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CLINICAL STUDY

Long non-coding RNA *MEG3* regulates proliferation, apoptosis, and autophagy and is associated with prognosis in glioma

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Abstract

Purpose Accumulating evidence indicates that dysregulated long noncoding RNAs (lncRNAs) play critical roles in tumorigenesis and cancer progression. LncRNA-maternally expressed gene 3 (*MEG3*) has been shown to be involved in the initiation and development of several cancers, including glioma. However, the clinical prognostic value of *MEG3* in glioma has not yet been fully elucidated.

Methods The expression levels of *MEG3* were detected in 79 glioma tissues and adjacent normal brain tissues, as well as, glioma cells and normal human astrocytes by qRT-PCR. Kaplan–Meier and Cox regression methods were utilized for the survival analysis. MTT assay, flow cytometry, and immunofluorescence assay were carried out to detect the impact of *MEG3* on glioma cell proliferation, apoptosis, and autophagy.

Result The current results showed that *MEG3* expression was significantly downregulated in glioma tissues and cell line and negatively correlated with WHO grade in glioma patients. Low *MEG3* expression was significantly associated with the advanced WHO grade, low Karnofsky performance score (KPS), IDH wild-type, and tumor recurrence. Patients displaying a low expression of *MEG3* contributed to poor overall survival. The downregulated level of *MEG3*, advanced WHO grade, low KPS, IDH wild-type, and tumor recurrence were independent poor prognostic indicators in glioma patients. The in vitro experiments demonstrated that the *MEG3* overexpression remarkably suppressed the proliferation while facilitating apoptosis and autophagy in glioma cells.

Conclusions These findings indicated a critical role of *MEG3* in glioma cell proliferation, apoptosis, and autophagy. Also, the gene was found to be significantly associated with the prognosis in glioma patients. Thus, it might provide a new target for predicting prognosis and therapeutic intervention in glioma.

Keywords LncRNAs · *MEG3* · Proliferation · Apoptosis · Autophagy · Prognosis · Glioma

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Introduction

Gliomas are the most common type of primary brain tumors, accounting for approximately 80% of the primary malignancies in the central nervous system of adults [1, 2]. According to the degree of malignancy, gliomas are categorized into four grades (WHO grade I–IV) [3]. Despite the recent improvements in tumor diagnosis and treatment including neurosurgery, radiotherapy, chemotherapy, and photodynamic therapy, the prognosis, especially in patients with glioblastoma remains poor with the median survival of only 9–12 months [4, 5]. Therefore, identifying the novel risk factors to predict the clinical prognosis and developing new therapeutic targets and biomarkers for this life-threatening disease is imperative.

Long noncoding RNAs (lncRNAs) are a class of noncoding RNAs (ncRNAs), exceeding 200 nucleotides in length with little protein-coding ability. Reportedly, lncRNAs are involved in the regulation of gene expression at the transcriptional and post-transcriptional levels and in the epigenetic mechanisms [6–9]. Numerous studies have shown that lncRNAs dysregulate in several tumors and play a critical role in the biological process during tumor initiation and progression, such as proliferation, migration, invasion, and apoptosis [10–12]. Recent evidence indicates that specific lncRNA expression patterns are associated with the clinical features and outcomes that might predict the prognosis in various types of human cancers such as gliomas. For instance, the levels of lncRNA AB073614, HOXA-AS3, MALAT1, and UCA1 in glioma tissues correlate closely with clinical staging and prognosis [13–16].

Herein, we focused on lncRNA-maternally expressed gene 3 (*MEG3*), an imprinted gene localized at 14q32, encoding a ncRNA associated with tumorigenesis [17, 18]. *MEG3* served as a tumor suppressor in several human cancers including gliomas [19–21]. However, to the best of our knowledge, the prognostic significance of *MEG3* in gliomas has not yet been fully evaluated. Thus, the present study aimed to detect the expression levels of *MEG3* in glioma tissues and adjacent normal brain tissues and analyze the correlation between the level of *MEG3* expression and the clinicopathological characteristics and prognosis of glioma patients. Finally, we also investigated the effect of *MEG3* on glioma cell proliferation, apoptosis, and autophagy.

