previously, miR-301a expression was found ascended obviously in glioma (Yue et al. 2016). Unfortunately, the clinical significance of miR-301a in glioma remains unknown. In the present experiment, we found that in contrast to corresponding normal brain tissues, the miR-301a expression was increased remarkably in glioma.

In this study, the relevance between high miR-301a expression and advanced WHO grade was observed. Thus, we conducted a follow-up in all the 100 patients with glioma to determine whether miR-301a expression has a prognostic value. The data indicated that the 5-year OS rate was significantly reduced in the high-expression group than the low-expression group. Furthermore, Multivariate analysis indicated that miR-301a was a prognostic variable for OS rate. Therefore, high miR-301a expression was associated with poorer prognosis.

MiR-301a has been proven for its multi-roles in cancer, including tumorigenesis, progression, invasion, and metastasis. In gastric cancer, miR-301a owned the ability to promote cell migration, cell invasion, as well as cell clone formation *in vitro* (Xu et al. 2013). Chen et al. (2012) found miR-301a to promote cell proliferation in pancreatic cancer through regulating Bim. In addition, miR-301a enabled to promote glioma cell invasion through Wnt/miR-301a/SEPT7 signaling axis (Yue et al. 2016). In this study, miR-301a promoted glioblastoma cell proliferation and TMZ resistance, suggesting that miR-301a functioned importantly in glioblastoma. As a tumor suppressor, BTG1 was involved in multiple cancers (Yuniati et al. 2019). Wang et al. (2019) found that PUM2 knockdown remarkably suppressed proliferation and migration of glioblastoma cells *via* increasing BTG1 expression (Wang et al. 2019). In the study by Zhu et al. (2013), BTG1 overexpression could attenuate cell proliferation and promote apoptosis in breast cancer. Zhang et al. (2017) showed that miR-511 overexpression enhanced hepatoma cells proliferation *via* regulating BTG1. In our experiment, BTG1 is the target of miR-301a. Moreover, BTG1 overexpression abolished the effect of miR-301a overexpression on cell proliferation and TMZ resistance of U251 and LN229 cells. In summary, miR-301a may be a potential noninvasive biomarker for predicting clinical outcome of glioma. Further multi-center prospective studies are warranted to confirm our findings. Also, miR-301a overexpression glioblastoma cell proliferation and TMZ resistance by targeting BTG1.

Table 2: Multivariate analyses of prognostic variables of overall survival in glioma patients

Variable	Hazard ratio	95% CI	P-value
Age	2.016	0.677-5.018	0.103
Gender	1.228	0.489-2.102	0.719
KPS score	3.019	1.979-8.335	0.038
Tumor size	3.693	1.472-8.933	0.017
grade WHO	5.294	2.759-13.923	0.001
miR-301a expression	3.384	1.383-10.021	0.021

4. Experimental

4.1. Patients and tissue samples

The glioma tissues of 100 patients with glioma treated in the Department of Neurosurgery of the General Hospital of Shenyang Military were collected. Patients received radiotherapy or chemotherapy before surgery were excluded. The general characteristics are shown in Table 2. All the patients signed informed consent voluntarily. The Ethics Committee of The First Affiliated Hospital of Soochow University approved our present study.

4.2. Real-time PCR

Total RNAs were isolated from cells or samples using Trizol (Invitrogen, USA). TaqMan miRNA real-time RT-PCR kit was helped to evaluate the miRNA levels while 7500 software v.2.0.1 was helped to analyze the data, and RNU6B was as the control. The SYBR Green Master Mix (Vazyme, Nanjing, China) was adopted to determine the gene levels, and β-actin was as the control. The $2^{-\Delta\Delta Ct}$ method was taken for determining miR-301a or genes relative expression.

4.3. Cell culture, lentiviral transduction, and transfection

U251 and LN229 cell lines were got from ATCC. Lentiviruses (LV) were achieved from Genepharma (Shanghai, China). For lentiviral transduction, LV-miR-301a or LV-NC was added into U251 and LN229 cells. After 24 h, 0.2 μg/ml puromycin was added into cells. BTG1 overexpression vector (BTG1 Vec) and control vector (Vec) that transfected U251 and LN229 cells using Lipofectamine 3000 reagent (Invitrogen) were also achieved from Genepharma (Shanghai, China).

4.4. CCK-8 assay

CCK-8 kits purchased form Dojindo (Kumamoto, Japan) were adopted for cell proliferation detection. Cells treated with LV-NC or LV-miR-301 were seeded 96-well plates at 1,000/well. After 24 h, 48 h, and 72 h, add 10 μl CCK-8 reagent to the cells for measuring the absorbance at 450 nm.

4.5. Cell viability detection

Seed cells into 96-well plates at the density of 5,000/well and then treated with varying concentrations of 0-1000 μM temozolomide (TMZ, Sigma, USA). Cells were harvested at 24 h and co- incubated for another hour with CCK-8 reagent. After then, measure the absorbance at 450 nm.

4.6. Cell apoptosis assay

After treated with TMZ (80 μM) for 24 h, cells were dyeing using 7-AAD and Annexin V-PE (Franklin Lakes, NJ, USA), as described previously (Ma et al. 2020).

4.7. Luciferase reporter assay

Psi-CHECK-2 luciferase reporter vector was generated in the light of wild type BTG1 3'UTR with miR-301a binding sites or mutant of each site. Lipofectamine 3000 reagent (Invitrogen) was helped for transfecting the luciferase reporter vectors w into cells. After 48h, the Dual-Luciferase Reporter Assay was carried out as previously (Lan et al. 2015).

4.8. Western blot

Proteins were extracted using lysis buffer, and the western blot assay was carried out subsequently (Zhang et al. 2020). The primary antibodies were as follows: anti-human BTG1 (Abcam, UK) and anti-human GAPDH (Abcam).

4.9. Statistical methods

SPSS 18.0 (Chicago, USA) was adopted to analyze data. For comparing two groups, t-test was used. Log-rank test was performed for comparing the survival curves. For evaluating the effect of each variable on survival, univariate Cox regression was adopted. $P<0.05$ meant the difference was statistically significant.

Conflict of interests: There was no conflict of interests.

Authors' contributions: Yanping Wang conceived the experiments and gave an experimental guidance in the lab; Shiming Zhang carried out the experiments; Feng Xiao finished data analysis and manuscript writing.

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