Materials and methods

Patients and tissue samples

Clinical glioma tissues and adjacent normal brain tissues were obtained from 79 patients who underwent surgery between January 2010 and June 2012 at the Department of Neurosurgery, Wuhan General Hospital of PLA, Wuhan, Hubei Province, China. The resected tissue samples were immediately frozen in liquid nitrogen and stored at − 80 °C for RNA extraction. None of these patients had received any anti-cancer treatments including radiotherapy and chemotherapy prior to surgery. After histopathological diagnosis by pathologists, 34 patients were classified as low-grade (15 WHO I: 15 pilocytic astrocytoma; 19 WHO II: 8 diffuse astrocytomas, 11 oligodendrogliomas) and 45 as high-grade (21 WHO III: 13 anaplastic oligodendrogliomas, 8 anaplastic astrocytomas; 24 WHO IV: 24 glioblastomas) according to the 2007 WHO classification. All patients were followed up for 5 years since the date of surgery. Data regarding the clinicopathological characteristics, including age, gender, Karnofsky performance score (KPS), WHO grade, surgical strategies, tumor size, tumor location, and tumor recurrence were collected. The present study was approved by the Research Ethics Committee of Wuhan General Hospital of PLA. Written informed consent was obtained from each patient before surgery.

LncRNA isolation and quantitative RT-PCR assay

Total RNA from tissue specimens was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently cDNA was synthesized, followed by quantitative real-time polymerase chain reaction (qRT-PCR) that was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Green real-time PCR Kit (Takara Biotechnology, Takara, Dalian, China); *GAPDH* was used as an endogenous control. The PCR primers for *MEG3* and *GAPDH* were as follows: *MEG3* forward, 5′-CTGCCCATCTACACCTCACG-3′ and reverse, 5′-CTCTCCGCCGTCTGCGCTAGGGGCT-3′; *GAPDH* forward, 5′-AGCCACATCGCTCAGACAC-3′ and reverse, 5′-GCCCAATACGACCAAATCC-3′. The relative expression of *MEG3* was calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to *GAPDH*.

Cell culture and transduction

Human glioma cell line (U251) and normal human astrocytes (NHA) were procured from the Cell Resource Center

of Shanghai Institute of Life Sciences (Shanghai, China). All cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific Inc.) and 100 mg/mL penicillin and cultured at 37 °C in a humidified chamber containing 5% CO_2. Subsequently, the U251 cells were transduced with pcDNA-MEG3 lentiviral vector and negative control (NC) in DMEM without FBS.

Cell proliferation assay

In order to evaluate the cell viability, in vitro cell proliferation of glioma cells was measured using MTT (Sigma, St. Louis, MO, USA) method according to the manufacturer's instructions. U251 cells were seeded into 96-well plates and transduced with pcDNA-MEG3 lentiviral vector and NC, respectively. The MTT assay was performed to test the cell viability at 24, 48, 72, and 96 h after transduction. The optical density (OD) was measured at 490 nm.

Apoptosis assay

Cell apoptosis was determined by FACS flow cytometry using an Annexin-V-FITC apoptosis detection kit (Becton–Dickinson) according to the manual. The rate of apoptosis was calculated using the Cell Quest Pro software (Becton–Dickinson). The annexin-V positive population was considered as apoptotic cells.

Immunofluorescence

U251 cells were seeded on coverslips in 6-well plate and cultured overnight. Subsequently, the cells were fixed in 3.5% paraformaldehyde, permeabilized in KB solution and 0.2% Triton X-100 at room temperature, followed by blocking. Then, the cells were incubated with a primary antibody (diluted in KB) for 30–45 min at 37 °C followed by secondary antibody for 30–45 min at 37 °C. Finally, the cells were mounted on the slide with a mounting solution medium 0.2 mg/mL DAPI, sealed with nail polish, stored at 4 °C in a dark box, and observed under a fluorescent microscope.

Statistical analysis

SPSS 20.0 software system (IBM, SPSS, Chicago, IL, USA) was used for statistical analysis and data represented as mean ± SE. The differential expression of *MEG3* among glioma tissues and adjacent normal brain tissues, as well as glioma cell and NHA was evaluated by one-way ANOVA test. The relationship between *MEG3* expression and clinicopathological characteristics was assessed by Pearson's Chi square test. The Kaplan–Meier method evaluated the overall survival rates, and data analyzed using the log-rank test. The Cox proportional hazards regression model was used to calculate the hazard ratio (HR) and the 95% confidence interval (CI); the risk factors that exert a significant influence on the overall survival were identified. $P < 0.05$ was considered as statistically significant.

Results

Expression level of *MEG3* was downregulated in human glioma tissues and cell line

Firstly, we examined the expression of *MEG3* in 79 pairs of glioma tissues and adjacent normal brain tissues using qRT-PCR. The current results demonstrated that the expression level of *MEG3* was significantly decreased in glioma tissues as compared to the adjacent normal brain tissues (3.89 ± 0.14 vs. 6.76 ± 0.15, $P < 0.001$) (Fig. 1a). In addition, the current

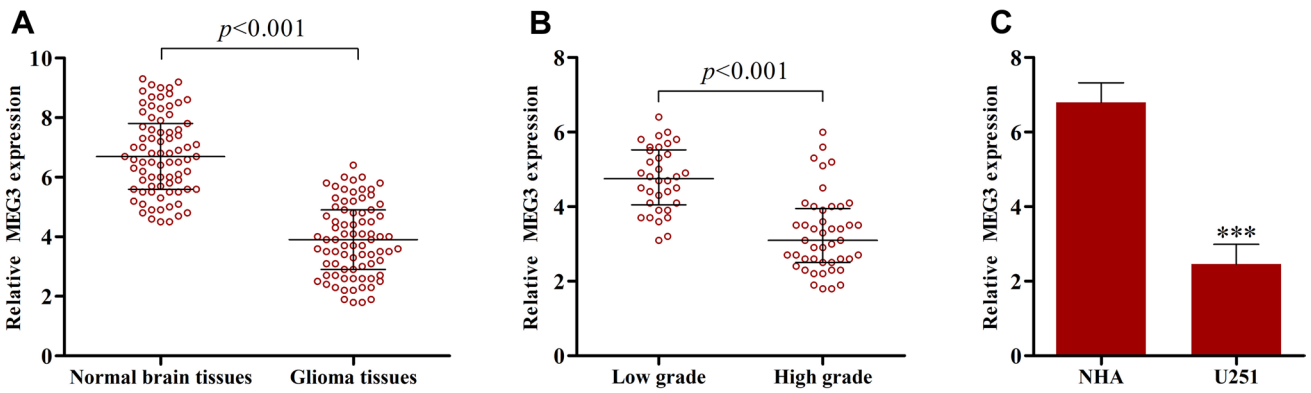

Fig. 1 The relative expression level of *MEG3* in 79 pairs of glioma tissues and adjacent normal brain tissues. **a** The expression level of *MEG3* was significantly decreased in glioma tissues as compared to the adjacent normal brain tissues (***$P < 0.001$). **b** The expression level of *MEG3* was much lower in high-grade (WHO III–IV) than that in low-grade (WHO I–II) gliomas (***$P < 0.001$)

data showed that the level of *MEG3* in high-grade (WHO III–IV) glioma was significantly lower than that in low-grade (WHO I–II) glioma (3.26 ± 0.16 vs. 4.74 ± 0.15, $P < 0.001$), indicating that the level of *MEG3* was inversely correlated to the malignancy degree of glioma (Fig. 1b). Furthermore, the expression level of *MEG3* in glioma cell line (U251) and NHA was examined. A similar phenomenon was observed in tissues, wherein the *MEG3* expression showed a significant decrease in U251 cells as compared to that in NHA (2.46 ± 0.53 vs. 6.80 ± 0.52, $P < 0.001$) (Fig. 1c).

Relationship between *MEG3* expression and clinicopathological characteristics in glioma patients

To further explore the association between *MEG3* expression and clinicopathological characteristics of glioma, 79 glioma tissue specimens were divided into two groups according to the *MEG3* level. 41 samples with *MEG3* expression level less than the median value were assigned to the low-expression group, while the remaining 38 samples with *MEG3* expression above the median value were assigned to the high-expression group. As shown in Table 1, the low level of *MEG3* expression was significantly associated with advanced WHO grade ($P < 0.001$), low KPS ($P = 0.019$), IDH wild-type ($P = 0.001$) and tumor recurrence ($P = 0.003$). However, a significant association of *MEG3* expression with age, gender, tumor location, tumor size, or surgical strategies was not observed ($P = 0.589$, 0.616, 0.873, 0.428, and 0.450, respectively). Taken together, these data suggested that *MEG3* might play a critical role in the progression of glioma.

Table 1 Relationship between *MEG3* expression level and clinicopathological characteristics in glioma patients

Characteristics	*n*	*MEG3* expression		*p*
		High expression	Low expression	
Age (years)				0.598
< 50	35	18	17	
≥ 50	44	20	24	
Gender				0.616
Male	48	22	26	
Female	31	16	15	
KPS				**0.019**
≥80	37	23	14	
<80	42	15	27	
Tumor location				0.873
Frontal	32	17	15	
Parietal	18	7	11	
Occipital	7	4	3	
Temporal	13	6	7	
Others	9	4	5	
Tumor grade				**< 0.001**
Low grade I–II	34	26	8	
High grade III–IV	45	12	33	
Tumor diameter (cm)				0.428
< 4.5	40	21	19	
≥ 4.5	39	17	22	
Surgical resection				0.450
GTR	55	28	27	
PR	24	10	14	
IDH mutation status				
IDH-mutated	30	22	8	**0.001**
IDH-wildtype	34	11	23	
Tumor recurrence				**0.003**
Yes	43	15	28	
No	36	23	13	

Bold indicate statistically significant values

KPS Karnofsky performance score, *GTR* gross total resection, *PR* partial resection

Low *MEG3* expression predicts poor prognosis in patients with glioma

To investigate the prognostic significance of *MEG3* in glioma, Kaplan–Meier method with log-rank test was employed to analyze the association between *MEG3* expression level and overall survival rates. As shown in Fig. 2, the glioma patients with low level of *MEG3* expression had distinctly lower overall survival rates than patients with high *MEG3* expression level, suggesting that the low expression significantly impacted the overall survival rates of glioma patients ($P < 0.001$). Furthermore, univariate and multivariate analyses assessed whether *MEG3* expression level and other clinicopathological characteristics were independent prognostic factors for glioma. The results from univariate Cox regression analysis showed that the low level of *MEG3* expression ($P = 0.004$), the advanced WHO grade ($P = 0.015$), low KPS ($P = 0.031$), and tumor recurrence ($P = 0.022$) were the leading variables for prognosis. The multivariate analysis using the Cox proportional hazards regression model demonstrated that the low level of *MEG3* expression (HR 3.423, 95% CI 2.052–6.779, $P < 0.001$), the advanced WHO grade (HR 2.921, 95% CI 1.562–6.095, $P < 0.001$), low KPS (HR 2.615, 95% CI 1.387–5.047, $P = 0.009$), IDH wild-type (HR 2.671, 95% CI 1.426–5.054, $P = 0.006$), and tumor recurrence (HR 2.485, 95% CI 1.311–4.702, $P = 0.013$) were significantly correlated with poor prognosis of glioma patients (Table 2). Taken together, the current findings suggested that the expression of *MEG3* was a novel and independent prognostic factor for glioma.

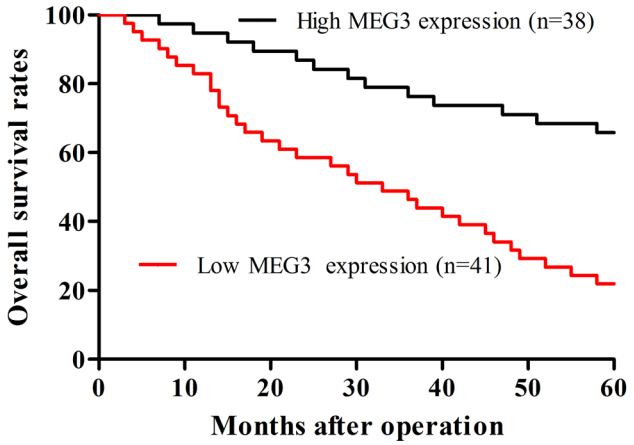

Fig. 2 Kaplan–Meier curves of the overall survival of 79 glioma patients with low- and high-expression levels of *MEG3*. The overall survival rates in glioma patients with low *MEG3* expression (red line) were distinctly lower than that in patients with high *MEG3* expression (black line, ***$P < 0.001$)

Overexpression of *MEG3* significantly suppressed the proliferation, while facilitated apoptosis and autophagy of glioma cells in vitro

In order to investigate the role of *MEG3* in glioma, U251 cells were transduced with NC or pcDNA-MEG3 lentiviral vector. qRT-PCR assay revealed that the endogenous expression of *MEG3* was obviously increased in *MEG3* overexpression group as compared to that in the NC group (Fig. 3a). After transduction, the MTT assay examined the impact of *MEG3* overexpression on the proliferation of glioma cells. Compared to the NC group, the overexpression of *MEG3* resulted in a significant decrease in the viability of U251 cells as monitored by the MTT assay (Fig. 3b). Furthermore, flow cytometry analysis was performed to define whether transduction with pcDNA-MEG3 lentiviral vector could induce glioma cell apoptosis. The current result showed that the rate of cell apoptosis was significantly increased after overexpression of *MEG3* (Fig. 3c). Finally, the immunofluorescence LC3 autophagy assay showed that LC3 was significantly increased in the *MEG3* overexpression group as compared to that in the NC group, indicating that the overexpression of *MEG3* could activate the in vitro autophagy in glioma cells (Fig. 3d).

Discussion

An increasing number of studies have focused on the role of lncRNAs in the development of human disease, especially cancers [22, 23]. Some lncRNAs are shown to be directly involved in various biological activities from nuclear organization to post-transcriptional influence on mRNA splicing, stability, and translation [24, 25]. Accumulating evidence also demonstrated that dysregulated lncRNA expression contributes to specific tumorigenic and continuous processes, including cell proliferation, cell growth, cell cycle progression, and apoptosis [26, 27]. These findings indicated that these specific lncRNAs might provide the missing

Table 2 Univariate and multivariate analyses of different clinicopathological variables and *MEG3* expression in patients with glioma

Characteristics	Univariate analysis	Multivariate analysis	
	P	HR (95% CI)	*P*
Age (< 50 vs. ≥ 50 years)	0.534	1.361 (0.726–2.357)	0.417
Gender (female vs. male)	0.426	0.723 (0.351–1.342)	0.697
KPS (< 80 vs. ≥ 80)	**0.031**	2.615 (1.387–5.047)	**0.009**
Tumor location			
Parietal vs. Frontal	0.722	0.866 (0.354–1.927)	0.651
Temporal vs. Frontal	0.358	1.417 (0.633–2.646)	0.363
Occipital vs. Frontal	0.463	0.924 (0.427–2.131)	0.632
Others vs. Frontal	0.274	1.347 (0.638–2.825)	0.403
Tumor grade (I–II vs. III–IV)	**0.015**	2.921 (1.562–5.595)	**< 0.001**
Tumor diameter (< 4.5 vs. ≥4.5 cm)	0.673	0.684 (0.313–1.207)	0.722
Surgery (GTR vs. PR)	0.704	0.565 (0.272–1.021)	0.816
IDH (mutated vs. wildtype)	**0.019**	2.671 (1.426–5.054)	**0.006**
Tumor recurrence (yes vs. no)	**0.022**	2.485 (1.311–4.702)	**0.013**
MEG3 expression (low vs. high)	**0.004**	3.423 (2.052–6.679)	**< 0.001**

Bold indicate statistically significant values

HR hazard ratio, *CI* confidence interval, *KPS* Karnofsky performance score, *GTR* gross total resection, *PR* partial resection

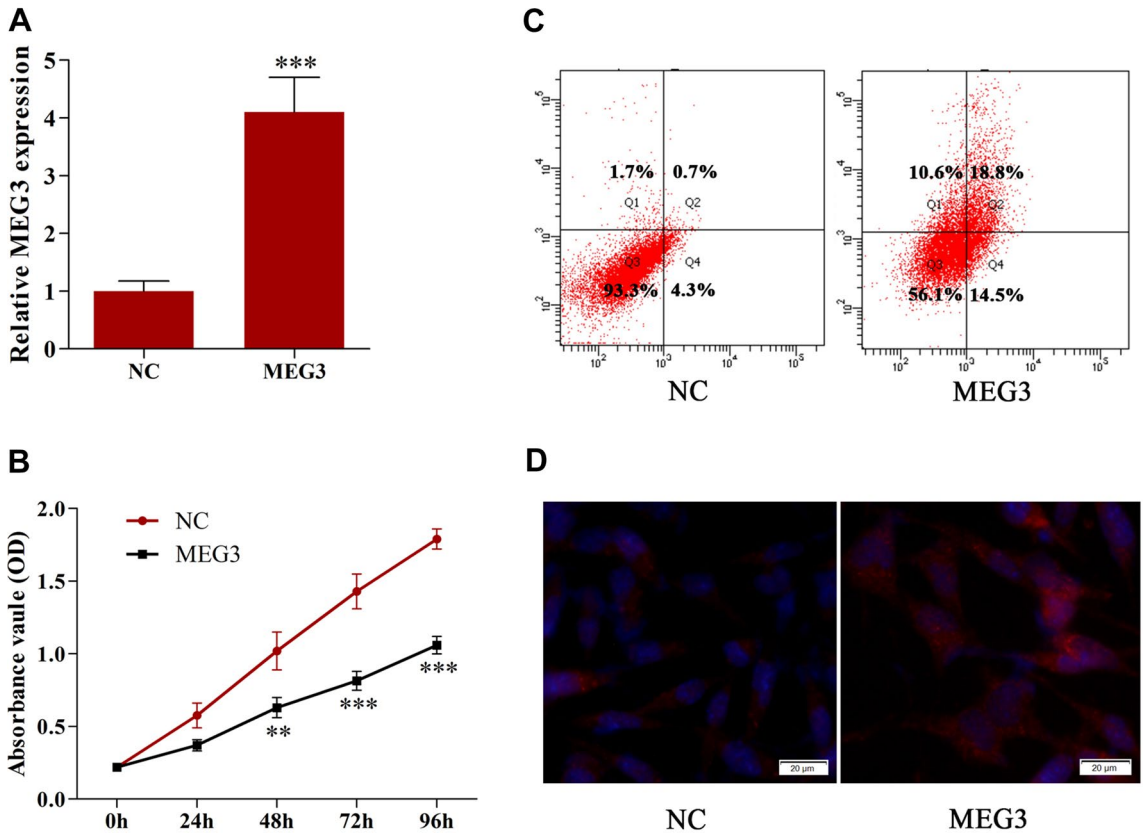

Fig. 3 Overexpression of *MEG3* significantly suppressed the proliferation, while facilitated the apoptosis of glioma cells in vitro. **a** The efficiency of *MEG3* overexpression in U251 cells transduced with NC or pcDNA-MEG3 lentiviral vector was measured by qRT-PCR. **b** MTT assay detected the proliferation of U251 cells after transduction with NC or pcDNA-MEG3 lentiviral vector. **c** Cell apoptosis of U251 cells transduced with NC or pcDNA-MEG3 lentiviral vector was determined by Annexin-FITC/PI assay. **d** LC3 autophagy of U251 cells transduced with NC or pcDNA-MEG3 lentiviral vector was determined by immunofluorescence assay (**$P < 0.01$; ***$P < 0.001$)

piece of the well-known tumor-suppressive and oncogenic network puzzle.

Several recent investigations have begun to unravel the vitality of lncRNAs in tumor initiation and development. Sun et al. [28] discovered that the increased expression of lncRNA 00511 promoted oncogenesis, tumor size, metastasis, and poor prognosis by binding to EZH2 and suppressing p57 in human non-small-cell lung cancer. Liu et al. [29] found that lncRNA BX357664 acted as a potential tumor suppressor by inhibiting the TGF-β1/p38/HSP27 signaling pathway and interfering with cancer cell proliferation, invasion, and metastasis in renal cell carcinoma. In addition, lncRNA RP5-833A20.1 inhibited tumor cell proliferation and cell-cycle progression and induced apoptosis by suppressing the expression of nuclear factor IA in gliomas [30]. Taken together, these findings provided evidence that some lncRNAs play a major role in tumorigenesis and progression, advocating that these lncRNAs may serve as novel biomarkers and therapeutic targets in a variety of malignancies.

The imprinted *MEG3* gene has attracted increasing research interest. Previous studies have proved that *MEG3* was downregulated and acted as a tumor suppressor in several human cancers including oral squamous cell carcinoma [31], cervical cancer [32], breast cancer [33], pancreatic cancer [34], and glioma [35]. Zhang et al. [36] demonstrated that *MEG3* suppresses breast cancer cell proliferation, invasion, and angiogenesis via the AKT pathway. Xiu et al. [37] demonstrated the tumor suppressor function of *MEG3* in epithelial ovarian cancer by regulating the ATG3 activity and inducing autophagy. Notably, Tong et al. [38] found that *MEG3* suppressed the proliferation, migration, and invasion of glioma cells when acting as competitive endogenous RNA of miR-19a. Another study demonstrated that *MEG3* promoted the proliferation of cells through targeting Wnt/β-catenin signaling pathway in glioma [39]. Moreover, some groups have shown that the decreased expression of *MEG3* predicted a poor prognosis in patients with colorectal cancer, cervical cancer, non-small cell lung cancer, and gastric cancer [21, 40–43]. However, to date, no study has reported the prognostic significance of *MEG3* in glioma.

In the present study, for the first time, we focused on investigating the correlation between the expression level

of *MEG3* and prognosis of glioma patients. In addition, the current findings demonstrated that *MEG3* expression was downregulated in glioma tissues as compared to the adjacent normal brain tissues. The *MEG3* level in high-grade glioma was significantly lower than that in low-grade glioma. Furthermore, we found that the decreased expression level of *MEG3* was significantly associated with advanced WHO grade, low KPS, IDH wild-type, and tumor recurrence. However, no correlation was established between *MEG3* expression and age, gender, surgical strategies, tumor size, or tumor location. Importantly, the low expression of *MEG3* was correlated with poor overall survival rates and could function as an independent prognostic factor for glioma. Moreover, this study also explored the impact of *MEG3* overexpression on glioma cell line. Interestingly, results consistent with the previous studies were also observed in our study; *MEG3* overexpression could suppress glioma cell proliferation in vitro. To the best of our knowledge, this study for the first time demonstrated that *MEG3* overexpression facilitated apoptosis and autophagy of glioma cells in vitro.

Nevertheless, the present study had some limitations. First, it was a retrospective study. Secondly, the sample size was relatively small; thus, a study containing a large sample size is essential to confirm these findings. Finally, although our data revealed that the overexpression of *MEG3* significantly suppressed the proliferation and facilitated apoptosis of glioma cells, the underlying mechanisms and the precise role of *MEG3* in glioma have not yet been elucidated, and further investigations of *MEG3* function and its downstream genes in glioma cell and animal models are essential.

In conclusion, the current findings demonstrated that the downregulation of *MEG3* was closely correlated with the lower overall survival rates in patients with glioma. The low expression of *MEG3* might function as an independent biomarker for poor prognosis. The overexpression of *MEG3* significantly suppressed the proliferation, while apoptosis and autophagy of glioma cells were facilitated in vitro. Therefore, further investigations are necessary to confirm these preliminary findings and illuminate the underlying molecular mechanisms.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethics approval The study was approved by the Research Ethics Committee of Wuhan General Hospital of PLA, and it complied with the Declaration of Helsinki. All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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